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Targeting Spare CCR5 as a Principle to Inhibit HIV-1 Entry*

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Keywords: G protein-coupled receptor (GPCR); HIV-1; antiviral agents; CCR5; conformation; virus entry; endocytosis

Background: CCR5 is a chemokine receptor and a co-receptor for HIV entry.

Results: A pool of spare CCR5 binds the potent anti-HIV chemokine analog PSC-RANTES, but not native chemokines.

Conclusion: Targeting a large number of CCR5 improves the anti-HIV property of PSC-RANTES.

Significance: Spare receptors represent a target for inhibiting HIV and might constitute a viral escape route.

CCR5 binds the chemokines CCL3, CCL4, CCL5 and is the major co-receptor for HIV-1 entry into target cells. Chemokines are supposed to form a natural barrier against HIV-1 infection. However, we showed that their antiviral activity is limited by CCR5 adopting low-chemokine affinity conformations at the cell surface. Here, we investigated whether a

pool of CCR5 that is not stabilized by chemokines could represent a target for inhibiting HIV infection. We exploited the characteristics of the chemokine analog PSC-RANTES, which displays potent anti-HIV-1 activity. We show that native chemokines fail to prevent (i) high-affinity binding of PSC-RANTES, (ii) the analog-mediated calcium release (in desensitization assays) and (iii) the analog-mediated CCR5 internalization. These results indicate that a pool of spare CCR5 may bind PSC-RANTES but not native chemokines. Improved recognition of CCR5 by PSC-RANTES may explain why the analog promotes higher amounts of β -arrestin2/CCR5 complexes, thereby increasing CCR5 down-regulation and HIV-1 inhibition. Together these results highlight that spare CCR5, which might permit HIV-1 to escape from chemokines, should be targeted for efficient viral blockade.

The CC chemokine receptor 5 (CCR5) is a G protein-coupled receptor (GPCR) for the chemokines RANTES/CCL5, MIP1 α /CCL3 and MIP1 β /CCL4 (1). CCR5 is also a CD4-associated co-receptor required for human immunodeficiency virus type 1 (HIV-1) entry into host cells (2). It plays a prominent role during transmission, progression of infection and evolution to AIDS. Individuals that do not express a functional CCR5 are highly resistant to HIV-1 infection without major pathological consequences (3). CCR5 is therefore a suitable therapeutic target for HIV-1 blockade. A number of non-competitive and allosteric antagonists have been developed, among which Maraviroc is the only one currently used in HIV treatment (4). However, viral resistance to this drug is a major drawback highlighting the need for new inhibitors (5).

GPCRs can oscillate between various inactive and active conformations at the cell surface (6). Structurally different ligands can stabilize and/or create distinct conformations (7) that interact with specific effectors including heterotrimeric G-proteins and β -arrestins, which are scaffolding proteins involved in receptor desensitization and endocytosis (8). Reciprocally, interaction of a particular receptor conformation with an effector can increase the affinity of the receptor for the ligand (6). CCR5 exists in multiple conformations displaying distinct pharmacological and antigenic properties (9,10). This conformational heterogeneity is exploited by HIV-1 to escape inhibition by native CCR5-binding chemokines and small-molecule CCR5 inhibitors (10-12). Therefore, taking into account the multiplicity of CCR5 conformations is essential for the development of new anti-HIV molecules.

In this study, we investigated whether a pool of CCR5 that is not accessible to native chemokines would be a target for inhibiting HIV-1 infection. For this purpose, we studied the molecular mechanisms whereby the N-terminally modified CCL5 analog PSC-RANTES shows improved antiviral activity compared to native chemokines (13,14). This drug protects macaques from HIV-1 infection after vaginal application (15). The exceptional capacity of PSC-RANTES to inhibit infection is related to its ability to increase CCR5 down-regulation (11,13). A super agonist activity of PSC-RANTES (16) and its ability to sequester CCR5 into the trans-Golgi network (17) have been

suggested to play a role in this context. However, we report here that PSC-RANTES also displays the unique property of recognizing a larger array of CCR5 conformational states than native chemokines, making it more efficient in recruiting β -arrestins and down-regulating CCR5. These findings provide evidence for the existence of a set of CCR5 that are not stabilized by native chemokines at the cell surface and highlight the targeting of this spare CCR5 as an anti-HIV principle.

EXPERIMENTAL PROCEDURES

Plasmids, siRNA and chemokines – The previously described dominant negative mutant of Eps15 GFP-E Δ 95/295 (18) was a gift from A. Benmerah (Institut Imagine, Paris, France). YFP tagged-CCR5, β -arrestin2 RLuc (β arr2-Rluc) and the construct encoding for GFP fusions of wild-type β -arrestin2 (β arr2-GFP) have been described (19,20). The double-stranded 5'-ACCUGCGCCUCCGCUAUG-3' siRNA sequence (Dharmacon) was used to simultaneously target both β arr1 and β arr2 as previously described (21). The β arr1/2 siRNA and a scrambled siRNA (control: 5'-UGGUUUACAUGUCGACUAA-3') (Dharmacon) were transfected by RNAimax (Invitrogen) according to the manufacturer's instructions. The chemokines MIP-1 α /CCL3 and RANTES/CCL5 were purchased from Peprotech (Tebu-bio). MIP1 β /CCL4 was chemically synthesized by F. Baleux (Institut Pasteur, Paris, France). ¹²⁵I-CCL3 was from PerkinElmer Life Sciences. The CCR5 antagonist TAK779 was obtained through the centre for AIDS reagents, NIBSC, UK, with permission of Takeda Chemical Industries Ltd (22). The chemokine analog 5P12 with antagonist feature was previously described (23). The chemokine analog PSC-RANTES (N- α -(n-nonanoyl)-des-Ser(1)-[L-thiopropyl(2), L cyclohexylglycyl(3)] RANTES(4-68)) was provided by the Centre for AIDS Reagents, NIBSC, UK. PSC-RANTES carrying a C-terminal biotin (PSC-RANTES-biot) was synthesized essentially as in (13), except that synthesis of the C-terminal fragment was carried out on a Boc-Gly-PAM resin, incorporating a Lys residue with an Fmoc-protected epsilon amino group. At the end of synthesis, the side chain of this Lys residue was deprotected and elongated successively with Fmoc-aminocaproic acid and Biotin-OSu.

Biotinylated PSC-RANTES shows indistinguishable activity to non-biotinylated material in an R5-tropic envelope-dependent cell fusion assay (O.H. et al., unpublished results).

Cell lines and primary CD4⁺ T lymphocytes - The human embryonic kidney HEK293 cells stably expressing FLAG-tagged CCR5 WT and CCR5-349 mutant have been described (24). The human A3.01 T cell line stably expressing FLAG-tagged CCR5 was generated by the Amaxa nucleofector technology (Amaxa Biosystems) and culture of cells for several weeks in 1 mg/ml G418 (Geneticin; Invitrogen). Cell clones were screened and sorted by flow cytometry (on a MoFlo Astrios, Beckman Coulter) using the M1 anti-FLAG (Sigma) or the anti-CCR5 2D7 (BD Pharmingen) monoclonal antibody. FLAG-CCR5 HEK293 cells stably expressing β arr2-GFP were generated by calcium phosphate co-precipitation and specific sorting. HEK293 cells were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. T cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Human CD4⁺ T lymphocytes were purified from PBMCs of healthy blood donors (Etablissement Français du Sang) by Ficoll centrifugation (PAA) followed by immunomagnetic selection (Miltenyi Biotec). They were maintained for 2 days in RPMI 1640 medium containing phytohemagglutinin (1 μ g/mL) and interleukin 2 (300 IU/mL) and then in medium containing IL-2 before use.

Viral production and HIV-1 virion-based fusion assay - HIV-1 particles containing BlaM-Vpr were produced by co-transfection of HEK293T cells with a NL4-3Ren-based plasmid expressing the envelope glycoprotein gp160 of the HIV-1 R5-tropic strain Bx08 (11) and pCMV-BlaM-vpr encoding β -lactamase fused to the viral protein Vpr (25). After 48 h of culture at 37°C, the virus-containing supernatant was centrifuged at low speed to remove cellular debris and then ultracentrifuged at 72,000 \times g for 90 min at 4°C to sediment viral particles. The virion-enriched pellet was resuspended in DMEM and aliquoted for storage at -80°C. Following 30 min incubation with chemokines, 1.5 \times 10⁵ primary CD4⁺ T cells were inoculated with the BlaM-Vpr-containing viruses (50 ng p24 Gag) by 1 h spinoculation at 4°C and incubated 2 h at 37°C. Cells were then

loaded with CCF2/AM, the BlaM-Vpr substrate (2 h at RT) and fixed. Enzymatic cleavage of CCF2/AM by β -lactamase (the readout of viral entry fusion) was measured by flow cytometry (FACSanto, BD) and data were analyzed with FlowJo software. The percentage of fusion corresponds to the percentage of cells displaying increased cleaved CCF2/AM fluorescence (447 nm).

Radioligand Binding Assays on A3.01-FLAG-CCR5 cell lines - Displacement of ¹²⁵I-CCL3 (0.5 nM) in the presence of CCL5, CCL4, CCL3 or PSC-RANTES was performed as in (11) except that incubations were done in Eppendorf tubes at 4°C. To remove unbound ¹²⁵I-CCL3, cells were pelleted at 4 °C (5 min) and then washed once with washing buffer (50 mM Hepes, pH 7.4, 5 mM MgCl₂, 1 mM CaCl₂, and 150 mM NaCl). Pellets were resuspended in the washing buffer and radioactivity was counted in a Multi Crystal LB2111 (Berthold). Displacement of PSC-RANTES-biot (1 nM) in the presence of CCL5, CCL3 or PSC-RANTES were done in eppendorf tubes at 4°C during 90 min. Unbound PSC-RANTES-biot were remove by centrifugation and pellets were incubated 30 min with PBS/BSA 0.2% containing streptavidin-PE (BD Bioscience). Cells were analyzed by flow cytometry (Gallios flow cytometer, Beckman Coulter). Analysis of the binding data was made using the Prism software (GraphPad).

[³⁵S]GTP γ S binding - [³⁵S]GTP γ S binding to crude membrane preparations of CCR5-expressing HEK293T cells was described previously (9). Briefly, membranes were incubated for 15 min at 30°C in 20 mM HEPES, pH 7.4, containing 100 mM NaCl, 10 μ g/ml saponin, 1 μ M GDP and 3 mM MgCl₂, in the presence or absence (basal [³⁵S]GTP γ S binding) of chemokines. Then, 0.1 nM [³⁵S]GTP γ S (PerkinElmer) was added to membrane-containing mixes, which were incubated for 30 min at 30°C. The incubation was stopped by centrifugation (800 \times g for 10 min) at 4°C and removal of supernatants. Microplates were counted 2 min per well in a PerkinElmer Wallac 1450 Microbeta Trilux (PerkinElmer Life and Analytical Sciences).

Calcium Measurements - FLAG-CCR5 expressing HEK293 cells or primary CD4⁺ T cells were incubated for 20 min at 37°C with 1.5 μ M

Fura-2/AM (Molecular Probes). Calcium measurements by spectrofluorimetry were performed in mammalian saline as previously described (26) with a Cary Eclipse spectrofluorimeter (Varian) (excitation: 340 and 380 nm; emission: 510 nm). For measurements by microscopy, cells were analyzed with a Nikon TE2000 microscope (Nikon, Melville, NY) equipped with a cooled CDD camera (cascade, princeton Instruments) and a Metafluor imaging system (Roper Scientific) as described (27). Calcium levels are represented as a ratio 340/380 fluorescence intensity.

Fluorescence flow cytometry assays of receptor internalization and recycling – Flow cytometry was used to quantitate the internalization and the recycling of CCR5 by measuring the levels of cell surface FLAG-tagged receptors as described previously (24). Mean values were used to compute the proportion of internalized receptors as indicated by a decrease of immunoreactive surface receptor with agonist compared to untreated cells. The percentage of receptor recycling was the proportion of receptors that was recovered at the cell surface out of the internalized receptors.

Cell surface biotinylation and assay of agonist-induced proteolysis - Western blot – The cell surface biotinylation assay was described previously (24). Briefly, cells were incubated with 300 µg/ml sulfo-NHS-biotin (Pierce) in PBS at 4°C for 30 minutes. Unreacted sulfo-NHS-biotin was quenched by washes with ice-cold Tris-buffered saline. Cells were incubated with media at 37°C for the indicated times in the absence or presence of agonists. They were chilled on ice, washed with PBS and extracted with lysis buffer (0.5% TX-100, 10 mM Tris-HCl, 120 mM NaCl, 25 mM KCl, protease inhibitors mixture). Extracts were centrifuged and equal amounts of proteins (Bradford analysis) were incubated with 25 µl of streptavidin beads (Pierce) overnight (O/N). Eluted proteins were resolved by SDS-PAGE and transferred to a PVDF membrane for Western blot analysis. For receptors detection, blots were incubated with M1 anti-FLAG antibody (Sigma) and then with an HRP-coupled goat anti-mouse IgG for enzyme-linked chemiluminescence detection (ECL system, Amersham Life Sciences). A control of the quantity of proteins per lane was performed after an acid strip and incubation of blots with an avidin-HRP complex (Sigma). Band

intensities were quantified by densitometry of films.

For detection of β -arrestin after siRNA transfection, equal amount of proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. Blots were incubated O/N with the anti- β -arrestin antibody D24H9 (Cell signaling). After washing, blots were incubated with an anti-rabbit-HRP antibody (Jackson) before detection by ECL. A control of the quantity of proteins per lane was performed after an acid strip and incubation of blots with anti-LDH5 (Biodesign) and anti-goat-HRP (Dako) antibodies.

siRNA rescue – For recombinant protein re-expression in FLAG-CCR5-349 expressing HEK293 cells, β arr2-GFP was mutated by site directed mutagenesis to ACC TGT GCC TTT AGG TAT G (underlined bases show synonymous mutations introduced) in order to assure resistance to knockdown by β arr1/2 siRNA (res- β arr2-GFP). siRNA (10 nM) and plasmid (50 ng) were co-transfected (with 48 h interval between the two) using RNAimax and Lipofectamine 2000 respectively. Experiments of internalization were conducted 3 days after siRNA transfection. After staining, GFP-positive cells were analyzed for CCR5 cell surface expression by flow cytometry (Gallios flow cytometer, Beckman Coulter).

BRET assays - BRET assays were performed as described previously (28). Briefly, 24 h post-transfection (with 30 ng β arr2-Rluc-encoding plasmid and increasing concentrations of plasmid encoding CCR5-YFP), cells were detached with DMEM without phenol red (Invitrogen) and distributed in white 96-well optiplates (Perkin Elmer). 50 nM of chemokines was added and incubated at 37°C for the indicated times. After incubation, Coelenterazine *h* (Interchim) was added to a final concentration of 5 µM, and incubated for 3 minutes at 25°C. BRET readings were collected using a Multilabel Reader Mithras LB 940 (Berthold Technologies). Substrate and light emissions were detected at 480 nm (Rluc) and 540 nm (YFP) for 1 second. The BRET signal corresponds to the ratio between fluorescence emitted by YFP and the light emitted by Rluc (YFP/Rluc). The ratio values were corrected by subtracting background BRET signals detected when β arr2-Rluc was expressed alone. mBRET values were calculated by multiplying these ratios

by 1000. For kinetics analysis, cells were transfected with 100 ng β arr2-Rluc and 250 ng CCR5-YFP. The day after, Coelenterazin *h* and the agonist were added simultaneously, and the BRET signal was measured over time.

Total Internal Reflection Fluorescence (TIRF) microscopy – 4×10^5 FLAG-CCR5/ β arr2-GFP expressing HEK293 cells plated on MatTek plates were incubated in the presence or absence of 2 nM CCL5 or PSC-RANTES in DMEM/1%BSA medium for the indicated time. Cells were put on ice and fixed with PFA 4% at 4°C during 40 min before 3 wash in PBS. Experiments were performed using a TIRF microscope (IX81F-3, Olympus) equipped with a 100XNA 1.45 Plan Apo TIRFM Objective (Olympus) and fully controlled by CellM (Olympus). Images were collected using an IxonEM+ Camera (DU885, Andor). All the TIRF images analyses were performed using ICY software (<http://icy.bioimageanalysis.org>). The number of spot detected per cells was normalized to the size of the cell surface (29).

RESULTS

Native chemokines and PSC-RANTES trigger similar G-protein signaling but different CCR5 intracellular trafficking- The antiviral activity of PSC-RANTES ($IC_{50} = 0.38 \pm 0.14$ nM), which is much more potent than that of CCL5 or CCL4 ($IC_{50} = 259 \pm 26$ nM and >1000 nM, respectively) (Fig. 1A), is attributed to its ability to induce a stronger CCR5 down-regulation (13). We investigated whether this effect could result from increased binding affinity of PSC-RANTES for CCR5 or from an increased ability to activate G-proteins (16). Native chemokines and PSC-RANTES displaced ^{125}I -CCL3 binding to CCR5-expressing A3.01 cells with comparable potencies ($IC_{50} = 2.6 \pm 0.11$, 2 ± 0.25 , 1.1 ± 0.14 nM and 1.2 ± 0.12 for CCL5, CCL4, CCL3, PSC-RANTES, respectively) (Fig. 1B), in line with previous observations (11). Of note, the extent of ^{125}I -CCL3 binding displacement by the unlabeled chemokines varied with the concentration of ^{125}I -CCL3, indicating that a competitive inhibition of ^{125}I -CCL3 binding occurred (30) (Fig. 1B, insert). These chemokines also displayed similar potencies and efficacies for activating G-proteins in ^{35}S -GTP γ S binding assays ($EC_{50} = 3.4 \pm 0.27$ and 7 ± 0.17 nM for CCL5 and PSC-RANTES,

respectively) (Fig. 1C) and for calcium responses ($EC_{50} = 2.3$ nM for CCL5 and PSC-RANTES) (Fig. 1D).

Despite the similar output of CCL5 and PSC-RANTES in terms of G-protein activation, the analog promoted stronger receptor sequestration than the native chemokine (Fig. 1, E and F), in line with observations on other cells (13). After 30 min stimulation with a saturating concentration (50 nM) of PSC-RANTES or CCL5 (Fig. 1E), cell surface CCR5 was reduced by 50% and 20%, respectively. We investigated whether these differences could be explained by differences in the rates of CCR5 endocytosis or recycling to the cell surface (24). In cells expressing CCR5-349, a receptor mutant unable to recycle (24), PSC-RANTES-induced internalization reached 90% at 30 min, whereas CCL5-dependent internalization only reached 30% (Fig. 1F). Endocytosis half-life was 50 min for CCL5 and 7 min for PSC-RANTES, indicating that PSC-RANTES induced faster receptor down-regulation than the native chemokine. CCR5 recycling after PSC-RANTES-induced endocytosis was maintained, although at a slower rate compared to CCL5 (Fig. 1G). In addition, regardless of the agonist used, the receptor was not directed to the degradation pathway (Fig. 1, H and I), in agreement with previous data (17). In conclusion, both improved receptor endocytosis and slower recycling likely explain the strong CCR5 down-regulation and the potent antiviral activity of PSC-RANTES.

Compared to native chemokines, PSC-RANTES induces increased β -arrestin2 recruitment to CCR5- We next investigated whether PSC-RANTES-dependent CCR5 trafficking would involve a distinct endocytic pathway. Both CCL5- and PSC-RANTES-induced internalization were impaired in the presence of the $\Delta 95/295$ dominant negative mutant of Eps15 (Fig. 2A), a component of clathrin-coated pits (CCPs) (18). Thus both agonists induced CCR5 internalization via the clathrin-driven endocytic pathway. As a control, $\Delta 95/295$ also inhibited the internalization of transferrin (not shown).

GPCR endocytosis most often requires β -arrestins, which bridge the receptor cargo to AP2 and clathrin (31). Silencing (70% reduction) β -arrestin1 and 2 (β arr1 and β arr2) endogenous expressions with siRNA similarly decreased

CCR5-349 internalization regardless of the agonist used (Fig. 2, *B-D*). To test specificity, we rescued CCR5 internalization by co-transfecting a β arr2-GFP mutant resistant to knockdown by β arr1/2 siRNA (Fig. 2*E*). These experiments indicate that β -arrestins contributed to CCR5 internalization whatever the agonist. These results are consistent with recent data showing intracellular clustering of β arr2 after CCR5 stimulation with PSC-RANTES (32).

We examined whether the improved CCR5 sequestration and slow recycling induced by PSC-RANTES were due to increased affinity of β arr2 for the PSC-RANTES-occupied receptor. β arr2 translocation to agonist-bound GPCRs can be monitored using Bioluminescence Resonance Energy Transfer (BRET) (28). We conducted BRET saturation assays in living HEK293 cells co-expressing plasmids encoding β arr2-Rluc as the BRET donor, and CCR5-YFP as the BRET acceptor, in the presence or absence of 50 nM PSC-RANTES or CCL5. We obtained hyperbolic saturation curves in the presence of both agonists, indicating a specific recruitment of β arr2 to agonist-bound CCR5 (Fig. 3*A*). BRET₅₀ values (YFP/Rluc value for half-maximal BRET), which reflect the propensity of β arr2-Rluc to associate with CCR5-YFP, were not significantly different after CCL5 and PSC-RANTES stimulation (0.58 ± 0.27 vs 0.22 ± 0.05 ; $p=0.24$ in unpaired two-tailed student t test), ruling out the hypothesis of an increased affinity of β arr2 for PSC-RANTES-occupied receptors, compared to CCL5-occupied receptors. Accordingly, kinetic analysis revealed similar half-time values for the association of β arr2 to CCL5- and PSC-RANTES-bound CCR5 (16.2 ± 2.7 vs 11.2 ± 2.7 min; $p=0.1$ in unpaired two-tailed student t test) (Fig. 3*B*).

These data suggest that PSC-RANTES and CCL5 stabilize CCR5 in a similar β arr2-interacting conformation. In this context, the 3-fold increase of the BRET_{max} values (the BRET values corresponding to the plateau of the hyperbola) in the presence of PSC-RANTES might indicate that a larger fraction of β arr2 (the BRET donor) would be associated with CCR5 (the acceptor) in the presence of PSC-RANTES compared to CCL5 (Fig. 3*A*). However, since BRET_{max} values also depend on the distance and on the relative orientation of the BRET donor and acceptor (28),

different BRET_{max} values could also correspond to different conformations of β arr2/CCR5 complexes elicited by CCL5 and PSC-RANTES. To discriminate between these two possibilities, we examined the amount of β arr2 recruited at the plasma membrane using Total Internal Reflection Fluorescence (TIRF) microscopy, which restricts the observation to the first 100–200 nm from the coverslip (33). TIRF acquisitions were performed in fixed cells stably expressing β arr2-GFP and FLAG-CCR5 (Fig. 3, *C* and *D*). β arr2-GFP fluorescence was not enriched at the plasma membrane in untreated cells (Fig. 3*C*). After receptor stimulation with PSC-RANTES or CCL5, β arr2-GFP was recruited into spots close to the plasma membrane, likely corresponding to CCR5-containing CCPs (Fig. 3*C*). The number of spots increased over time after agonist exposure: at all time points the number of β arr2-GFP spots in PSC-RANTES-treated cells was at least three times higher than in CCL5-treated cells (Fig. 3*D*). Note that the surface of spots was not significantly different between both treatments at 10 min ($\text{surface}_{\text{CCL5}}=5 \pm 0.8$ pixels; $\text{surface}_{\text{PSC-RANTES}}=6.25 \pm 0.6$ pixels) and slightly increased for PSC-RANTES-treated cells at 15 min ($\text{surface}_{\text{CCL5}}=4.7 \pm 0.6$ pixels; $\text{surface}_{\text{PSC-RANTES}}=7.5 \pm 0.6$ pixels). This indicates that the difference in the amount of β arr2-GFP spots detected after PSC-RANTES treatment compared to CCL5 is mainly due to an increase in the quantity of β arr2 recruited to the plasma membrane rather than to a difference in the size distribution of β arr2-GFP.

These results indicate that PSC-RANTES may trigger the formation of a larger number of β arr2-CCR5 complexes compared to CCL5. This may explain why after stimulation with CCL5 only a fraction of β arr2-Rluc molecules interacted with CCR5-YFP in saturation BRET experiments (see Fig. 3*A*). The increased recruitment of β arr2 to CCR5 after PSC-RANTES exposure likely contributed to the increased internalization of CCR5 (Fig. 1), compared to that observed after CCL5 stimulation.

PSC-RANTES stabilizes a pool of CCR5 that is not accessible to native chemokines- The data above are consistent with a model where PSC-RANTES can activate a larger pool of CCR5 than native chemokines. To explore the hypothesis that some CCR5 conformations would bind PSC-

RANTES but not native chemokines, we conducted competition experiments, in which increasing concentrations of unlabeled chemokines (CCL5, CCL3, PSC-RANTES) were used to inhibit the binding of biotinylated PSC-RANTES (PSC-RANTES-biot) to CCR5 (Fig. 4A). Whereas PSC-RANTES competed with PSC-RANTES-biot for binding to the receptor ($IC_{50} = 7 \pm 0.67$ nM), native chemokines, even at micromolar concentration could not (Fig. 4A). This result, showing that PSC-RANTES-biot prevents the binding of native chemokines to CCR5, contrasts with the competitive inhibition of 125 I-CCL3 binding by unlabeled chemokines shown Fig. 1B, and suggests that the receptors binding PSC-RANTES-biot and those binding 125 I-CCL3 represent two different receptor populations. We measured a slow dissociation rate of PSC-RANTES-biot from CCR5 (Fig. 4B), which could contribute to this result. Alternatively, but not exclusively, native chemokines might display marginal affinity for this particular fraction of CCR5, thereby explaining why they failed to displace PSC-RANTES-biot. Considered altogether, the results from Fig. 1B and 4A suggest that CCR5 exist in two distinct pools, one with high affinity for both PSC-RANTES and native chemokines, and another, the larger one, with high affinity for PSC-RANTES and minimal affinity for native chemokines.

If PSC-RANTES might stabilize an additional pool of CCR5, a significant fraction of receptors internalized in response to PSC-RANTES would not be accessible to native chemokines. This would explain why PSC-RANTES induces a stronger CCR5 down-regulation than native chemokines. To investigate this possibility, we tested both in HEK293 cells (Fig. 4C) and in A3.01 T cell line (Fig. 4D) whether a saturating concentration of native chemokines could prevent subsequent PSC-RANTES-induced internalization. Pre-incubating cells 90 min at 4°C with 100 nM CCL5 (or 300 nM CCL4) neither influenced the potency nor efficacy of PSC-RANTES to induce CCR5 internalization (Fig. 4, C and D), demonstrating that PSC-RANTES can induce the internalization of receptors that are not targeted by native chemokines.

The binding of PSC-RANTES to a pool of spare receptors was also supported by experiments of agonist-induced CCR5 desensitization. A first

stimulation with CCL5 [C] promoted a CCR5-dependent calcium release, whereas a second challenge with the same chemokine failed to induce any further response (Fig. 4, E and F - [CC] on graphs). In contrast, a second challenge by PSC-RANTES [P] could elicit a calcium response (Fig. 4, E and F-[CP]), suggesting that a proportion of CCR5, not desensitized by the first stimulation with CCL5, could still be activated by PSC-RANTES. In a parallel experiment, the initial stimulation by PSC-RANTES desensitized all receptors, since no further calcium signal could be elicited by challenge with PSC-RANTES or the native chemokine (Fig. 4, E and F-[PP; PC]). These data were obtained both at a single cell level (Fig. 4E) and in assays on cell populations (Fig. 4F). These results were confirmed in primary CD4⁺ T cells (Fig. 4G).

DISCUSSION

In this study, we investigated whether a pool of CCR5 that is not occupied by native chemokines could represent a target for inhibiting HIV-1 infection. Our results are consistent with the existence of spare forms of CCR5 on the cell surface, which bind PSC-RANTES with high-affinity but not native chemokines. These results support the concept that CCR5 exists in multiple conformations that are differentially stabilized by functionally different ligands. They are also relevant in light of our recent findings showing that HIV-1 can escape inhibition by native chemokines by exploiting low-chemokine affinity conformations of CCR5 (11). PSC-RANTES would bypass this limitation by targeting spare CCR5. This would lead to a larger number of β arr2/CCR5 complexes, stronger CCR5 down-regulation and potent HIV-1 inhibition compared to native chemokines.

Spare CCR5 receptors. GPCRs are dynamic proteins adopting an array of inactive and active conformations (6,7). Recent NMR experiments suggest that agonists turn the receptor into heterogeneous ligand-bound intermediate states, which are stabilized into fully active conformations once bound to downstream effectors (6). In this context, our BRET experiments (showing similar affinity of β arr2 for CCL5-bound and PSC-RANTES-bound CCR5) suggest that PSC-RANTES and native chemokines stabilize CCR5 into a similar intermediate

conformational state likely to interact with β arr2. Consequently, the higher amount of β arr2/CCR5 complexes formed by PSC-RANTES could result from a higher affinity of PSC-RANTES with this intermediate conformation compared to native chemokines. Alternatively, the higher amount of β arr2/CCR5 complexes could depend on the selection by PSC-RANTES of additional resting receptor states. Consistent with these two hypotheses, we found that native chemokines failed to prevent biotinylated PSC-RANTES binding to CCR5 (Fig. 4A), while they were as potent as unlabeled PSC-RANTES in displacing 125 I-CCL3 binding (Fig. 1B). This suggests that CCR5 may exist in at least two different conformational states, one with high-affinity for both PSC-RANTES and native chemokines, and the other, the larger one, binding preferentially the analog. We previously showed that CCR5 coupling to nucleotide-free forms of G-proteins (NF G-proteins) is necessary for high-affinity binding of native chemokines (11). It is thus possible that the pool of CCR5 that binds PSC-RANTES but not native chemokines corresponds to NF G-protein-uncoupled receptors, whereas the pool of CCR5 capable of binding both native chemokines and PSC-RANTES would correspond to NF G-protein-coupled receptors. In agreement with previous experiments showing that the recruitment of β arr2 to CCR5 and CCR5 endocytosis are independent of G-proteins (9,11), G-protein-uncoupled and PSC-RANTES-bound CCR5 would maintain the capacity of recruiting β -arrestins.

The above results imply that in the range of concentrations at which native chemokines and PSC-RANTES produced comparable effects on G-protein activation (Fig. 1), native chemokines occupy a smaller fraction of receptors than PSC-RANTES. In other words, this indicates that not all CCR5 are occupied by native chemokines to produce full G-protein activation. Such a property of an agonist is also observed in other receptor systems (34), where reserve receptors (also named spare receptors) exist and are not needed for agonists to evoke a maximal functional response. Here, we provide evidence indicating that a population of CCR5 is poorly accessible to native chemokines, while being targeted by PSC-RANTES, supporting the concept of spare CCR5. First, we observed that saturating concentrations of

native chemokines failed to prevent β -arrestin-dependent, PSC-RANTES-mediated CCR5 internalization (Fig. 4, C and D). Second, while PSC-RANTES fully desensitized further CCR5 activation mediated by native chemokines, the reverse situation was not observed (Fig. 4, E-G). This latter observation also suggests that a fraction of spare receptors, which are targeted by PSC-RANTES, would represent receptors capable of interacting with G-proteins. This result might appear at odds with the observation that PSC-RANTES and native chemokines activate G-protein dependent signals with comparable efficacy (Fig. 1). However, PSC-RANTES might stabilize intermediate CCR5 conformations likely to couple to G-proteins less efficiently than those induced by native chemokines. This suggests that PSC-RANTES-occupied CCR5 displays a bias toward β -arrestin recruitment over G-protein coupling compared to native chemokines. This hypothesis is in line with recent data showing that biased agonism, which results in differential activation of different signaling pathways, is a common property of chemokines (35).

Molecular determinants and mechanisms featuring PSC-RANTES functionality. The classical model for chemokine binding to CCR5 and receptor activation proposes that the chemokine core binds extracellular regions of the receptor while the N-terminal tail of the chemokine interacts with the transmembrane domains (36). The length and the amino-acid composition of the N-terminal tail of CCR5-binding chemokines affect their binding and signaling properties as well as their anti-HIV potency (37,38). Moreover, different classes of N-terminal-modified CCL5 analogs displaying improved anti-HIV properties have different pharmacological profiles (23), suggesting that they may stabilize different conformations of CCR5. Mutations in CCR5 transmembrane domains differentially impact the antiviral activities of these chemokine analogs including PSC-RANTES (39). Interestingly, these mutations did not alter the ability of the analogs to displace 125 I-CCL3 binding to the receptor (39). We can hypothesize that the mutations may change the relative proportions of receptors likely to establish high-affinity interactions with the analogs. In line with this hypothesis, recent computational docking approaches showed that mutations in the

transmembrane domains stabilize different sets of CCR5 conformations, each of which differs in their ability to bind different ligands and/or different effectors (40).

Overall, our results are consistent with the view that the robust CCR5 down-regulation following PSC-RANTES stimulation results from the stabilization by the N-terminal tail of PSC-RANTES of a higher amount of β arr2/CCR5 complexes but not from faster CCR5 internalization. Indeed, we observed similar kinetics of CCR5 internalization (Fig. 1E) and of β arr2 recruitment (Fig. 3B) regardless of the agonist used, in agreement with previous studies (11,17). We also showed different rates of CCR5 recycling after PSC-RANTES- and CCL5-induced endocytosis (Fig. 1G) (17). While the strength of β arr2/receptors interactions appears to control the extent of receptor recycling and the post-endocytic fate of receptors (41), our results showing similar β arr2 affinities for PSC-RANTES-bound and CCL5-bound CCR5 (Fig. 3A) do not support this hypothesis in the case of CCR5.

In contrast to wild-type (WT) CCR5, results on the recycling-defective mutant CCR5-349 show different rates of internalization depending on PSC-RANTES or CCL5 (Fig. 1F). CCL5 caused a slower rate of CCR5 internalization compared to PSC-RANTES. Note that all of the CCR5-349 will finally be removed from the cell surface, which is not the case of WT receptor, where a plateau was observed. This suggests that CCR5-349 internalization involves both high-chemokine affinity receptors and spare receptors (that are of high-affinity for PSC-RANTES but bind poorly native chemokines). In this context, the low affinity of CCL5 for spare CCR5-349 and/or a slow isomerization rate between low-CCL5 affinity and high-CCL5 affinity conformations of CCR5-349 may explain why the native chemokine induces a slower receptor internalization compared to PSC-RANTES. Conversely, similar kinetics of internalization of WT CCR5 by PSC-RANTES and CCL5 suggest that both ligands engage the same high-affinity population of WT CCR5.

Role of spare receptors in HIV-1 infection and inhibition. The observation that chemokines display high-affinity binding to CCR5 and anti-HIV-1 activity *in vitro* led to the proposal that they

act as a natural barrier against HIV-1 *in vivo*, but their role in HIV-1 transmission and progression of infection remains debated (42). CCR5 chemokines show antiviral potencies lower than would be expected based on their CCR5 binding affinity constants, indicating that HIV-1 could escape inhibition by chemokines (16,38,43). The existence of a set of CCR5, which are poorly accessible to chemokines, but could be exploited by HIV-1, might contribute to the low efficiency of CCR5 chemokines as a HIV-1 barrier and constitute a viral escape route. We previously demonstrated that different cell surface conformations of CCR5, which depend on whether the receptor interacts or not with heterotrimeric G-proteins, are differentially used by native CCR5 chemokines and HIV envelope glycoproteins (11). In particular, we revealed that G-protein uncoupled CCR5 represent low-chemokine affinity receptors, while constituting a portal for HIV-1 entry. Our current findings show that the pool of CCR5 with high-chemokine affinity probably represents only a minor fraction of the total receptor pool. This is in agreement with the small amount of nucleotide free forms of G-proteins present in a cell, which confer to the receptor a high-affinity for agonists (44).

Finally, our results are consistent with the fact that targeting spare receptors allows PSC-RANTES to form an increased amount of β arr2/CCR5 complexes, leading to a stronger down-regulation of CCR5 and potent HIV entry inhibition. Thus spare CCR5 might represent a pool of co-receptors capable of supporting HIV-1 entry. This is in line with previous studies showing that HIV-1 entry is a cooperative process that requires the recruitment of several CD4 and co-receptor molecules into a fusion complex (45). In this context, HIV-1 could use these spare receptors in addition to high-chemokine affinity receptors to enter into host cells more efficiently. Furthermore, targeting spare receptors would allow PSC-RANTES to bypass CCR5 populations with higher affinity for the viral envelope glycoprotein (11). The example of PSC-RANTES thus illustrates a new principle to inhibit HIV-1 infection and provides a basis for future strategies on the development of new inhibitors.

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Footnotes

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Author contributions

JJ, PC, IS, EL-F, CF, AD, SR, CR, NS, BL and AB performed experiments and analyzed data. OH, CR, MGHS, SM and NS contributed materials and technical support. MGHS, SM, NS, FAS, BL and AB

designed research and analyzed data. SM, FAS, BL and AB wrote the manuscript. All authors reviewed and edited the manuscript.

Figure legends

Figure 1: Functional properties of PSC-RANTES and native chemokines. *A*, Chemokine-mediated inhibition of fusion between Bx08Ren viruses containing BlaM-Vpr and CCF2-AM-loaded primary CD4⁺ T lymphocytes. *B*, Displacement of 0.5 nM ¹²⁵I-CCL3 binding to CCR5-expressing A3.01 cells by increasing concentrations of the indicated unlabeled chemokines. Insert -Displacement of increasing concentrations of ¹²⁵I-CCL3 binding to CCR5-expressing A3.01 cells by 1.5 nM CCL3 or PSC-RANTES. *C*, Chemokine-induced ³⁵S-GTPγS binding to CCR5-expressing HEK293T cell membranes. *D*, Calcium responses elicited in CCR5 Fura2-loaded HEK293 cells by indicated chemokines and measured by spectrofluorimetry (ratio: F₃₄₀/F₃₈₀). In panels *A* to *D*, data (mean±s.e.m of 2 or 3 independent experiments) were fitted according to a sigmoidal dose-response model with a variable slope. *E* and *F*, CCR5 internalization. Cell surface expression of FLAG-CCR5 (*E*) or FLAG-CCR5-349 (*F*) was monitored by flow cytometry in stable HEK293 cell clones after 50 nM chemokine stimulation for the indicated time. Percentage of total bound antibody was calculated from mean fluorescence intensity relative to untreated cells (mean±s.e.m from at least 2 independent experiments). *G*, CCR5 recycling. FLAG-CCR5 expressing HEK293 cells fed with anti-FLAG antibody were treated with 200 nM CCL5 or 10 nM PSC-RANTES to obtain 40% of CCR5 internalization for both agonists. Receptor recovery at the cell surface, in the presence of an excess of TAK779, was monitored by flow cytometry (mean±s.e.m, n=3). Panels *E* to *G*, *p<0.05; **p<0.01 compared to CCL5-treated cells in unpaired two-tailed student t test. *H*, Post-endocytic sorting of CCR5. Biotin degradation assay of FLAG-CCR5 in cells treated with CCL5 (100 nM) or PSC-RANTES (10 nM) for 1, 2 or 4 hours. Representative anti-FLAG blots out of 6 are shown. *I*, Quantification of blots from experiments performed in (*H*). The amount of cell surface receptors was quantified by densitometry and plotted relative to the amount of receptor detected in untreated cells (mean±s.e.m, n=6). The amounts of cell surface receptors were not statistically different (p=0.39 in unpaired two-tailed student t test) in the presence of CCL5 or PSC-RANTES.

Figure 2: PSC-RANTES internalization follows a clathrin- and β-arrestin-dependent pathway. *A*, Eps15 dominant negative mutant GFP-Δ95/295 inhibits CCR5 internalization. Cell surface expression of CCR5 in GFP-Δ95/295- or GFP- (control) transfected HEK293 cells was monitored after 30 min of 50 nM chemokine stimulation. GFP positive cells were analyzed by flow cytometry (mean±s.d of 2 independent experiments). *B-D*, β-arrestins1/2 are involved in CCR5 internalization. *B*, Western blot of total cellular levels of βarr1/2 in CCR5-349-expressing HEK293 cells transfected (72 h) with either nonsilencing siRNA (siCTL) or βarr1/2 siRNA (siβarr) (10 nM). Lysates were also blotted for LDH5 to test the specificity of knockdown. Representative blots out of 3 independent experiments are shown (NT, non transfected cells). *C* and *D*, Cell surface expression of CCR5-349 was monitored by flow cytometry after stimulation with 100 nM CCL5 (*C*) or PSC-RANTES (*D*) of siRNA treated cells for the indicated time. *E*, Rescue of siRNA-mediated β-arrestin knockdown. CCR5-349-expressing HEK293 cells were co-transfected, or not (no plasmid), with βarr1/2 siRNA (10 nM) and a plasmid encoding GFP (50 ng) or a siRNA-resistant βarr2-GFP construct (res-βarr2-GFP) (50 ng) and stimulated 20 min with 100 nM PSC-RANTES (n=4). *p <0.05, **p <0.01 compared to siRNA control in unpaired two-tailed student t test.

Figure 3: PSC-RANTES triggers a massive recruitment of β-arrestin2 to CCR5. *A*, Saturation BRET experiments in cells expressing βarr2-Rluc and increasing amounts of CCR5-YFP in basal conditions (untreated) or after 10 min chemokine (50 nM) stimulation (fitted according to a one site binding model, 4 independent experiments). *B*, Kinetics of BRET after chemokine (50 nM) stimulation (fitted according to a one phase association model). Values correspond to the mean±s.e.m of 4

independent experiments. *C* and *D*, TIRF microscopy on HEK293 cells stably expressing β arr2-GFP and FLAG-CCR5 obtained 5, 10 and 15 min after stimulation with 2 nM CCL5 or PSC-RANTES. *C*, β arr2 spots were detected on TIRF images. Scale bar: 5 μ m. *D*, Quantification of the spots over time using the ICY software (mean \pm s.d; n \geq 10 cells). **p<0.01, ***p<0.001 compared to CCL5-treated cells in unpaired two-tailed student t test.

Figure 4: PSC-RANTES stabilizes spare CCR5 conformations. *A*, Displacement of 1 nM PSC-RANTES-biot binding to CCR5-expressing A3.01 cells by increasing concentrations of the indicated chemokines. Data with unlabeled PSC-RANTES used as competitor were fitted according to one site competitive binding model (mean \pm s.e.m, n=4). *B*, PSC-RANTES-biot or 125 I-CCL3 dissociation from CCR5. CCR5-expressing A3.01 cells were incubated 90 min with 1 nM PSC-RANTES-biot or 120 min with 0.5 nM 125 I-CCL3. Dissociation was initiated by addition of 100 nM of the high-affinity CCR5 antagonist 5P12. PSC-RANTES-biot or 125 I-CCL3 remaining bound to CCR5 was measured over time and analyzed as in Fig. 4*A* and 1*B* (one representative experiment out of 2). *C* and *D*, Dose-dependent CCR5 internalization in CCR5-349-expressing HEK293 cells (*C*) or in CCR5-expressing A3.01 cells (*D*) mediated by 20 min (*C*) or 5 min (*D*) stimulation with PSC-RANTES at 37°C. *C*, Receptor internalization was measured after incubation, or not, of CCR5-349-expressing HEK293 cells with 100 nM CCL5 at 4°C for 90 min (data were fitted according to a sigmoidal dose-response model with a variable slope) (mean \pm s.e.m, n=4). *D*, Receptor internalization was measured after incubation, or not, of A3.01 cells with 300 nM CCL4 at 4°C for 90 min (data were fitted according to a sigmoidal dose-response model with a variable slope) (mean \pm s.e.m, n=3). *E-G*, Calcium responses measured in Fura2-loaded CCR5-expressing HEK293 cells (*E* and *F*) or primary T cells (*G*) stimulated twice (2 min intervals between the first and the second stimulation) with 15 nM chemokines. *E*, Results were obtained by imaging on cells coated on a glass coverslip. Each trace is the mean of at least 25 single-cell calcium responses. *F* and *G*, Results were obtained by spectrofluorimetry on the entire resuspended HEK293 cell population (*F*) (n=3 independent experiments) or primary T cell population (*G*) (one representative experiment out of 2 performed on 2 different donors). *p<0.05, **p<0.01, ***p<0.001 in unpaired two-tailed student t test. (C: CCL5, P: PSC-RANTES).

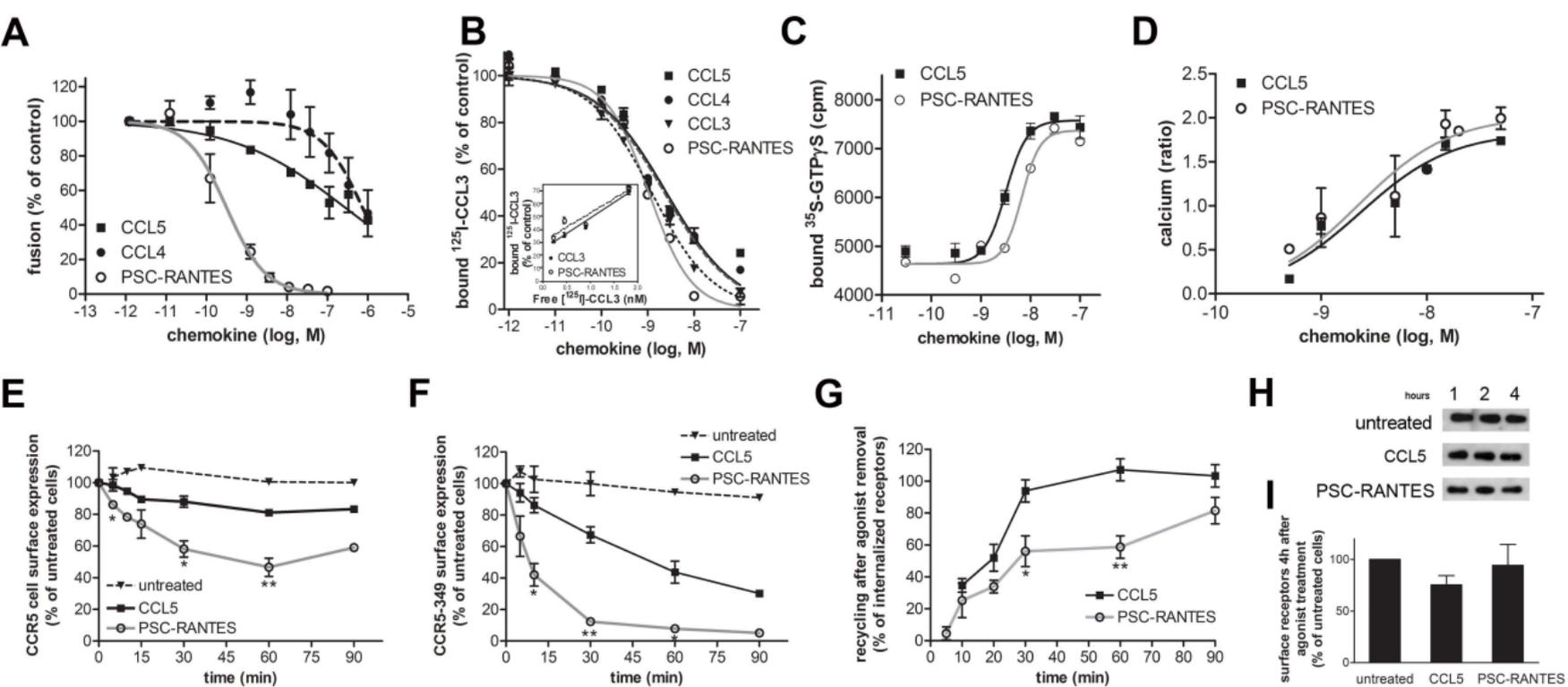
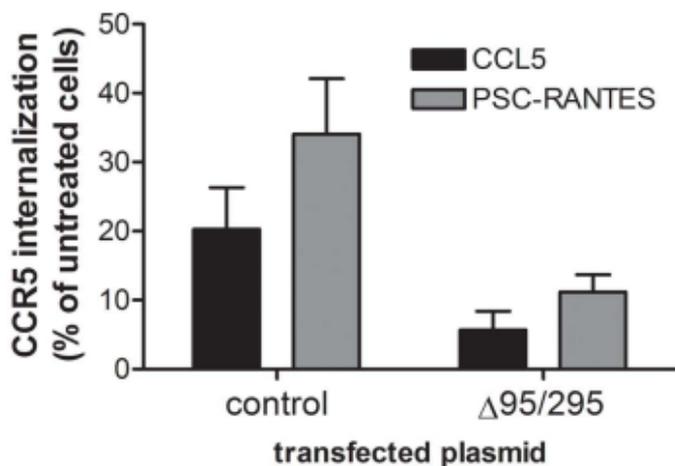
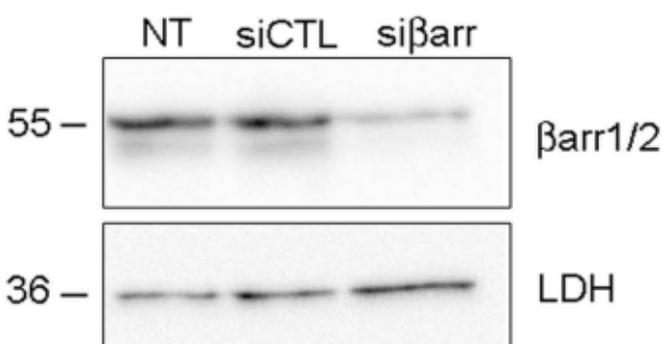


Figure 1

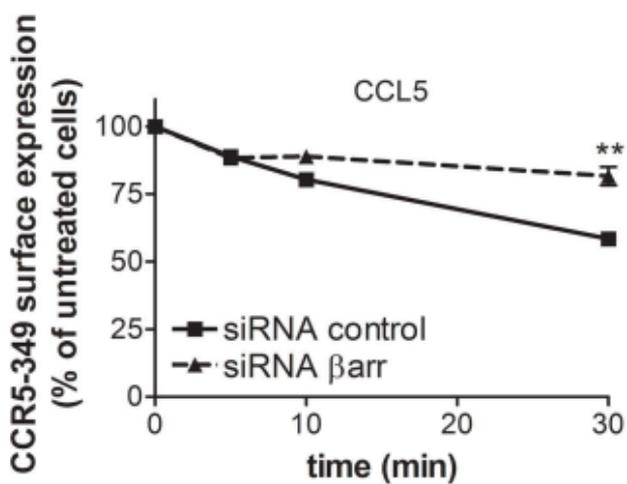
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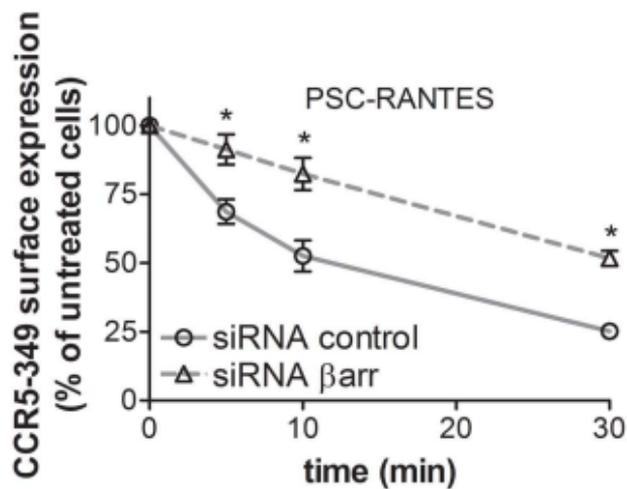
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C



D



E

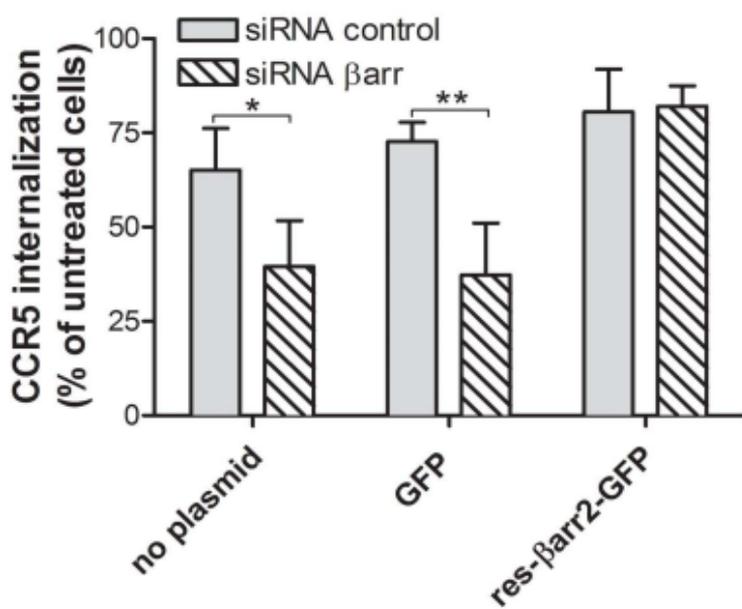
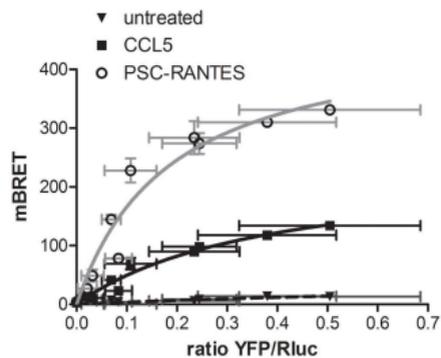
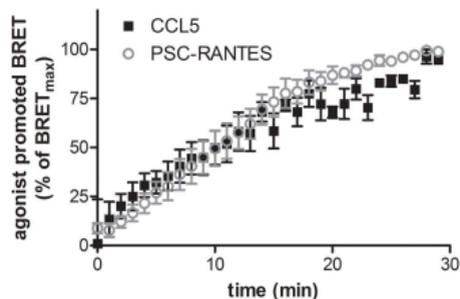


Figure 3

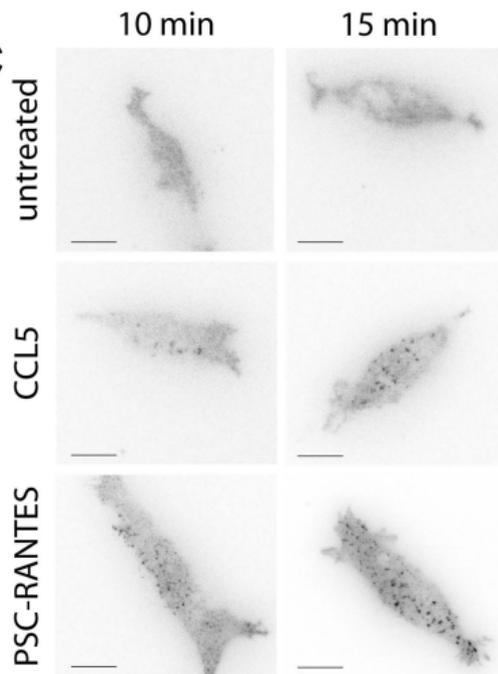
A



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C



D

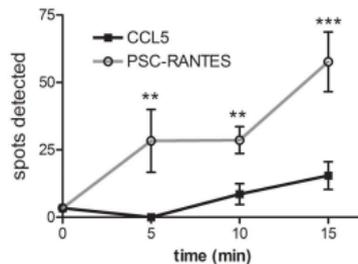


Figure 4