The EMBO Journal (2004) 23, 66–76 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
www.embojournal.org

THE EMBO JOURNAL

Blocking HIV-1 infection via CCR5 and CXCR4 receptors by acting in *trans* on the CCR2 chemokine receptor

José Miguel Rodríguez-Frade, Gustavo del Real, Antonio Serrano, Patricia Hernanz-Falcón, Silvia F Soriano, Antonio J Vila-Coro, Ana Martín de Ana, Pilar Lucas, Ignacio Prieto, Carlos Martínez-A and Mario Mellado*

Department of Immunology and Oncology, Centro Nacional de Biotecnología, UAM Campus de Cantoblanco, Madrid, Spain

The identification of chemokine receptors as HIV-1 coreceptors has focused research on developing strategies to prevent HIV-1 infection. We generated CCR2-01, a CCR2 receptor-specific monoclonal antibody that neither competes with the chemokine CCL2 for binding nor triggers signaling, but nonetheless blocks replication of monotropic (R5) and T-tropic (X4) HIV-1 strains. This effect is explained by the ability of CCR2-01 to induce oligomerization of CCR2 with the CCR5 or CXCR4 viral coreceptors. HIV-1 infection through CCR5 and CXCR4 receptors can thus be prevented in the absence of steric hindrance or receptor downregulation by acting in *trans* on a receptor that is rarely used by the virus to infect cells.

The EMBO Journal (2004) **23,** 66–76. doi:10.1038/sj.emboj.7600020; Published online 11 December 2003
Subject Categories: microbiology and pathogens; membranes and transport
Keywords: AIDS; chemokine; chemokine receptor; HIV-1

Introduction

The chemokines are a family of structurally related proinflammatory cytokines that control activation and chemotaxis in specific types of leukocytes, including monocytes, lymphocytes, natural killer (NK) cells, basophils, eosinophils and neutrophils (Mackay, 2001). They mediate their biological effects via interaction with a family of seven-transmembrane glycoprotein receptors coupled to a G-protein signaling pathway. These receptors consist of a single polypeptide chain with an extracellular amino-terminal domain and three extracellular loops that participate in receptor–ligand interactions, as well as a cytoplasmic carboxy-terminal domain and three intracellular loops that cooperate to bind and activate G proteins (Rossi and Zlotnik, 2000; Moser and Loetscher, 2001) and other signaling molecules.

In addition to binding chemokines, chemokine receptors are the primary receptors for the human immunodeficiency virus (HIV-1) (Littman, 1998; Garzino-Demo *et al*, 2000). The dichotomy in HIV-1 viral tropism, based on its ability to grow in transformed T cells or in peripheral blood mononuclear cells, was related to its use of the CXCR4 or CCR5 chemokine receptors (Alkhatib *et al*, 1996; Deng *et al*, 1996; Doranz *et al*, 1996; Berger *et al*, 1998). The identification of mutations that prevent or delay AIDS onset led to several important advances in understanding the role of chemokine receptors as HIV-1 receptors. Some of the best-studied mutations are a 32 bp deletion mutation in CCR5 that renders homozygous individuals highly resistant to viral infection (Samson *et al*, 1996; O'Brien and Moore, 2000), a single conservative amino-acid substitution (Val 64 to Ile) in the first transmembrane domain of the CCR2 receptor (Smith *et al*, 1997a, b), and a CCR5 promoter mutation (McDermott *et al*, 1998). The CCR5 polymorphism results in the absence of cell surface CCR5 expression, whereas the CCR2bV64I mutation confers resistance to AIDS progression, probably due to the ability of this mutant receptor to heterodimerize with the CCR5 and CXCR4 receptors (Mellado *et al*, 1999). Chemokine receptor homo- or heterodimerization has proven to be the critical starting point for chemokine signaling (Mellado *et al*, 2001a, b); oligomerization may also have a function in blocking HIV-1 infection (Vila-Coro *et al*, 2000). Since the substitution in the CCR2bV64I mutant permits heterodimerization with CCR5 or with CXCR4, we produced monoclonal antibodies (mAb) to CCR2, a chemokine receptor not generally used by HIV-1 to infect cells. We screened for mAb that induced receptor dimerization and identified CCR2-01, an mAb that best recognizes the receptor in the presence of ligands. By targeting this receptor with the CCR2-01 mAb, we induced oligomers between CCR2 and CCR5 or CXCR4 receptors, and blocked HIV-1 entry through these two receptors.

Results

CCR2-01 mAb does not interfere with chemokine responses

We produced a set of mAb to the CCR2 extracellular domains (Frade *et al*, 1997a), and further characterized one of these (CCR2-01). Wild-type HEK 293 cells constitutively express CXCR4; after transfection with CCR5 (CCR5 HEK 293) or CCR5 plus CCR2 (CCR2/CCR5 HEK 293), they also express the appropriate receptor(s), as determined by reverse transcriptase (RT)–PCR, western blot analyses and fluorescence-activated cell sorting (Figure 1A). Specific CCR2-01 mAb recognition of CCR2 was unaffected by an excess of receptor-bound CCR2 ligands (100 nM; CCL2, CCL7, CCL13) (Figure 1B, Supplementary materials A), indicating that CCR2-01 and the chemokines analyzed do not compete for binding. As a control for receptor-bound CCL2, CCR2-04 or

*Corresponding author. Department of Immunology and Oncology, Centro Nacional de Biotecnología/CSIC, UAM/Campus de Cantoblanco, E-28049 Madrid, Spain. Tel.: +34 91 585 4660; Fax: +34 91 372 0493; E-mail: mmellado@cnb.uam.es

Received: 5 June 2003; accepted: 5 November 2003; Published online: 11 December 2003

 ©2004 European Molecular Biology Organization

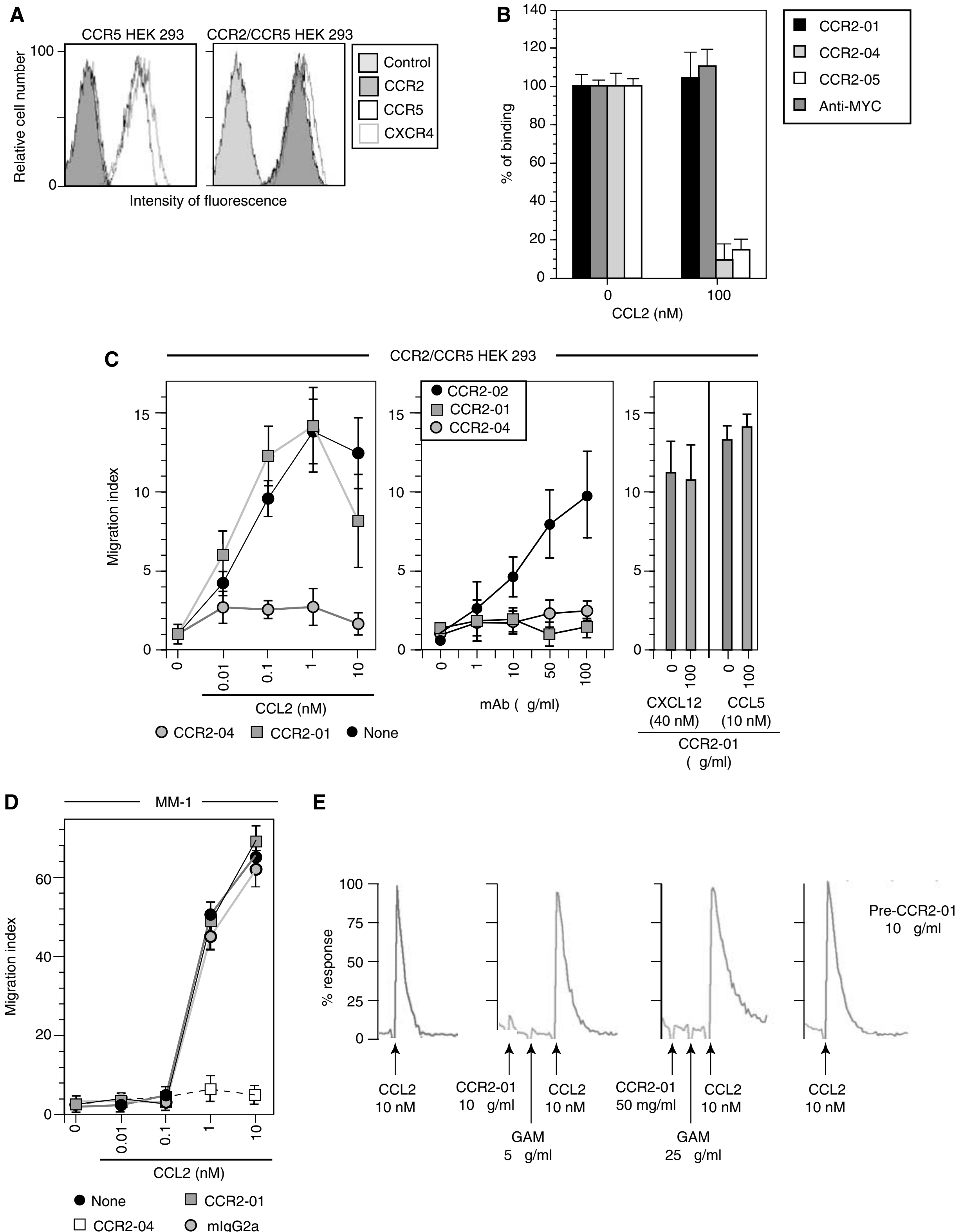

Figure 1 CCR2-01 mAb binds specifically to the human CCR2 receptor and does not compete with the CCL2 ligand. (**A**) CCR5 or CCR2/CCR5 HEK 293 cells were incubated with biotin-labeled CCR2-01, CCR5-03, CXCR4-01 mAb or their respective isotype-matched control mAb, followed by FITC-labeled streptavidin. (**B**) MycCCR2 HEK 293 cells alone or with a CCL2 excess (100 nM) were incubated at 4°C with biotin-mAb CCR2-01, CCR2-04, CCR2-05, or anti-Myc, followed by FITC-streptavidin. (**C**) Chemotaxis of CCR2/CCR5 HEK 293 cells was assessed using various CCL2, CCR2-01, CCR2-02 or CCR2-04 concentrations. To block CCL2-mediated chemotaxis, CCR2-01 or CCR2-04 (10 μg/ml) was added to the lower well simultaneously with the chemokines. Chemotaxis in response to CCL5 (10 nM) or CXCL12 (40 nM), and the effect of CCR2-01 (100 μg/ml) were assessed in a similar manner. Data represent the mean ± s.d. of triplicate determinations. (**D**) Chemotaxis of untreated and CCR2-01-, CCR2-04- or mIgG2a-treated Mono Mac 1 cells was assessed using the CCL2 concentrations indicated. Data represent the mean ± s.d. of duplicate determinations. (**E**) Ca^{2+} flux in untreated or CCR2-01-pretreated Mono Mac 1 cells was analyzed after sequential stimulation with CCR2-01 followed by goat anti-mouse Ig crosslinking and CCL2. The result is shown of one of three experiments. Results are expressed as a percentage of the maximum chemokine response.

CCR2-05 mAb staining was used to track the receptor; these two mAb are antagonists and cannot bind the receptor in the presence of CCL2 (Frade *et al*, 1997b). In immunofluorescence studies of CD14 antigen expression, CCR2-01 recognizes the CCR2 receptor expressed in all human monocytes. It also detects the CCR2 receptor in a significant fraction (40–70%) of activated peripheral blood B and T cells, and in a minor population of resting $CD4^+$ T cells (Frade *et al*, 1997b). To elucidate the CCR2-01 binding characteristics, CCR2/CD4 HEK 293 cells were analyzed in flow cytometry and displacement assays. CCR2-01 shows similar recognition of cell surface CCR2 (antibody binding capacity (ABC): 27,811±1220) compared to other anti-CCR2 mAb (ABC: 29,211±1870), as determined in flow cytometry. Scatchard analysis indicates no major affinity difference compared to other anti-CCR2 mAb (Kd in the range of 0.2–0.6 nM).

CCL2 induces Ca^{2+} mobilization and transmigration in several cell types, including monocytes. CCR2-01 mAb functional activity was studied in transmigration assays using collagen-coated filters in 96-well microchambers. CCR2/CCR5 HEK 293 cells migrated in response to CCL2, CXCL12, CCL5 and the agonist mAb CCR2-02, but not to CCR2-01 or CCR2-04 mAb (Figure 1C). This CCL2-induced migration was blocked in a dose-dependent manner following incubation with the antagonist CCR2-04, but not with CCR2-01 mAb (Figure 1C). CCR2-01 does not affect CXCL12- or CCL5-induced CCR2/CCR5 HEK 293 cell migration (Figure 1C), indicating that the mAb does not alter CXCR4 or CCR5 expression and/or function. These results were confirmed using Mono Mac1 cells, a human monocytic cell line that migrates in response to CCR2 ligands (CCL2, CCL7, CCL13), but not to CCR2-01. In addition, CCL2-, CCL7- and CCL13-induced migration was blocked by CCR2-04 but not by CCR2-01 (Figure 1D, Supplementary materials A). Similar results were obtained when Ca^{2+} flux was analyzed in Mono Mac1 cells. CCR2-01 alone or crosslinked with goat anti-mouse immunoglobulin (Ig) neither triggered a response nor altered the CCL2-induced response (Figure 1E). The results allow us to conclude that CCR2-01 does not block CCL2/CCR2 interaction, does not trigger CCR2 activation, and does not prevent ligand-mediated CCR2 activation.

CCR2-01 mAb blocks HIV-1 infection in the absence of receptor downregulation or signaling

The HIV-1 suppressive activity of CCL2 was described using human peripheral blood mononuclear cells (PBMC) as target cells (Doranz *et al*, 1996; Frade *et al*, 1997a), and we reported similar activity for the agonist anti-CCR2 mAb, CCR2-02 (Frade *et al*, 1997a). Like the CCR2-02 mAb, CCR2-01 showed suppressive activity against X4 HIV-1 NL4-3 (Figure 2A). These results were extended to the infection of monocyte-derived macrophages (MDM) by the HIV-1 monotropic R5 strain BaL (Figure 2B), as measured by RT activity. To establish the mechanism by which CCR2-01 blocks HIV-1 entry into the cell, cell–cell fusion experiments were performed using HEK 293 target cells transfected with CD4 alone (CD4 HEK 293) or together with CCR2 (CD4/CCR2 HEK 293), and BSC40 cells infected with recombinant vaccinia virus expressing env (IIIB) as effectors. In CD4 HEK 293 cells, CXCL12 inhibited gp120-mediated fusion, whereas no effect was observed with CCR2-01 (Figure 2C). Nonetheless, both CXCL12 and mAb CCR2-01 blocked fusion in CCR2/CD4 HEK 293 cells, as determined by luciferase activity (Figure 2C). No blockage was seen using mIgG2a or CCL2 (not shown). To discard possible toxic effects of CCR2-01 mAb, we tested non-chemokine-related receptor-mediated HIV-1 entry. CCR2-01- or mIgG2a-pretreated MT-2-transfected cells that express CCR2, CCR5, CXCR4 and CD4 receptors (not shown) were transduced with viral supernatants from a replication-defective NL4-3 virus pseudotyped with MLV envelope or NL4-3 envelope. CCR2-01 did not affect entry of the MLV envelope-pseudotyped virus, whereas it blocked entry of the virus pseudotyped with NL4-3 envelope (Figure 2D). Preincubation with CCR2-01 did not affect viral gp120 binding to its coreceptor at any concentration analyzed, as determined by flow cytometry (Supplementary materials B). These data indicate that the CCR2-01 mAb specifically inhibits HIV-1 entry via a CCR2-dependent mechanism that is unrelated to steric hindrance.

Several models might explain the CCR2-01 suppressive activity. The first is that CCR2-01 induces downregulation of CCR2, although neither Ca^{2+} influx nor chemotaxis was observed. Flow cytometry analysis showed that CCL2 promoted CCR2 internalization in Mono Mac 1 cells in a time-dependent manner, whereas CCR2-01 did not (Figure 3A). Both 10 nM CCL5 and 50 nM CXCL12 induced downregulation of CCR5 and CXCR4 receptors, respectively, in CCR2/CCR5 HEK 293 cells (not shown); CCR2-01 mAb treatment (10 μg/ml, 30 min, 37°C) did not affect expression of these receptors (Figure 3B), consistent with its lack of effect on CXCL12- and CCL5-induced migration (Figure 1C). The results indicate that CCR2-01 does not promote CCR2 downregulation, and confirm the unique features of this mAb.

An alternative is that CCR2-01 preferentially recognizes a CCR2 conformation associated with ligand binding. Flow cytometry analysis using CD4/CCR2 HEK 293 cells shows that CCR2-01 and CCR2 ligands (CCL2, CCL7, CCL13) bind simultaneously to the receptor, and that CCR2 ligands significantly increase CCR2-01 binding to the receptor (Figure 3C). A similar CCR2-01 binding increase was observed when these cells were preincubated with gp120 (Figure 3C); this indicates interaction between CD4 and chemokine receptors on the cell surface and confirms the unique characteristics of this mAb.

Chemokines induce chemokine receptor homodimerization, followed by rapid association and activation of JAK Tyr kinases (Mellado *et al*, 1998). As a consequence, the chemokine receptor is rapidly tyrosine phosphorylated and the G_i protein pathway is activated (Mellado *et al*, 2001a; Rodríguez-Frade *et al*, 2001). To determine whether CCR2-01-mediated HIV-1 inhibition is receptor activation-dependent, we analyzed whether CCR2-01 triggers any of these biochemical pathways. In contrast to CCL2, CCR2-01 induced neither JAK2 (Figure 3D) nor $G\alpha_i$ (Figure 3E) association to CCR2, and did not trigger tyrosine phosphorylation of JAK2 (Supplementary materials C) or CCR2 (Figure 3F).

CCR2-01 triggers CCR2 homo- and heterodimerization with CCR5 and CXCR4 receptors

We showed that CCL2 and the CCR2-02 agonist mAb, but not CCR2-02 Fab fragments, trigger CCR2 dimerization (Rodríguez-Frade *et al*, 1999). We now tested the ability of the CCR2-01 mAb to mediate this effect, using Mono Mac 1 cells expressing a functional CCR2 receptor (Mellado *et al*,

 ©2004 European Molecular Biology Organization

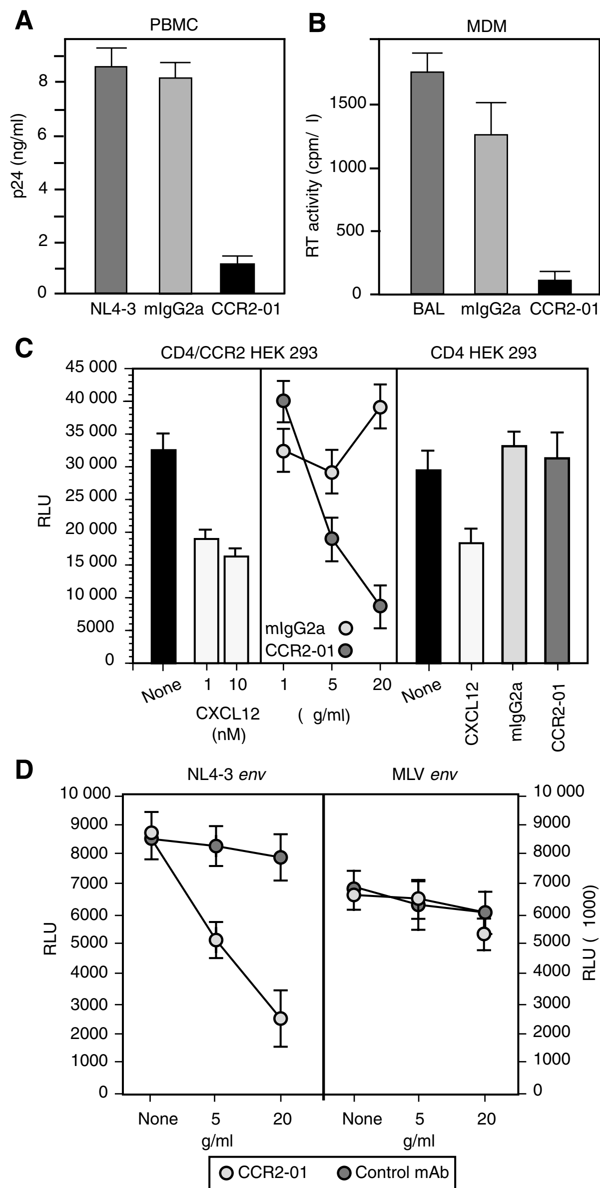

Figure 2 CCR2-01 mAb prevents HIV-1 replication in human PBMC and MDM cells. (**A**) CCR2-01 or mIgG2a mAb were tested for HIV-1 suppressive activity using the X4 NL4-3 viral strain and activated PBMC target cells. Untreated culture supernatants were used as control (NL4-3). Data represent the mean±s.d. of triplicate analyses. (**B**) The suppressive activity of CCR2-01 and mIgG2a mAb (2 μg/ml) was tested against the R5 HIV-1 BaL strain in MDM. Untreated culture supernatants were used as control (BaL). Data represent the mean±s.d. of triplicate determinations. (**C**) Luciferase reporter cell–cell fusion assay showing the inhibitory effect of 20 μg/ml CCR2-01. HEK 293 target cells containing the luciferase gene under the control of vaccinia virus promoter 7.5 were transfected with plasmids for CD4 and CCR2. CD4 HEK 293 cells were used as a control. As effectors, BSC40 cells were infected with a recombinant vaccinia virus expressing the env gene of HIV-1 strain IIIB. Cell fusion experiments included the use of CXCL12 as a positive control and mIgG2a as a negative control. Cell–cell fusion was assessed after mixing target and effector cells by quantitation of luciferase activity in relative light units (RLU). Data represent the mean±s.d. of triplicate determinations in a single experiment of three performed, with similar results. (**D**) CCR2-01 or mIgG2a mAb were tested for suppressive activity in single-round experiments using a replication-defective NL4-3 virus pseudotyped with MLV or NL4-3 envelopes. After 2 days, infected cells were determined as luciferase activity (RLU). Data represent the mean±s.d. of duplicate points.

1998). After stimulation with 10 nM CCL2 or 5 μg/ml CCR2-01, disuccinimidyl suberate (DSS) crosslinking and CCR2 immunoprecipitation, we detected a high molecular weight receptor species that corresponds to the predicted molecular weight of two CCR2 molecules (Figure 4A). In contrast, when Mono Mac 1 cells were stimulated with 5 μg/ml of the CCR2-05 mAb, this high molecular weight receptor species was not detected (Figure 4A). The CCR2-05 mAb reacts with the CCR2 third extracellular loop (amino acids 273–292) and blocks CCL2-induced responses including Ca^{2+} mobilization, chemotaxis and chemokine-mediated HIV-1 neutralization, with no intrinsic activity (Frade *et al*, 1997a, b). We show that both CCL2 and CCR2-01 trigger CCR2 receptor dimerization and prevent HIV-1 cell entry, suggesting that chemokine receptor dimerization is sufficient to prevent HIV-1 infection in the absence of any other signal.

The NL4-3 and BaL viral strains, whose infection is prevented by the CCR2-01 mAb, use CXCR4 and CCR5 receptors, respectively, neither of which is recognized by this antibody. We showed that after costimulation by their respective ligands, CCR5 but not CXCR4 heterodimerizes with CCR2 (Mellado *et al*, 1999, 2001b). To explain this HIV-1-blocking effect, we postulated that CCR2-01 triggers CCR2 oligomerization with CXCR4 and/or the CCR5 receptor. CCR2/CCR5 HEK 293 cells were stimulated with CCR2-01 or mIgG2a (5 μg/ml, 5 min, 37°C), then lysed and immunoprecipitated with anti-CCR2 mAb. CCR2-01 induced dimerization of CCR2 with CCR5, shown by the presence of CCR5 in CCR2 immunoprecipitates only when cells were stimulated with CCR2-01 (Figure 4B, top left). In the reverse experiment, CCR2 appeared in CCR5 immunoprecipitates only when cells were stimulated with CCR2-01 (Figure 4B, top right). As a control, membranes were reprobed with the immunoprecipitating antibody (Figure 4B, bottom).

We examined possible CCR2-01-induced heterodimerization of CCR2 and CXCR4. CCR2/CCR5 HEK 293 cells were stimulated with CCR2-01 or mIgG2a as above, then lysed and immunoprecipitated with anti-CCR2 antibodies. Western blot was developed using anti-CXCR4 (Figure 4C, left). CCR2-01 induces CCR2 dimerization with CXCR4, shown by the presence of CXCR4 in CCR2 immunoprecipitates only when cells were stimulated with CCR2-01 (Figure 4C, left). As a control, the membrane was reprobed with anti-CCR2 mAb (Figure 4C, right). Similar data were obtained using Mono Mac 1 cells expressing equivalent CCR2 and CXCR4 levels (not shown). To exclude that CCR2-01 immunoprecipitated CCR5 or CXCR4 receptors in the absence of CCR2, we analyzed CCR2-01 immunoprecipitates of CCR5 HEK 293 cells in western blot and showed that this mAb does not precipitate CXCR4 or CCR5 (Figure 4D, left). When the assay was performed in CCR2/CCR5 HEK 293 cells, CCR2-01 did not precipitate CXCR4, but precipitated a small but reproducible amount of CCR5 (Figure 4D, right). The effect promoted by CCR2-01 is CCR2-dependent; no crossreactivity was detected with other chemokine receptors.

CCR2-01-induced CCR2/CCR5 and CCR2/CXCR4 heterodimerization was confirmed using fluorescence resonance energy transfer (FRET) in CCR2/CCR5 HEK 293 cells. Unstimulated or CCL2 plus CCL5 (1 nM each)-stimulated cells were fixed prior to staining with anti-CCR2 (CCR2-03, $mIgG_1$) and anti-CCR5 (CCR5-03, mIgM) mAb, followed by Cy3-labeled anti-mouse IgM Fab (donor) or Cy2-anti-mouse

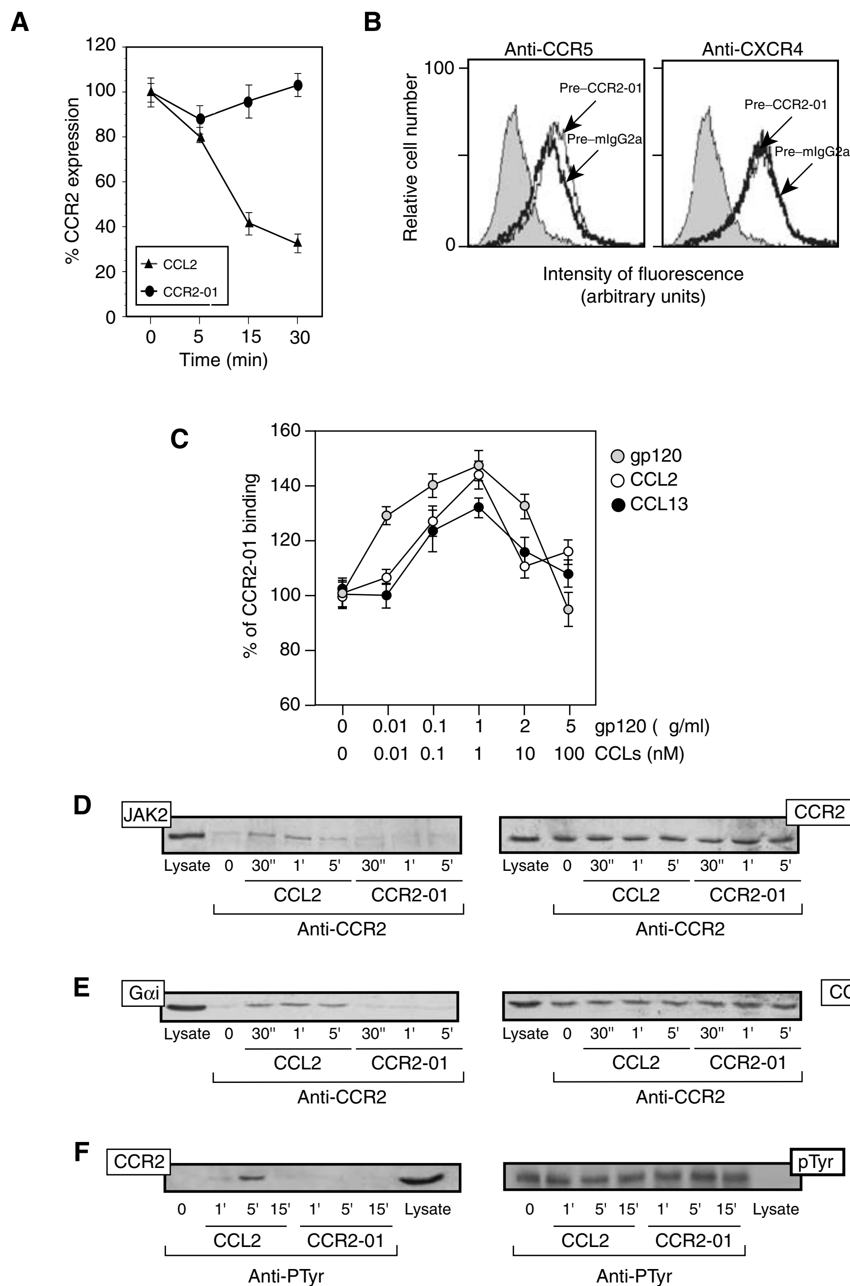

Figure 3 CCL2 but not mAb CCR2-01 triggers CCR2 receptor internalization and receptor signaling. (**A**) Time-course analysis of CCR2 internalization was assessed in serum-starved Mono Mac 1 cells incubated for the times indicated with CCL2 or CCR2-01. Surface CCR2 was detected in flow cytometry using biotin-labeled CCR2-03 mAb followed by FITC-streptavidin. Results are expressed as the mean percentage of maximum binding in the absence of stimulation, with s.d. indicated. (**B**) CCR2/CCR5 HEK 293 cells were preincubated with CCR2-01 or mIgG2a, followed by biotin-CCR5-03 or -CXCR4-01 mAb, and FITC-streptavidin. (**C**) CCR2/CD4 HEK 293 cells were preincubated with the indicated concentration of X4 gp120, CCL2 or CCL13, followed by incubation with CCR2-01 mAb and goat anti-mouse IgG. Binding was assessed by flow cytometry and expressed as a percentage of maximum CCR2-01 binding in the absence of chemokines or gp120. Data represent the mean$\pm$s.d. of triplicate determinations. (**D**) Serum-starved Mono Mac 1 cells were untreated or stimulated with CCL2 or CCR2-01. Lysates were precipitated with CCR2-03 mAb and the western blot developed with anti-JAK2 antibody. As a control, an unstimulated, unprecipitated Mono Mac 1 cell lysate was analyzed in western blot with anti-JAK2 antibody (left panel). Protein loading was controlled by reprobing membranes with CCR2-05. (**E**) CCL2- or CCR2-01-induced Mono Mac 1 cell lysates were immunoprecipitated with CCR2-03 mAb and the western blot developed with anti-Gα_i Ab. As a positive control, Mono Mac 1 lysates were tested in western blot with the same anti-Gα_i Ab (left panel). CCR2 protein loading was controlled by reprobing membranes with CCR2-05 (right panel). (**F**) Serum-starved Mono Mac 1 cells were untreated or stimulated with CCL2 or CCR2-01. Lysates were precipitated with anti-PTyr antibody and the western blot developed with CCR2-05. As a control, an unstimulated, unprecipitated Mono Mac 1 cell lysate was analyzed in western blot with CCR2-05 (left panel). Protein loading was controlled by reprobing membranes with anti-PTyr antibody (a 40 kDa tyrosine-phosphorylated protein is shown, right panel).

IgG Fab (acceptor) (Figure 5A, top panels). Staining was restricted to cell membrane receptors, as confirmed by the absence of intracellular receptor staining and Hoechst 33342 exclusion (not shown). FRET quantitation by enhanced donor emission after photobleaching (Bastiaens and Jovin, 1996) showed a basal energy level, indicating preformed heterodimers in the absence of ligands (Figure 5B). The G-protein-coupling receptors (GPCR) appeared to be in

©2004 European Molecular Biology Organization

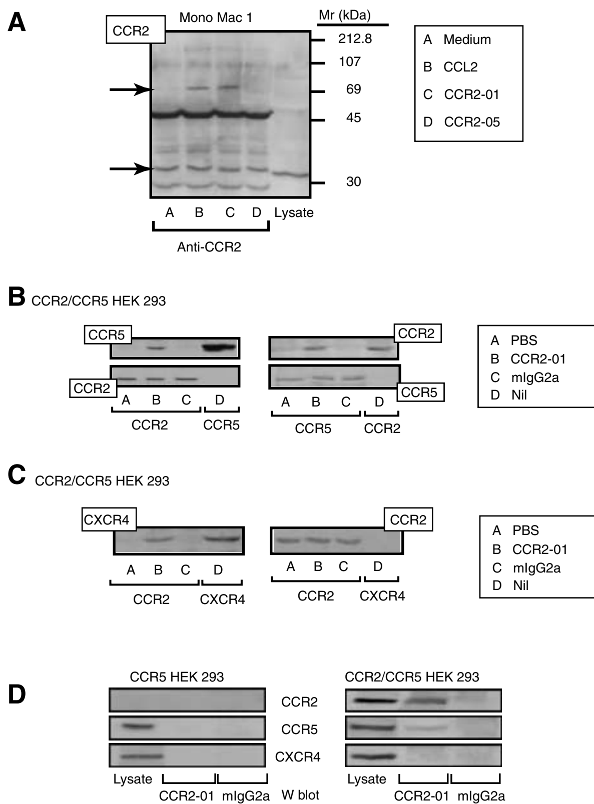

Figure 4 CCR2-01 mAb triggers CCR2/CCR2, CCR2/CCR5 and CCR2/CXCR4 receptor oligomerization. (**A**) Serum-starved Mono Mac 1 cells were stimulated (5 min) with CCL2, CCR2-01 or CCR2-05 mAb, and then DSS crosslinked. Cell lysates were immunoprecipitated with CCR2-03 and analyzed in western blot with CCR2-05. As a control, lysates from uncrosslinked Mono Mac 1 cells were immunoblotted with CCR2-05. Arrows indicate the receptor monomer and dimer. (**B**) CCR2/CCR5 HEK 293 cells were stimulated with CCR2-01 or mIgG2a (5 min, 37°C). Cell lysates were immunoprecipitated with CCR2-03 and analyzed in western blot with anti-CCR5 mAb; as a positive control, cell lysates were immunoprecipitated with anti-CCR5 mAb (top left). The membrane was reprobed with CCR2-05 as a protein loading control (bottom left). The reverse experiment was performed in the same cells, using anti-CCR5 mAb to immunoprecipitate stimulated cell lysates, and CCR2-05 (top right) or anti-CCR5 mAb (bottom right) for western blot analysis; as a positive control, cell lysates were immunoprecipitated with CCR2-03. (**C**) CCR2/CCR5 HEK 293 cells were processed as in (B); the western blot was analyzed with anti-CXCR4 mAb, using anti-CXCR4 mAb immunoprecipitates as a positive control (left). The blot was reprobed with CCR2-05 as a protein loading control (right). (**D**) Lysates from CCR2/CCR5 HEK 293 or CCR5 HEK 293 cells were immunoprecipitated with CCR2-01 or mIgG2a mAb and analyzed in western blot with CCR2-05 (top), CCR5-01 (middle) or CXCR4-01 mAb (bottom). Unprecipitated cell lysates were used as control.

monomer, dimer and heterodimer equilibrium (Angers *et al*, 2000), and the ligand may stabilize the active receptor conformation. Concurring with this model, FRET efficiency increased when cells were stimulated simultaneously with both chemokines, indicating heterodimer stabilization (Figure 5B).

We evaluated the CCR2/CXCR4 pair using the same approach, staining cells with CCR2-03 and CXCR4-01 (mIgM) mAb. Under these conditions, CXCR4 and CCR2 did not form heterodimers in basal conditions or after stimulation with 10 nM CXCL12 plus 1 nM CCL2 (Figure 5A, bottom panels). This confirms previous data (Mellado *et al*, 1999), demonstrates FRET analysis specificity, eliminates the possibility that the energy transfer detected was due to receptor clustering (Signoret *et al*, 2000), and emphasizes the functional relevance of chemokine receptor dimerization. We evaluated FRET after CCR2-01 stimulation (5 μg/ml) using CCR2/CCR5 HEK 293 cells and Cy2-CCR2-03, Cy3-CCR5-03 or Cy3-CXCR4-01 mAb. Concurring with the biochemical data, CCR2-01 stabilized the CCR2/CCR5 complex and triggered CCR2/CXCR4 oligomer formation (Figure 5B). Detailed cell-by-cell analysis of CCR2-01-induced FRET is shown in Figure 5C. We conclude that the CCR2-01 mAb induces CCR2 homodimers, stabilizes CCR2/CCR5 and CCR2/CXCR4 heterodimers, and that this process appears to be critical in the prevention of HIV-1 infection.

Discussion

Several models have been postulated by which chemokines inhibit HIV-1 infection (Doranz *et al*, 1997; Berger *et al*, 1999). The steric hindrance model sustains that chemokine binding to its receptor blocks interaction of the HIV-1-env/CD4 complex with the receptor (Doranz *et al*, 1997; Murakami *et al*, 1997; Schols *et al*, 1997). Another model attributes this effect to a signaling mechanism, and a third suggests that chemokines induce receptor desensitization and internalization, preventing the virus from interacting with and infecting the target cell (Mack *et al*, 1998). In accordance with our previous results, we show that HIV-1 infection can also be prevented in the absence of chemokine signaling, steric hindrance or receptor internalization. The fact that HIV-1 infection through CCR5 could be blocked by induction of receptor dimerization (Vila-Coro *et al*, 2000), and that CCR2 can form heterodimers with CCR5 points to CCR2 as a potential target of HIV-blocking compounds. The anti-CCR5-02 mAb (Vila-Coro *et al*, 2000) blocks infection by induction of CCR5 homodimerization, and is thus R5 HIV-1 strain-specific. In contrast, data presented here indicate that the CCR2-01 mAb blocks HIV-1 by interacting with receptors different from those used during infection, probably by specific CXCR4 and CCR5 recruitment to the CCR2. This leads to the formation of receptor heterodimers or clusters that disable viral penetration of the cells. Since CCR2-01 does not recognize any receptor other than CCR2, this mAb must block virus–cell fusion through a CCR2-dependent mechanism.

The dimerization process described here is triggered via a receptor that is not used by either of the viral strains tested to infect the cells. The capacity of the CCR2bV64I mutant receptor to delay AIDS progression may be associated with its ability to heterodimerize with both CCR5 and CXCR4 (Mellado *et al*, 1999). We thus propose that chemokine receptor oligomerization might be a mechanism through which chemokines prevent HIV-1 infection in the absence of receptor internalization.

Receptor occupancy modifies the signaling properties and the conformational state of the receptor (Chidiac *et al*, 1996). A number of studies of agonist–receptor protein interactions have attempted to distinguish between active and inactive receptor conformations (Kenakin, 1997; Mellado *et al*, 1997; Blanpain *et al*, 2002). Evidence suggests that, after binding to GPCR, agonists induce an active state or select a receptor-activated state that leads to signaling. Our FRET analysis data show chemokine receptor dimerization, and

©2004 European Molecular Biology Organization

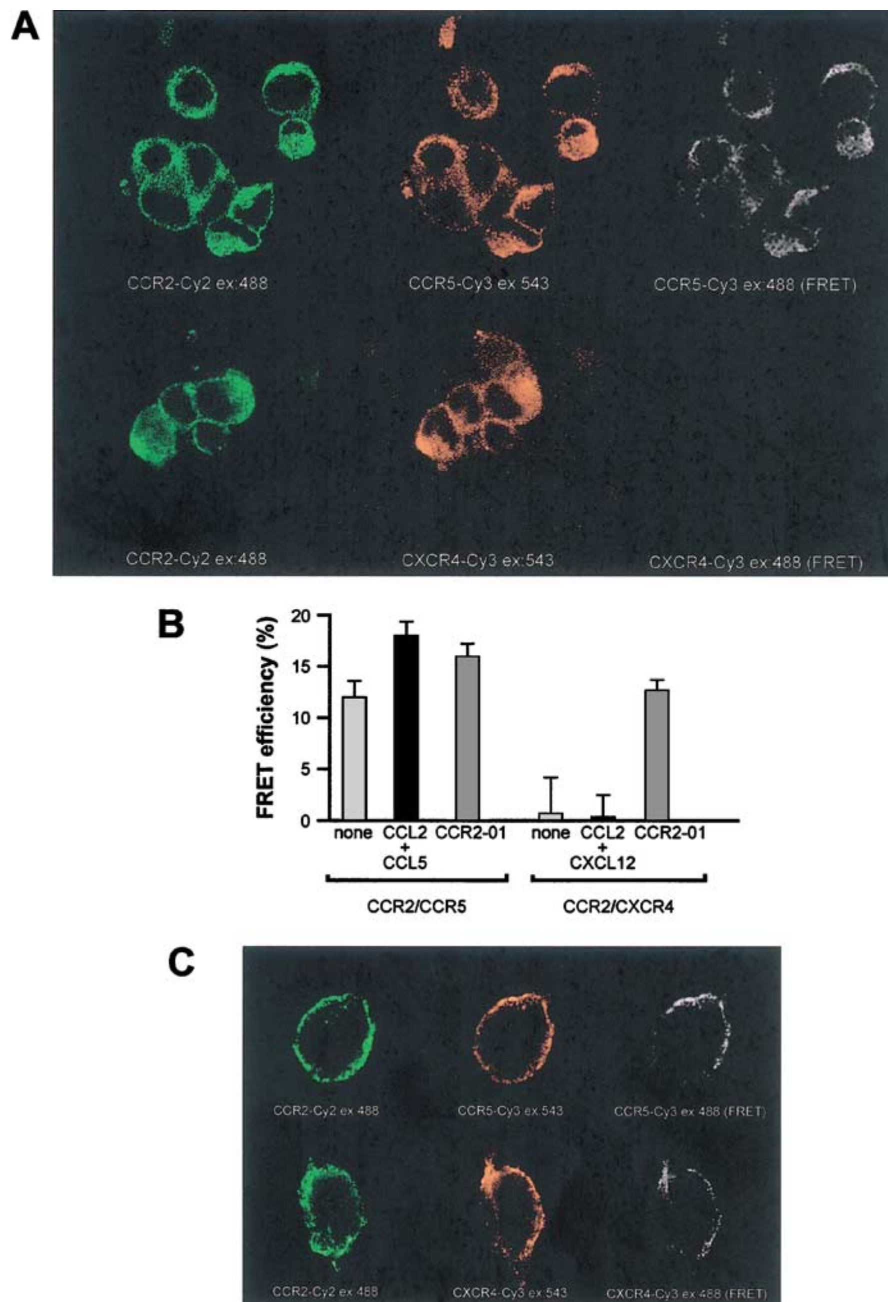

Figure 5 FRET analysis of ligand-induced CCR2/CCR5 and CCR2/CXCR4 receptor heterodimerization. (**A**) Unstimulated CCR2/CCR5 HEK 293 cells were fixed and stained with anti-CCR2, -CCR5 or -CXCR4 mAb followed by Cy2-mIgGFab (excitation at 488 nm) or Cy3-mIgMFab (excitation at 543 nm), as indicated. FRET images were visualized by Cy3 (acceptor) emission due to Cy2 (donor). (**B**) CCR2/CCR5 HEK 293 cells, unstimulated or stimulated with CCL2 plus CCL5, CCL2 plus CXCL12, or CCR2-01, were fixed, stained and energy transfer efficiency was calculated as described (Materials and methods). (**C**) CCR2-01 (5 μg/ml)-stimulated CCR2/CCR5 HEK 293 cells were treated as in (A) and stained with Cy2-labeled CCR2-03 and Cy3-CCR5-03 or Cy3-CXCR4-01. FRET images were visualized by Cy3 (acceptor) emission due to Cy2 (donor).

that it is a very specific phenomenon. Dimers are also detected in the absence of ligands, however, indicating that the role of the ligand is not to form the complex, but to stabilize the active conformation. These results concur with a model previously defined for other GPCR (Angers *et al*, 2000), in which the chemokine receptors appear to be in an equilibrium of monomers, dimers and heterodimers, and the ligands may stabilize the appropriate receptor conformation. The existence of ligand association-independent CCR5 oligomers was recently reported in studies using transfected cells and bioluminescence resonance energy transfer (BRET) technology (Issafras *et al*, 2002).

The CCR2-01 effect may be related to chemokine receptor localization in lipid rafts. The importance of these membrane microdomains for HIV-1 entry and for modulation of ligand activity was recently described (Mañes *et al*, 2000). Although further experiments are needed to define the specific mechanism, our data suggest that CCR2 and CCR5 coexist in the same raft domain and that CCR2-01 stabilizes their interaction. This concurs with the observation that CCR2-01 precipitates small

 ©2004 European Molecular Biology Organization

amounts of CCR5 only when cells coexpress both receptors. Increased CCR2-01 recognition of CCR2 after stimulation with either chemokines or gp120 raises interesting possibilities as to the mechanism by which this antibody blocks HIV-1 infection. HIV-1 chemokine coreceptors and CD4 localize to the same raft domains (Mañes *et al*, 2000); their physical association has been established even in the absence of ligand binding (Dimitrov *et al*, 1999; Xiao *et al*, 1999), which may explain this differential recognition. As lipid rafts act as platforms for efficient signaling (Fragoso *et al*, 2003), the chemokines, gp120 or anti-receptor antibodies may alter the presence and/or function of receptors in the raft domain.

Selective ligand-induced stabilization of one of the diverse receptor conformations present on the cell membrane leads to differential receptor availability for participation in biological responses. As shown, CCR2-01 recognizes a CCR2 conformation different from that detected in the unbound receptor, which appears to be associated with the binding of ligands that trigger dimerization. Stabilization of distinct CCR5 conformations was recently described using anti-CCR5 mAb (Blanpain *et al*, 2002).

The potentially immunosuppressive effect of chemokines raises concern about their possible therapeutic use to block HIV-1 infection. The search for specific agents that impede HIV-1 infection but do not interfere with chemokine physiology has yielded low molecular weight compounds that block T-tropic strains by preventing gp120 interaction with CXCR4 (Baggiolini and Moser, 1997). This and the results described here indicate that a tool such as the CCR2-01 mAb, which does not compete with chemokine binding but suppresses HIV-1 infection through the two main receptors, may be useful for exploring new approaches in AIDS prevention and treatment without inducing undesired inflammatory side effects (Ward *et al*, 1998).

Materials and methods

Cell culture, transfection, antibodies and flow cytometry analysis

Mono Mac 1 (DSM ACC252) and HEK 293 cells (ATCC TIB202) were from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and the American Type Culture Collection (Manassas, VA), respectively. MT-2-transfected cells were from Dr J Alcamí (Instituto Salud Carlos III, Madrid, Spain). CCR2-tagged, CCR2 and CCR5 receptors were prepared, expressed and tested as described (Rodríguez-Frade *et al*, 1999; Mellado *et al*, 2001b). CCL2, -5, -7, -13 and CXCL12 were from Peprotech (London, UK). Antibodies included mAb CCR2-01, -02, -03, -04, -05, CCR5-03 and CXCR4-01 generated in our laboratory (Frade *et al*, 1997b; Mellado *et al*, 1999; Vila-Coro *et al*, 2000), anti-β_2-microglobulin (Pharmingen, San Diego, CA), anti-Myc (clone 9E10, Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-PTyr (Promega, Madison, WI), anti-JAK2 (Upstate Biotechnology, Lake Placid, NY) and anti-$G\alpha_i$ antibody (Calbiochem, La Jolla, CA).

For flow cytometry analysis, cells were preincubated (20 min, 4°C) with different concentrations (0.1–100 nM) of the chemokines indicated, followed by CCR2-01, CCR2-04 or control mIgG2a mAb and PE-labeled goat anti-mouse IgG antibody (Southern Biotechnologies, Birmingham, AL). Binding of biotin-labeled CCL2 was measured using Fluorokine (R&D Systems, Minneapolis, MN). Viral gp120 interaction was analyzed by incubation with various gp120 concentrations (0.01–5 μg/ml; Intracell, London, UK), followed by rabbit anti-gp120 and FITC-anti-rabbit antibody (Southern Biotechnologies) in the presence of the mAb indicated.

Scatchard plot analysis was performed by competitive inhibition of ^{125}I mAb binding to native CCR2 expressed on CD4/CCR2 HEK 293 cells with various concentrations of unlabeled mAb, as described (Chuntharapai *et al*, 1994).

Immunoprecipitation, SDS–PAGE and western blot analysis

Cells (10^7 cells/ml) were stimulated as indicated with CCL2 (10 nM), CCR2-01 mAb (5 μg/ml), CCR2-05 mAb (5 μg/ml) or mIgG2a (5 μg/ml), after which the cell pellet was resuspended and processed as described (Rodríguez-Frade *et al*, 1999). In all cases, protein loading was controlled using a protein detection kit (Pierce, Rockford, IL) and, when necessary, by reprobing the membrane with the appropriate immunoprecipitating antibody.

Receptor crosslinking assays

Serum-starved Mono Mac 1 cells were unstimulated or stimulated (5 min, 37°C) with CCL2 (10 nM), CCR2-01 or CCR2-05 mAb (5 μg/ml) as indicated (Rodríguez-Frade *et al*, 1999). After washing twice with cold PBS, 10 μl of 100 mM DSS (Pierce) was added (10 min, 4°C, with continuous rocking). Cell lysates were immunoprecipitated as indicated (Mellado *et al*, 1998) using anti-CCR2 or anti-CCR5 mAb, electrophoresed and transferred to nitrocellulose membranes as above. The western blot was analyzed with anti-CCR2, anti-CCR5 or anti-CXCR4 antibodies.

Chemotaxis

Migration of CCR2/CCR5 HEK 293 cells was studied in a 96-well microchamber (NeuroProbe, Gaithersburg, MD). Chemokines (CCL2, CCL5, CXCL12) and antibodies (CCR2-01, CCR2-04, CCR2-02) were added to the lower well in RPMI containing 0.25% BSA; cells, untreated or preincubated with mAb CCR2-04 and CCR2-01 (10 μg/ml, 60 min, 37°C), were loaded in the upper chamber. Polyvinylpyrrolidone-free 10-μm-pore filters (NeuroProbe), precoated (20 μg/ml, 2 h, 37°C) with type IV collagen (Sigma, St Louis, MO), were used. The chamber was incubated (4 h, 37°C, 5% CO_2), after which filters were removed and cells wiped off the upper filter surface; filters were then fixed and stained (0.5% crystal violet, 20% methanol). Migration was quantified by densitometry of stained cells and expressed as a migration index (Rodríguez-Frade *et al*, 1999).

Mono Mac 1 cells (0.25×10^6 cells in 0.1 ml), untreated or preincubated with CCR2-04 or CCR2-01 mAb (10 μg/ml, 30 min, 37°C), were placed in the upper well of 24-well transmigration chambers (5 μm pore; Transwell; Costar, Cambridge, MA). CCL2, CCL7, CCL13, CCR2-01 or CCR2-04 (in 0.6 ml RPMI, 0.25% BSA) was then added to the lower well. Plates were incubated (120 min, 37°C), and cells that migrated to the lower chamber were counted as described (Frade *et al*, 1997a). Cell migration was calculated as the *x*-fold migration increase compared to medium controls.

Calcium determination

Mono Mac 1 cells (2.5×10^6 cells/ml), untreated or CCR2-01 treated (10 μg/ml, 30 min, 37°C), were suspended in RPMI 1640 medium containing 10% FCS and 10 mM HEPES, and incubated with Fluo-3AM (Calbiochem; 300 μM in DMSO, 10 μl/10^6 cells, 30 min, 37°C). Cells were washed, resuspended in RPMI 1640 containing 2 mM $CaCl_2$, and maintained at 4°C before adding CCL2 or CCR2-01 followed by goat anti-mouse Ig. Ca^{2+} flux was measured at 525 nm in an EPICS XL flow cytometer (Coulter). Fluo-3AM loading was controlled using ionomycin (5 μg/ml).

HIV-1 infectivity of PBMC: p24 determination

Human PBMC were activated with PHA (10 μg/ml, 48 h, 37°C, 5% CO_2). After washing, cells were preincubated for 1 h with mAb and then infected with HIV-1 NL4-3 strain (2 ng p24/10^6 cells per assay, 37°C, 2 h). Cells were washed extensively with PBS and cultured in complete RPMI 1640 medium containing rhIL-2 (10 ng/ml) alone or with CCR2-01 or mIgG2a mAb (10 μg/ml). Every 2 days after infection, half the culture supernatant was removed and replaced with fresh medium containing IL-2 and antibodies at initial concentrations (Frade *et al*, 1997a). Viral replication was monitored in cell-free supernatants by quantitating gag p24 antigen levels (ng/ml) at day 7 postinfection using a commercial ELISA test (Coulter).

Infection of human monocyte-derived macrophages and HIV-RT assay

Monocytes were isolated from buffy coats of healthy HIV seronegative blood donors by Ficoll–Hypaque and Percoll gradients (Pharmacia, Uppsala, Sweden). Purity was ⩾90%, as determined

©2004 European Molecular Biology Organization

by FACS analysis for CD14 expression using a mouse anti-human CD14 mAb (IgG2a; Caltag, Burlingame, CA) followed by FITC-goat anti-mouse Ig mAb (Jackson ImmunoResearch, West Grove, PA). Monocytes were seeded (2×10^5 cells/ml/well) in 48-well plates in DMEM supplemented with 10% FCS and 5% pooled human serum. As control, 5-day-old MDM were preincubated for 2 h with CCR2-01 or murine IgG at two concentrations (2 μg/ml and 200 ng/ml), and then infected with the R5 HIV-1 BaL strain at a 0.1 multiplicity of infection (m.o.i.). After overnight incubation, the medium was removed and fresh antibody-containing medium was added. At days 5 and 9 postinfection, half the medium was removed and replenished with fresh medium plus antibody. Half the culture medium was then changed twice weekly with no further antibody addition. Viral replication was monitored at day 25 postinfection by RT activity as described (Mengozzi *et al*, 1999) and expressed as cpm/μl.

Gene reporter assay

To quantitate cell–cell fusion events, we used a gene reporter fusion assay adapted in our laboratory. pSCluc plasmid (Rodríguez *et al*, 1988), harboring the luciferase gene under the control of the vaccinia virus 7.5 promoter, and the coreceptor construct pcDNA3.1-CCR2 were stably introduced into target HEK 293 cells expressing CD4. Transfection was performed in 48-well plates with 2 μg of each plasmid using Lipofectamine (Gibco-BRL, Gaithersburg, MD). Transfected cells (2×10^4) were incubated (37°C, 24 h). Env (IIIB) (Rodríguez *et al*, 1989) protein was introduced into effector African green monkey kidney BSC40 cells by infection with recombinant vaccinia virus (VV-env-1) (1 h, m.o.i. of 5). At 24 h postinfection, 10^5 effector cells cultured in rifampicin (100 μg/ml) were trypsinized and added to each well. Fusion was allowed to take place (37°C, 6 h) before lysis in 100 μl luciferase buffer (25 mM Tris-phosphate (pH 7.8), 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 8 mM $MgCl_2$, 15% glycerol). Luciferase activity was measured in a luminometer (Berthold, Wildbad, Germany). For fusion blocking, CXCL12 (10 nM) or antibodies (20 μg/ml) were added to target cells at the indicated concentrations 30 min before effector cell addition, and maintained in the cultures for the fusion period.

Single-round assays

For single-round infections, pNL4-3.Luc.R-E- (a gift of N Landau, AIDS Research and Reference Reagent Program, NIAID/NIH, Bethesda, MD) was pseudotyped with HIV-1NL4-3 env and MLVenv as described (Mengozzi *et al*, 1999). MT-2-transfected cells were transduced with viral supernatants (1 and 0.1 m.o.i.; 2 h, 37°C) and infectivity was determined after 48 h in a luminometer. For entry suppression, antibodies (20 and 5 μg/ml) were added to target cells 30 min before the addition of viral supernatants, and maintained in the cultures for the infection period.

Receptor downregulation

Serum-starved Mono Mac 1 cells (1×10^6) in 600 μl RPMI containing 0.25% BSA were stimulated with 10 nM CCL2 or 5 μg/ml CCR2-01 (30 min, 37°C, 5% CO_2) and analyzed by flow cytometry using biotin-labeled CCR2-03 mAb, followed by FITC-streptavidin. Surface expression of CCR5 and CXCR4 was analyzed by flow cytometry in CCR2/CCR5 HEK 293 cells treated with CCR2-01 mAb or mIgG2a (10 μg/ml; 30 min, 37°C) using biotin-anti-CCR5 or -anti-CXCR4 mAb followed by FITC-streptavidin.

FRET detection and analysis

Cells, unstimulated or stimulated with chemokines (5 min, 37°C), washed with cold PBS (4°C) and fixed with cold 4% PFA in PBS (5 min, 4°C), were stained with 10 ng/ml of CCR2-03 (IgG), CCR5-03 (IgM) and/or CXCR4-01 mAb (IgM). Staining was developed with Cy2-goat anti-mouse IgG Fab fragments and Cy3-goat anti-mouse IgM Fab (both from Jackson Immunoresearch). For DNA visualization, antibody staining was followed by cell permeabilization using 0.1% Triton X-100 in PBS; cells were then stained with Hoechst 33342 and excited using a multiphoton laser (760 nm; HQ390/70 emission filter). For quantitation studies, cells were treated with CCL2 (1 nM) plus CCL5 (1 nM) or CCL2 (1 nM) plus CXCL12 (10 nM), and then stained as above. Alternatively, CCR2-01 mAb (5 μg/ml; 5 min, 37°C)-stimulated cells were stained directly with Cy2-CCR2-03 and Cy3-CCR5-03 or Cy3-CXCR4-01. mAb were labeled using FluoroLink Cy2 and FluoroLink Cy3 (Amersham Biosciences) according to the manufacturer's protocols.

FRET was analyzed by sensitized acceptor fluorescence (SAF) and quantitated by increase of donor fluorescence after acceptor photobleaching (IDF). Images were captured with the Olympus ×60 Plan Apo objective of a confocal BioRad Radiance 2000 MP microscope with four lasers, Ar (488 nm), He–Ne (543 nm), red laser diode (637 nm) and infrared multiphoton (Mira 690–1000 nm), mounted on an Olympus IX70.

For FRET-SAF, Cy2 fluorescence (donor) was captured with an HQ515/30 emission filter after laser excitation of the sample (488 nm, 0.4 mW). Cy3 fluorescence (acceptor) was captured after excitation (488 nm, 0.4 mW) with an HQ 600/50 emission filter (dichroic mirror DCLPXR 560). Cy3 reference images were captured by exciting the sample (543 nm, 0.4 mW) with dichroic mirror DCLPXR 560 and an HQ 600/50 emission filter. Negative control images and those labeled with a single fluorochrome (Cy2 or Cy3) were acquired to define background and bleed-through (Gordon *et al*, 1998; Sorkin *et al*, 2000).

For quantification, six images were captured for each condition by IDF, using more than 20 cells each time. The reference fluorescence donor (Cy2) was captured by exciting the sample (488 nm, 0.4 mW) with an HQ515/30 emission filter; the Cy3 reference image was then captured using a DCLPXR 560 dichroic mirror and an HQ 600/50 emission filter after sample excitation (543 nm, 0.4 mW).

After reference image capture, the acceptor fluorophore was photobleached by scanning (6 min, 543 nm, maximum power). A second donor image was obtained under the same conditions (laser power, photomultiplier gain and optical section) used in the first capture. FRET efficiency was calculated by the increase in donor fluorescence intensity on a pixel-by-pixel basis (Wouters *et al*, 1998). A constant background value was subtracted from each image. A correction factor was calculated for indirect donor photobleaching during acceptor photobleaching using an image labeled only with donor fluorochrome, acquired and processed in parallel (Kenworthy, 2001). The correction factor was validated for each image by comparing donor pre- and postbleaching images in areas showing donor staining and no acceptor staining (Cy3 reference image). The FRET coefficient was represented as the percentage of $[D2-D1]/D2$, where D2 is postbleaching fluorescence and D1 is prebleaching fluorescence. Software Laserpix 4.0 (BioRad, Hemel Hempstead, UK) and Image Tool (Department of Dental Diagnostic Science, University of Texas, San Antonio, TX) were used to process and analyze images.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We are indebted to Dr J Alcamí for MT-2-transfected cells, Drs M Mengozzi and G Poli for help with HIV-RT assays and helpful comments, MC Moreno-Ortíz for flow cytometry analysis, A Checa for help with confocal microscopy, and C Bastos and C Mark for secretarial and editorial assistance, respectively. This work was supported by grants from Spanish CICyT, FEDER, Spain. The Department of Immunology and Oncology was founded and is supported by the Spanish Council for Scientific Research (CSIC) and by Pfizer.

References

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA (1996) CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* **272:** 1955–1958

Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M (2000) Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc Natl Acad Sci USA* **97:** 3684–3689

©2004 European Molecular Biology Organization

Baggiolini M, Moser B (1997) Blocking chemokine receptors. *J Exp Med* **186:** 1189–1191

Bastiaens PI, Jovin TM (1996) Microspectroscopic imaging tracks the intracellular processing of a signal transduction protein: fluorescent-labeled protein kinase C beta I. *Proc Natl Acad Sci USA* **93:** 8407–8412

Berger EA, Doms RA, Fenyö EM, Korber BT, Littman DR, Moore JP, Sattentau QJ, Schuitemaker H, Sodroski J, Weiss RA (1998) A new classification for HIV-1. *Nature* **391:** 240

Berger EA, Murphy PM, Farber JM (1999) Chemokine receptors as HIV-1 co-receptors: roles in viral entry, tropism and disease. *Annu Rev Immunol* **17:** 657–700

Blanpain C, Vanderwinden JM, Cihak J, Wittamer V, Le Poul E, Issafras H, Stangassinger M, Vassart G, Marullo S, Schlöndorff D, Parmentier M, Mack M (2002) Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies. *Mol Cell Biol* **13:** 723–737

Chidiac P, Nouet S, Bouvier M (1996) Agonist-induced modulation of inverse agonist efficacy at the beta 2-adrenergic receptor. *Mol Pharmacol* **50:** 662–669

Chuntharapai A, Lee J, Burnier J, Wood WI, Hébert C, Kim KJ (1994) Neutralizing monoclonal antibodies to human IL-8 receptor A map to the NH2-terminal region of the receptor. *J Immunol* **152:** 1783–1789

Deng H, Liu R, Ellmeier W, Choe S, Unutmaz D, Burkhart M, Di Marzio P, Marmon S, Sutton RE, Hill CM, Davis CB, Peiper SC, Schall TJ, Littman DR, Landau NR (1996) Identification of a major co-receptor for primary isolates of HIV-1. *Nature* **381:** 661–666

Dimitrov DS, Norwood D, Stantchev TS, Feng Y, Xiao X, Broder CC (1999) A mechanism of resistance to HIV-1 entry: inefficient interactions of CXCR4 with CD4 and gp120 in macrophages. *Virology* **259:** 1–6

Doranz BJ, Grovit-Ferbas K, Sharron MP, Mao SH, Goetz MB, Daar ES, Doms RW, O'Brien WA (1997) A small molecule inhibitor directed against the chemokine receptor CXCR4 prevents its use as an HIV-1 coreceptor. *J Exp Med* **186:** 1395–1400

Doranz BJ, Rucker J, Yi Y, Smyth RJ, Samson M, Peiper SC, Parmentier M, Collman RG, Doms RW (1996) A dual-tropic primary HIV-1 isolate that uses fusin and the β-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85:** 1149–1158

Frade JMR, Llorente M, Mellado M, Alcami J, Gutierrez-Ramos JC, Zaballos A, Real G, Martinez-A C (1997a) The N-terminal domain of the CCR2 chemokine receptor acts a co-receptor for HIV-1 infection. *J Clin Invest* **100:** 497–502

Frade JMR, Mellado M, Lind P, Gutierrez-Ramos JC, Martínez-A C (1997b) Characterization of the CCR2 chemokine receptor; functional CCR2 receptor expression on B cells. *J Immunol* **159:** 5576–5584

Fragoso R, Ren D, Zhang X, Su MW, Burakoff SJ, Jin YJ (2003) Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. *J Immunol* **170:** 913–921

Garzino-Demo A, DeVico AL, Conant KE, Gallo RC (2000) The role of chemokines in human immunodeficiency virus infection. *Immunol Rev* **177:** 79–87

Gordon GW, Berry G, Liang XH, Levine B, Herman B (1998) Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys J* **74:** 2702–2713

Issafras H, Angers S, Bulenger S, Blanpain C, Parmentier M, Labbe-Jullie C, Bouvier M, Marullo S (2002) Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors. *J Biol Chem* **277:** 34666–34673

Kenakin T (1997) Agonist-specific receptor conformations. *Trends Pharmacol Sci* **18:** 416–417

Kenworthy AK (2001) Imaging protein–protein interactions using fluorescence resonance energy transfer microscopy. *Methods* **24:** 289–296

Littman DR (1998) Chemokine receptors: keys to AIDS pathogenesis. *Cell* **93:** 677–680

Mack M, Luckow B, Nelson PJ, Cihak J, Simmons G, Clapham PR, Signoret N, Marsh M, Stangassinger M, Borlat F, Wells TN, Schlondorff D, Proudfoot AE (1998) Aminooxypentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J Exp Med* **187:** 1215–1224

Mackay CR (2001) Chemokines: immunology's high impact factor. *Nat Immunol* **2:** 95–101

Mañes S, del Real G, Lacalle R, Lucas P, Gómez-Moutón C, Sanchez-Palomino S, Delgado R, Alcamí J, Mira E, Martínez-A C (2000) Membrane raft micro-domains mediate lateral assemblies required for HIV-1 infection. *EMBO Rep* **1:** 190–196

McDermott DH, Zimmerman PA, Guignard F, Kleeberg CA, Leitman SF, Murphy PM (1998) CCR5 promoter polymorphism and HIV-1 disease progression. *Lancet* **352:** 866–870

Mellado M, Rodríguez-Frade JM, Aragay A, del Real G, Martin AM, Vila-Coro AJ, Serrano A, Mayor Jr F, Martinez-A C (1998) The chemokine monocyte chemotactic protein 1 (MCP-1) triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2b receptor. *J Immunol* **161:** 805–813

Mellado M, Rodríguez-Frade JM, Kremer L, von Kobbe C, de Ana AM, Merida I, Martinez-A C (1997) Conformational changes required in the human growth hormone receptor for growth hormone signaling. *J Biol Chem* **272:** 9189–9196

Mellado M, Rodríguez-Frade JM, Mañes S, Martínez-A C (2001a) Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu Rev Immunol* **19:** 397–421

Mellado M, Rodríguez-Frade JM, Vila-Coro AJ, Fernandez S, Martin de Ana A, Jones DR, Toran JL, Martinez-A C (2001b) Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *EMBO J* **20:** 2497–2507

Mellado M, Rodríguez-Frade JM, Vila-Coro AJ, Martín de Ana A, Martínez-A C (1999) Chemokine control of HIV-1 infection. *Nature* **400:** 723–724

Mengozzi M, De Filippi C, Transidico P, Biswas P, Cota M, Ghezzi S, Vicenzi E, Mantovani A, Sozzani S, Poli G (1999) Human immunodeficiency virus replication induces monocyte chemotactic protein-1 in human macrophages and U937 promonocytic cells. *Blood* **93:** 1851–1857

Moser B, Loetscher P (2001) Lymphocyte traffic control by chemokines. *Nat Immunol* **2:** 123–128

Murakami T, Nakajima T, Koyanagi Y, Tachibana K, Fujii N, Tamamura H, Yoshida N, Waki M, Matsumoto A, Yoshie O, Kishimoto T, Yamamoto N, Nagasawa T (1997) A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. *J Exp Med* **186:** 1389–1393

O'Brien SJ, Moore JP (2000) The effect of genetic variation in chemokines and their receptors on HIV transmission and progression to AIDS. *Immunol Rev* **17:** 99–111

Rodríguez D, Rodríguez JR, Rodríguez JF, Trauber D, Esteban M (1989) Highly attenuated vaccinia virus mutants for the generation of safe recombinant viruses. *Proc Natl Acad Sci USA* **86:** 1287–1291

Rodríguez JF, Rodríguez D, Rodríguez JR, McGowan E, Esteban M (1988) Expression of the firefly luciferase gene in a vaccinia virus: a highly sensitive gene marker to follow dissemination in tissues of infected animals. *Proc Natl Acad Sci USA* **85:** 1667–1671

Rodríguez-Frade JM, Mellado M, Martínez-A C (2001) Chemokine receptor dimerization: two are better than one. *Trends Immunol* **22:** 612–617

Rodríguez-Frade JM, Vila-Coro AJ, Martín de Ana A, Albar JP, Martínez-A C, Mellado M (1999) The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc Natl Acad Sci USA* **96:** 3628–3633

Rossi D, Zlotnik A (2000) The biology of chemokines and their receptors. *Annu Rev Immunol* **18:** 217–242

Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M (1996) Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382:** 722–725

Schols D, Struyf S, Van Damme J, Este JA, Henson G, De Clercq E (1997) Inhibitor of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J Exp Med* **186:** 1383–1388

©2004 European Molecular Biology Organization

Signoret J, Pelchen-Matthews A, Mack M, Proudfoot AE, Marsh M (2000) Endocytosis and recycling of the HIV coreceptor CCR5. *J Cell Biol* **151:** 1281–1293

Smith MW, Carrington M, Winkler C, Lomb D, Dean M, Huttley G, O'Brien SJ (1997a) CCR2 chemokine receptor and AIDS progression. *Nat Med* **3:** 1052–1053

Smith MW, Dean M, Carrington M, Winkler C, Huttley GA, Lomb DA, Goedert JJ, O'Brien TR, Jacobson LP, Kaslow R, Buchbinder S, Vittinghoff E, Vlahov D, Hoots K, Hilgartner MW, O'Brien SJ (1997b) Contrasting genetic influence of CCR2 and CCR5 variants on HIV-1 infection and disease progression. *Science* **277:** 959–965

Sorkin A, McClure M, Huang F, Carter R (2000) Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr Biol* **10:** 395–398

Vila-Coro AJ, Mellado M, Martin de Ana A, Lucas P, del Real G, Martinez-A C, Rodriguez-Frade JM (2000) HIV-1 infection through the CCR5 receptor is blocked by receptor dimerization. *Proc Natl Acad Sci USA* **97:** 3383–3393

Ward SG, Bacon K, Westwick J (1998) Chemokines and T lymphocytes: more than an attraction. *Immunity* **9:** 1–11

Wouters FS, Bastiaens PI, Wirtz KW, Jovin TM (1998) FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acid 737 oxidation enzymes in peroxisomes. *EMBO J* **17:** 7179–7189

Xiao X, Wu L, Stantchev TS, Feng YR, Ugolini S, Chen H, Shen Z, Riley JL, Broder CC, Sattentau QJ, Dimitrov DS (1999) Constitutive cell surface association between CD4 and CCR5. *Proc Natl Acad Sci USA* **96:** 7496–7501

©2004 European Molecular Biology Organization