CMLS, Cell. Mol. Life Sci. 60 (2003) 2492–2500
1420-682X/03/112492-09
DOI 10.1007/s00018-003-3295-1

CMLS Cellular and Molecular Life Sciences

Research Article

Induced recovery of defective membrane expression of a CC chemokine receptor 5 mutant by phytohemagglutinin

Q.-Y. Qi [a], F. Wang [a], H.-T. Zhang [a], J.-C. Wang [a], H.-P. Xiao [b], M.-H. Wang [a], Y.-F. Han [a], R.-M. Zhang [a], S.-H. Tao [a] and Z. W. Luo [a,c,*]

[a] Laboratory of Population & Quantitative Genetics, The State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Sciences, Morgan-Tan International Center for Life Sciences (MTIC), Fudan University, 220 Handan Road, Shanghai 200433 (China)
[b] Department of Tuberculosis, 507 Zhengmin Road, Shanghai Pulmonary Hospital, Shanghai 200433 (China)
[c] School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT (United Kingdom),
Fax: + 44 121 414 5429, e-mail: z.luo@bham.ac.uk

Received 23 July 2003; accepted 18 August 2003

Abstract. CC chemokine receptor 5 (CCR5) is a member of the G-protein-coupled receptor superfamily. It plays an important role in macrophage tropic human immunodeficiency virus-1 entry and in some inflammatory reactions. CCR5-893(–) is a single-nucleotide deletion that results in complete truncation of the C tail of the gene product. We detected CCR5-893(–) in a sample of patients infected with non-tuberculosis mycobacteria and found that it was maintained heterozygously with a frequency of 2%. There is no association between this mutation and any immunodeficiency. Membrane expression of CCR5-893(–) was substantially reduced compared to the wild type, but this defective surface presentation recovered greatly recovered in the presence of 2 mg l^{-1} phytohemagglutinin (PHA). However, PHA inducement did not affect the total intracellular expression of CCR5-893(–) or wild-type CCR5. Thus we suggest there exist some PHA-induced factor(s) that could mediate the presentation of truncated CCR5.

Key words. CCR5-893(–); immunodeficiency; membrane expression; PHA inducement.

CC chemokine receptor 5 (CCR5) is a member of the chemokine receptor subclass of the G-protein-coupled receptor (GPCR) superfamily [1]. It is involved in recruitment of monocytes and macrophages into inflamed tissues in infection or immune responses responding to its ligands, MIP-1α, MIP-1β (macrophage inflammatory peptides) and RANTES (regulated on activation, normal T cell expressed and secreted) [2]. It also serves as an essential coreceptor for cellular entry of macrophage-tropic human immunodeficiency virus-1 (HIV-1) viruses (R5), which are predominant in the early stages of HIV-1 infection [3–6]. At more advanced stages, T-cell-line-tropic virus (X4) strains emerge and use chemokine receptor 4 (CXCR4) as a coreceptor [7–9]. Although other chemokine receptors are also involved in entry of various strains of virus, these two chemokine receptors are generally recognized as the most important factors in AIDS progression [10]. There is evidence that ligands of CCR5 could block the replication of macrophage-tropic viruses in vitro [11, 12].

Surface expression of the CCR5 coreceptor may influence susceptibility to HIV-1 infection and AIDS progression. Several mutant alleles of CCR5 in natural populations, which cause low membrane expression of the protein, were found to be highly associated with retardation

* Corresponding author.
Q.-Y. Qi and F. Wang contributed equally to this work.

of AIDS progression [13, 14]. In particular, a single substitution of A to G in the CCR5 promoter was observed to reduce promoter activity by about 45% and to delay AIDS progression by an average of 3.8 years [15]. Mutations in the coding region sequence have provided a good opportunity for understanding expression of this receptor. Among them, a 32-bp deletion mutant (CCR5Δ32), which leads to a frameshift and early termination of the receptor, was found to be abundant in Caucasians. The CCR5Δ32 mutant is sequestered in the cytosol. Homozygotes of the mutant allele showed complete resistance to macrophage-tropic HIV-1 infection, while the heterozygotes exhibited delayed progression of AIDS by about 2 years on average [16, 17]. More recently, another CCR5 deletion mutant, CCR5-893(–), was screened in Asians [18]. The single-nucleotide deletion causes a frameshift at codon 299 and results in premature termination of translation. A difference in the frequency of the allele between HIV-infected and non-infected individuals showed that the mutant carriers tended to be partially resistant to initial HIV-1 attack [19]. Furthermore, another naturally occurring 24-bp deletion (CCR5Δ24) was discovered in sooty mangabey and red-capped mangabey monkeys, and the mutant was also seen to affect infection of the simian immunodeficiency virus [20, 21].

All these mutants share a common feature of intracellular C terminus truncation. Studies have shown that CCR5 shares with other GPCR members some unique motifs in the C-terminal cytoplasmic domain for ligand-dependent receptor signaling, internalization and desensitization [22, 23]. The impact of the C terminus on membrane expression of this protein has focussed interest on residues in the C terminus that affect surface expression [24, 25].

To date, two anti-HIV strategies have been proposed based on intervention at the chemokine receptor. Using chemokines or their analogues to block the binding site on chemokine receptors for HIV was demonstrated to be efficient in inhibiting HIV entry, but the redundancy of the chemokine system limited the application of this therapy [12]. The other alternative is to interfere with the surface expression of CCR5. An intra-antibody against CCR5 arrested the receptor in the cytosol and prevented it from being transported to the cell membrane [26]. Investigation of individuals carrying those truncated mutants elicited another anti-HIV therapy based on intervention at CCR5. Since CCR5Δ32 did not confer susceptibility to any immunodeficiency [7], this opens the opportunity for an anti-HIV strategy based on intervention at CCR5.

CCR5 has also been shown to regulate leukocyte chemotaxis in inflammation and to play a dominant role in the development of liver injuries associated with graft-versus-host disease, in multiple sclerosis lesions and in rheumatoid arthritis [27–29]. Specifically, CCR5Δ32 was associated with a reduced risk of severe symptoms in patients with multiple sclerosis and asthma [30, 31]. *Mycobacterium tuberculosis* infection stimulates human alveolar macrophages and peripheral blood monocytes to produce RANTES and some other CCR5 ligands. Thus CCR5+ Th1 effector cells can be recruited to the site of infection by these chemokines, and enlisted in establishment and maintenance of granulomas which are the characteristic histopathologic lesions of tuberculosis. This hints at a potential role for CCR5 in the immune response to intracellular pathogens.

On the other hand, there was experimental evidence showing remarkable effect of various microbial pathogens or their products on HIV replication in vivo [32–34]. Among them, Gram-negative bacterial cell wall constituents (e.g. lipopolysaccharide, LPS) could down-regulate CCR5 gene promoter activity and up-regulate RANTES promoter activity, thus yielding a deficient condition for R5 strains entering into macrophages [35]. This raises the question whether chemokine inducers, for example, phytohemagglutinin (PHA) or LPS [36, 37], could influence the reduced membrane expression of CCR5 mutants.

In this report, we demonstrated that CCR5-893(–), which was detected in the Chinese population, was not seen to be associated with any obvious immunodeficiency and showed that the cell surface expression of CCR5-893(–) was substantially reduced compared to wild type. Moreover, the lowered expression was recovered in the presence of PHA, an efficient chemokine inducer. This research provides convincing evidence for the existence of limiting factor(s) that may regulate CCR5 trafficking.

Materials and methods

Patients and controls

The peripheral blood samples were collected from 28 Chinese patients affected with non-tuberculosis mycobacteria (NTM) and other 102 healthy people. The patients suffered recurrent infections of poorly virulent mycobacterial species such as *M. avium*, *M. kansasii* and *M. chelonae*, but they did not show vulnerability to viral, bacterial or fungal infection. An immune work-up revealed a normal complement of C3 and C4, normal Ig levels (including IgM, IgG and IgA subtypes) and normal T-cell-mediated cellular immunity in these patients.

The genomic sense and antisense primers for genomic DNA, which was extracted from the peripheral blood mononuclear cells, were designed as 5′-CCTAAGCTTGGGGATCCGGTGGAACAAGATGGAT-3′ and 5′-CAGGAGCTCCCCTCGAGCCACTTGAGTCCGTGTCACA-3′. The target segment was amplified by 35 cycles of denaturation at 95 °C for 60 s, annealing at 55 °C for 55 s and extension at 72 °C for 100 s. The PCR products were directly sequenced or cloned into

pGEMD-3Zf(+) vector (Applied Biosystems, Foster City, USA). Sequencing of PCR products or recombinant plasmids was performed on an ABI PRISM377 DNA sequencer with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystem). Restriction enzyme digestion analysis was obtained in an independent PCR amplification reaction using genomic DNA. Thermal cycling was performed by 35 cycles of 95 °C for 60 s, 55 °C for 30 s, and 72 °C for 40 s, with the use of the primers 5′-ACCAAGCTATGCAGGTGAC-3′ (forward) and 5′-AGGCTGTGTATGA AAACTAAGC-3′ (reverse). PCR products were digested with *Bsi*YI (MBI Fermentas, Burlington, Canada).

Construction of the expression plasmids

HA-tag was appended to the N terminus of the wild-type or mutant CCR5 by use of PCR with another forward primer: 5′-CGTGGAACTTAAGCTTACC*ATGGGGTA-CCCATACGATGTTCCA*GATTACGCTGATTATCAAG-TGTCAAGTCC-3′, where the italicized letters indicate the sequence encoding the HA-tag. The reverse primer was as above. The PCR products were digested by *Hind*III and *Xho*I, and inserted into pcDNA3.0 or pIND (Invitrogen, Frederick, Md.).

DNA transfection

Monolayers of CV-1 were cultivated in RPMI-1640 medium (Life Technologies, Grand Island, N.Y.) containing 10% fetal calf serum (FCS; Hyclone, Logan, Utah), and that of COS-7 in DMEM medium (Life Technologies) containing 10% FCS. The monolayer cells were transfected with the plasmids using Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's manual. To construct an inducible expression system, the cells were cotransfected with both pIND-CCR5/CCR5-893(–)/LacZ and pVgRXR (1:1), and then induced by Ponasterone A in concentrations ranging from 0 to 10 μmol l^{-1} together with or without 2 mg l^{-1} PHA after 6 h of the transfection. The cells were incubated for another 24 h and then harvested for the flow cytometry or ELISA experiments to be described below.

Flow cytometry

The HA-tag antibodies used to identify the HA-appended receptor were rat monoclonal antibodies specific for the HA-tag (Roche Applied Diagnostics, Hongkong) and FITC-conjugated goat anti-rat antibodies (SBA Corp., Fountain Hills, Ariz.). The transfected cells from each of the transfection treatments (~10^5) were digested from the plate with 2% EDTA solution, collected by centrifugation and washed with PBS. To detect the surface antigen, the cells were incubated with HA antibody for 30 min at 4 °C in PBS containing 0.02% sodium azide. They were then washed and stained with the secondary antibodies for 30 min at 4 °C. After being stained and washed, the cells were resuspended in PBS containing 2% (v/v) paraformaldehyde and then analyzed on a Becton Dickinson flow cytometer (FACScalibur) installed with CELLQUEST software. The mean fluorescence intensity was used as a measurement of membrane expression at every unit of cell number. To detect the intracellular antigen, the cells were permeabilized with PBS containing 2% paraformaldehyde and 0.05% saponin (Sigma) for 10 min before being stained. The rest steps were the same as those for detecting the cell surface antigen except that the washing buffer was replaced with PBS containing 0.05% saponin.

Confocal immunofluorescence microscopy

The transfected cells were plated on coverslips and fixed in 2% paraformaldehyde for 15 min at 4 °C, rinsed five times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 5 min at 25 °C, and then incubated together with rat anti-HA antibody and rabbit anti-calnexin antibody (Stressgen Biotechnologies Corp. Victoria, Canada) for 30 min at 4 °C. After incubation, the cells were rinsed five times with PBS and stained with FITC-conjugated goat anti-rat antibody or phycoerythrin (PE)-conjugated goat anti-rabbit antibody (SBA). The cells were further rinsed five times and bathed in PBS. Images were recorded on a Zeiss LSM510 (Jena, Germany) confocal microscope.

Enzyme-linked immunosorbent assay

The monolayer cells were also transfected with pcDNA3-LacZ or pIND-LacZ. Transfection with pIND-LacZ was followed by inducement with Ponasterone A. After 24 h of transfection, the concentration of LacZ was determined by ELISA (Roche, Basel, Switzerland) according to the manufacturer's manual.

Results

Detection of the CCR5 mutant from patients affected with NTM

To assess the effect of CCR5 in predisposition to any immunodeficiency, especially the rare disease, NTM infection, we directly sequenced the coding region of the CCR5 gene in NTM subjects. We found that 1 of the 28 NTM patients was heterozygous for CCR5-893(–). Except for the CCR5-893(–) allele, no other sequence variation was detected. To examine association between the mutant and the disease, we were able to collect blood samples of the other family members of the proband. The family consisted of the proband (II-1), her parents and other two sibs (fig. 1 A). The father of the proband was diagnosed twice as being infected with pulmonary tuberculosis. Genotyping at the polymorphic site indicated that the mutant allele was transmitted from the proband's nor-

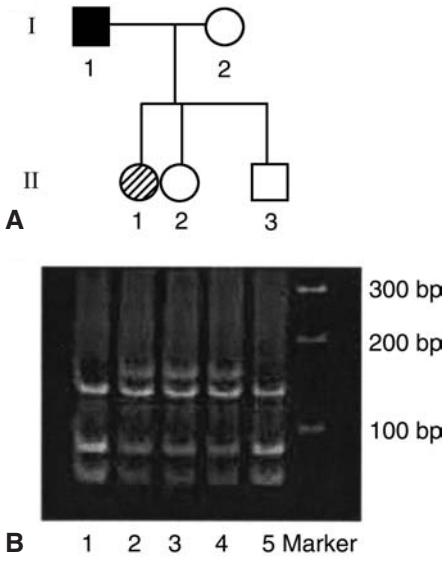

Figure 1. (*A*) A Chinese nuclear family affected with NTM. II-1 infected with *M. avium* was the proband and carrier of CCR5-893(–). Her father was diagnosed twice as a pulmonary tuberculosis patient. Her mother and sibs had no any history of mycobacterial infections. (*B*) Genotypes of the NTM family members at the CCR5 locus. The deletion of a C at the site of 893 causes the loss of a *Bsi*YI cleavage site. The DNA fragments generated by BsiYI were identified from 10% acrylamide gel electrophoresis. Lane 1, I-1; lane 2, II-1; lane 3, II-2; lane 4, I-2; lane 5, II-3.

mal mother who was also heterozygous for the mutant gene. The proband's sister was also a carrier of the mutant allele but showed no symptoms of lung infection. Thus, there is no convincing evidence for genetic association between the mutant and the lung disease.

To explore the population distribution of CCR5-893(–), we genotyped 102 randomly selected Chinese adults at the polymorphic site. These individuals had no record of hospitalization for pulmonary tuberculosis. Four of the 102 individuals were detected as heterozygotes for the mutant gene, indicating that CCR5-893(–) had an approximate frequency of 2%. This prevalence of the mutant allele in the natural population suggests that it might not necessarily be associated with any serious genetic disorders.

Impaired membrane expression of CCR5-893(–) and its recovery with PHA inducement

To investigate the effect of C tail truncation on expression, flow cytometry analyses were carried out to detect pcDNA3-CCR5 or pcDNA3-CCR5-893(–) by live (fig. 2A, C) or fixed (fig. 2B, D) staining. Two cell lines, COS-7 (fig. 2A, B) and CV-1 (fig. 2C, D) were used for the expression analysis. The cells transfected with pcDNA3-LacZ were treated as a control in this experiment (red curves in all the plots).

Figure 2A, C shows that the membrane expression of CCR5-893(–) (green curves) was greatly reduced when compared to its wild type (light-blue curves). However, the total expression of CCR5-893(–) (green curves in fig. 2B, D) was comparable to its wild type (light-blue curves in the correspondingly figures). This observation suggests that the impaired membrane expression of CCR5-893(–) might be due to defective trafficking rather than to changes in its intracellular expression.

In the above system, the fluorescence intensity of CCR5-893(–) on the cell membrane was remarkably enhanced when 2 mg l^{-1} PHA was added to the culture medium (yellow curves in fig. 2A, C). On the other hand, cell surface expression of wild-type CCR5 (dark-blue curves in fig. 2A, C) was not seen to change significantly under PHA treatment. Comparing the dark-blue curves in fig. 2B, D to the light-blue ones shows that the PHA inducement did not influence the intracellular expression of CCR5-893(–). This thus indicates that the impaired membrane expression of CCR5-893(–) was recovered in the presence of PHA.

The pIND/pVgRXR inducible system was established to explore the change in surface expression with varying concentrations of Ponasterone A. Fig. 3 illustrates the membrane expression of CCR5-893(–) and its wild type in the cell lines COS-7 (fig. 3A) and CV-1 (fig. 3B). The pattern of change in the expression level over various Ponasterone A concentrations showed that the expression level saturated after the concentration had increased up to 5 µmol l^{-1}. Again, the PHA inducement was shown to be an effective factor in recovering the impaired membrane expression of CCR5-893(–), while the saturating level of membrane expression of wild-type CCR5 was not enhanced under PHA treatment, excluding the possibility that the accessibility of the HA-tag to its detecting antibody was enhanced by PHA inducement. In addition, we carried out ELISA to explore the effect of PHA and Ponasterone A concentration on expression of LacZ. Figure 4A demonstrates expression of LacZ in the cell lines CV-1 and COS-7, both of which were transfected with pcDNA3-LacZ but treated with (+) or without (–) PHA. The expression was not affected by the PHA treatment, indicating that the inducible expression system established in the present study was not influenced by the presence or absence of PHA. Figure 4B shows the trend for an increase in expression of LacZ with increasing concentration of Ponasterone A in the two cell lines transfected with pIND-LacZ. Together with the previous observation of the saturated intracellular expression of CCR5, we would anticipate the existence of other factors that play important roles in regulating the membrane expression of the gene.

Subcellular distribution of CCR5-893(–)

The subcellular distributions of CCR5-893(–) and the wild-type CCR5 were examined using immunofluorescence confocal microscopy (IFCM). Figure 5 depicts views

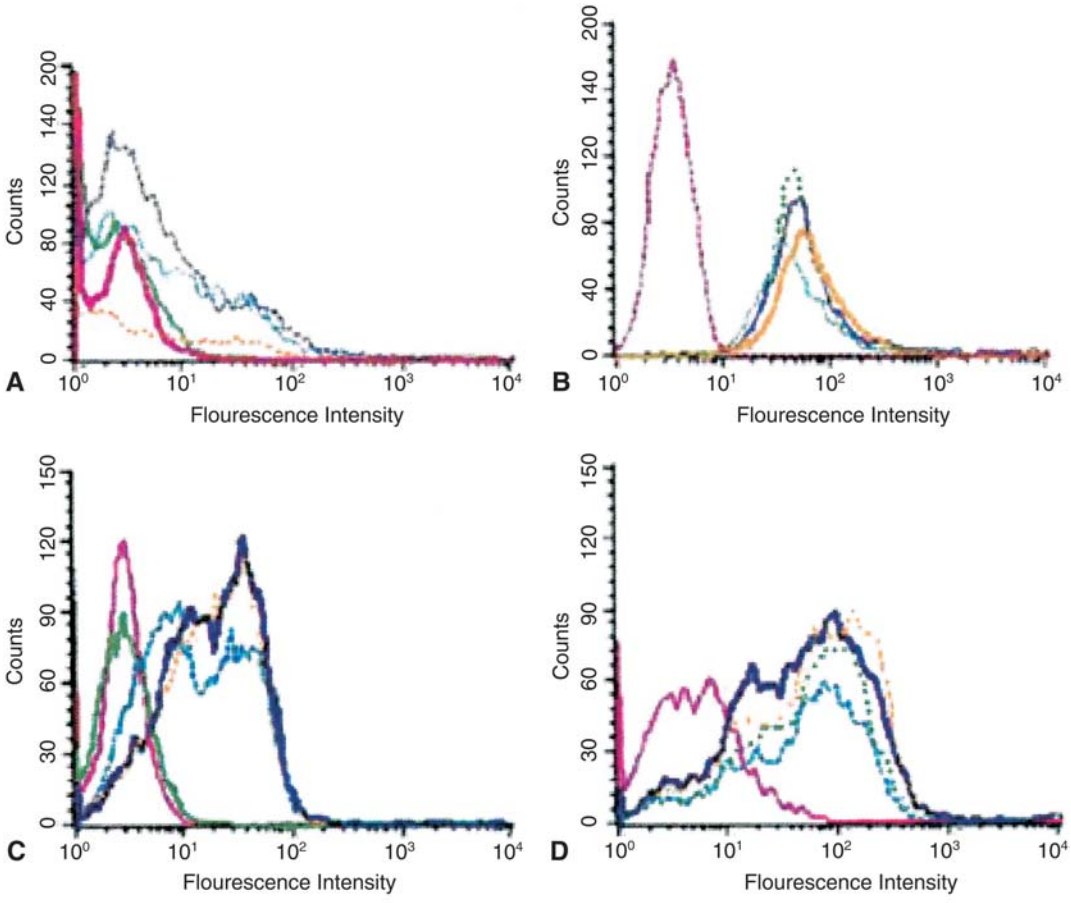

Figure 2. Surface and intracellular expression of wild-type CCR5 and CCR5-893(–) in COS-7 (*A*, *B*) and CV-1 (*C*, *D*) cells. For detection of surface expression of CCR5 (*A*) or CCR5-893(–) (*C*), cells transfected with pcDNA3-CCR5 or pcDNA3-CCR5-893(–) were treated with (light blue or yellow for the two transfection treatments, respectively) or without (dark blue or green for the two transfection treatments, respectively) 2 mg l^{-1} PHA. For detection of intracellular expression of CCR5 (*B*) or CCR5-893(–) (*D*), transfected cells were permeabilized with saponin before staining. Cells were also treated with (light blue and yellow for the two transfection treatments, respectively) or without (dark blue and green, for the two transfection treatments, respectively) 2 mg l^{-1} PHA. Cells transfected with pCDNA3-LacZ were used as controls (red).

of IFCM, which were obtained from the two cell lines, COS-7 (left panel) and CV-1 (right panel). Each of the panels was divided into two groups: A and B representing the IFCM views for the subcellular location of wild-type CCR5, C and D for CCR5-893(–), while A and C represent the IFCM views obtained from the cells without PHA treatment, and B and D represent the views obtained from the cells with PHA treatment. The three columns of the views S-HA, S-Calnexin and Merge represent the views of locations of endoplasmic reticulum (ER), the receptor and their merge, respectively. Calnexin, an ER chaperon that takes part in folding of proteins in the ER, was observed in the perinuclear regions of the cells.

The IFCM view A shows that the subcellular location of CCR5 partially overlaps with that of ER, indicating that only part of the CCR5 was retained in ER and part was transported onto the membrane. The IFCM view C reveals a different scenario for CCR5-893(–), which was mainly arrested in ER. However, comparing IFCM view C to D, CCR5-893(–) was clearly released from ER to the membrane when PHA was present in the culture medium (fig. 5D). Comparing view A to B shows that PHA did not cause a significant change in the subcellular distribution of wild-type CCR5.

Discussion

CCR5 is widely accepted as playing an important role in human infection by macrophage-tropic HIV virus. Various mutants of this receptor have been discovered in natural populations and found in association with altered responses to HIV infection or AIDS progression when compared to their wild type [7].

Among the patients affected with NTM, we detected a carrier of the CCR5-893(–) mutant, which causes a premature termination of translation and leads to deletion of 35 but gain of 10 new amino acid residues within the

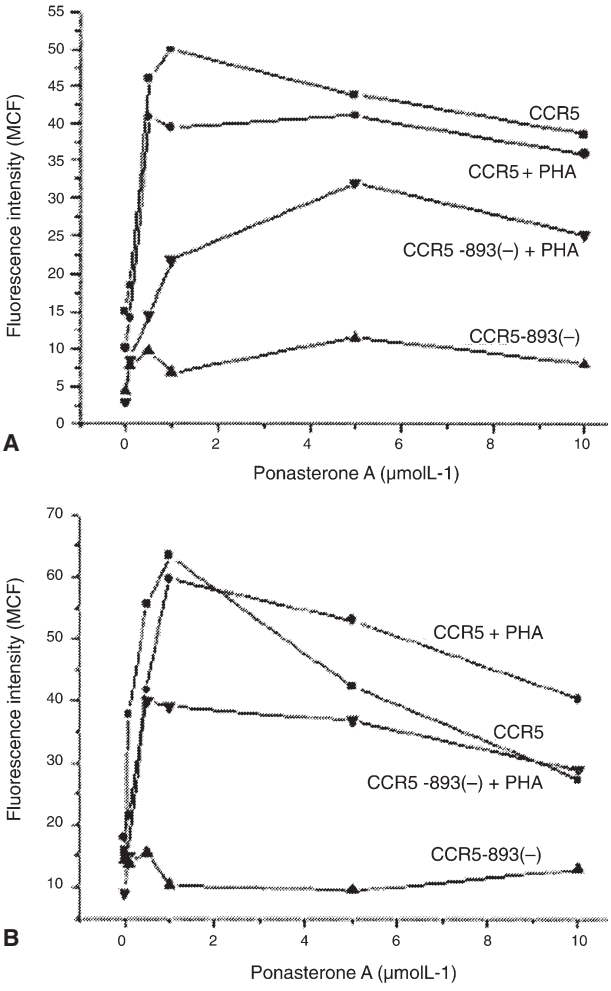

Figure 3. The dynamic change in membrane expression of wild-type CCR5 and CCR5-893(–) in COS-7 (*A*) and CV-1 (*B*) cells with increasing concentrations of Ponasterone A inducement. Cells were transfected with pIND-CCR5/pIND-CCR5-893(–) and pVgRXR, and 6 h later induced with Ponasterone A in increasing concentrations from 0 to 10 μmol l^{-1} in the presence (●, ▼) or absence (■, ▲) of 2 mg l^{-1} PHA.

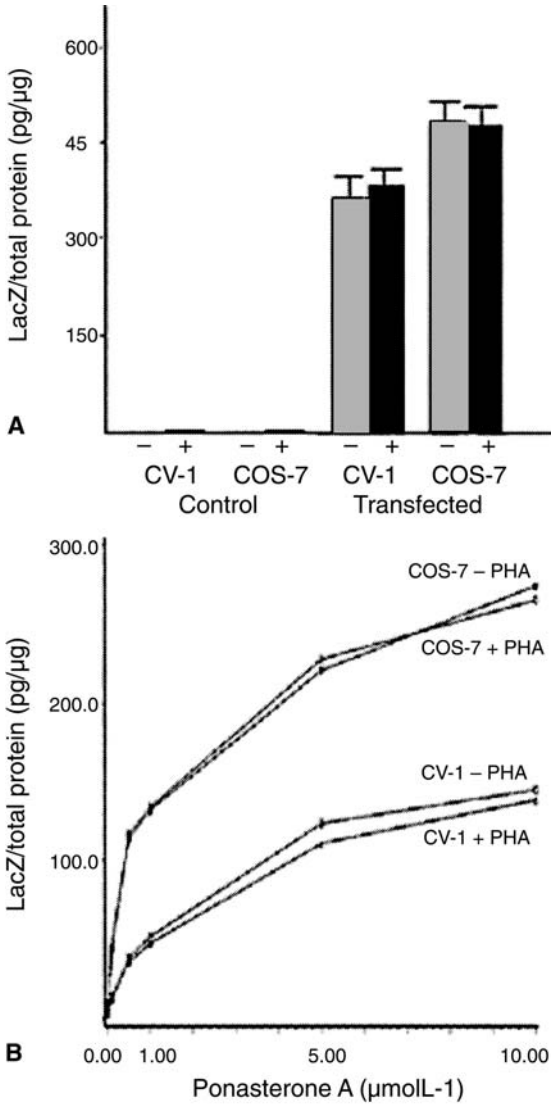

Figure 4. Expression of LacZ in COS-7 and CV-1 cell lines. The cells were transfected with pcDNA3-LacZ and incubated with (black) or without (gray) 2 mgl^{-1} PHA (*A*). The cells were transfected with pIND-LacZ, induced by Ponasterone A at varying concentrations from 0 to 10 μmol l^{-1} and incubated with or without 2 mg l^{-1} PHA (*B*). The cells without transfection treatment were used as controls.

C terminus intracellular domain of the receptor. Pedigree analysis of the mutant carrier and a population survey showed that there is no sufficient evidence supporting a pathogenic association of this mutant gene with susceptibility to NTM infection, a rare disease caused by an unknown immunodeficiency [38]. Although a population frequency of the mutant allele was estimated as 2%, homozygotes for the mutant gene were not seen in our samples and have not been reported in the literature. This may suggest that the mutant allele would be maintained in the natural population as heterozygotes.

Discovery of the CCR5 mutant opens an opportunity for understanding the molecular mechanism of the delayed progression of symptoms in AIDS patients who carry CCR5-893(–) [18, 19] and for exploring the significance of the C terminus of CCR5 for its membrane expression.

Infection of HIV is usually accompanied by coinfection of certain opportunistic germs, for example *M. tuberculosis*, *Giardia*, *M. avium* complex, herpes virus, or *Pneumocystis carinii* are common in HIV infection [39–41]. In addition, PHA was found to possess similar roles to these bacteria in inducing production of chemokines and in regulating activity of the chemokine receptor promoters. Thus, PHA in the present study plays a similar role to bacterial infection in creating a favorable/unfavorable environment for HIV entry.

Employing the mammal inducible system established in the present study, the flow cytometry experiment demon-

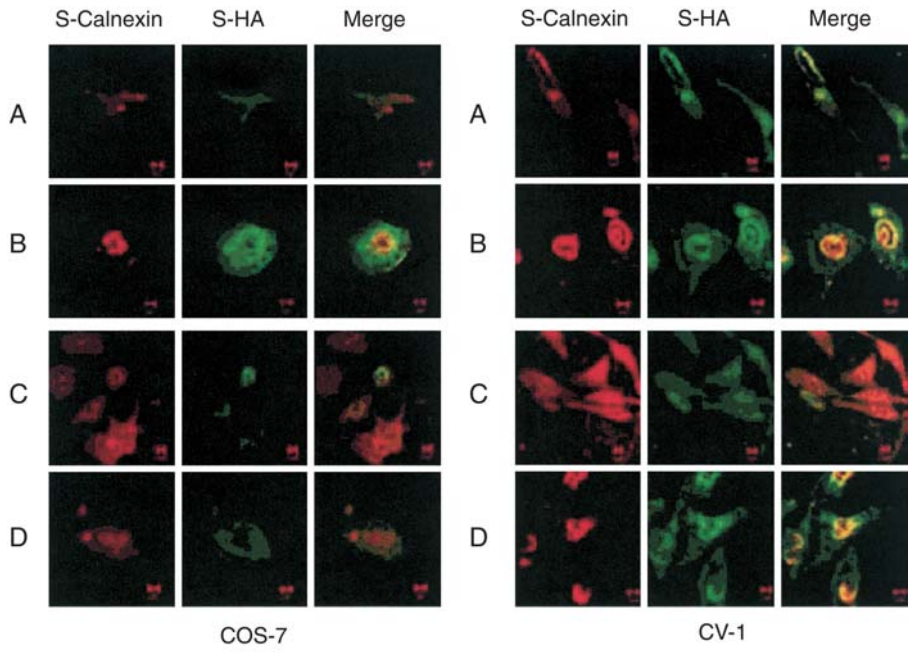

Figure 5. Confocal views of the subcellular distribution of wild-type CCR5 and CCR5-893(–) in COS-7 (left) and CV-1 (right) cells. The transfected cells expressing wild-type CCR5 or CCR5-893(–) were treated with (row B and D) or without (row A and C) 2 mgl^{-1} PHA. Cells transfected with pcDNA3-CCR5 or pcDNA3-CCR5-893(–) were permeabilized with saponin, then stained with rat anti-HA mAb and FITC-conjugated goat anti-rat secondary antibody. Endoplasmic reticulum (ER) was stained with rabbit anti-calnexin mAb followed by PE-labeled anti-rabbit IgG. Green indicates the location of CCR5 expression and red, ER.

strated that membrane expression of CCR5-893(–) was almost undetectable but its intracellular expression was comparable to that of its wild type (fig. 2). Venkatestan et al. [24] have demonstrated that the other CCR5 C terminus mutant could react normally with the antibodies directed against the conformational epitope and possess an unchanged steady-rate of endocytosis. Thus, we anticipate that the impaired expression of CCR5-893(–) was due to defective trafficking rather than to its intracellular expression. This at least partially explains the retarded response of the mutant carriers to HIV infection. Moreover, the experiment interestingly revealed that the defective membrane expression of CCR5-893(–) recovered to wild-type level by inducement with PHA; however, the presence of PHA did not influence either the membrane or intracellular expression of the wild-type CCR5.

IFCM analysis also clearly demonstrated a comparable level of intracellular expression of CCR5 and CCR5-893(–) but reduced membrane expression of CCR5-893(–) and recovery of the defective expression of the mutant under PHA inducement. Shioda et al. [19] also observed defective CCR5-893(–) membrane expression in a different system in which virus was used as vector. Another interesting phenomenon observed by using the expression system employed in the present study was the saturated expression of CCR5-893(–) and its wild type over increasing concentrations of Ponasterone A. This is in sharp contrast to the ELISA experiment in which expression of LacZ in both COS-7 and CV-1 cell lines was observed to increase with increasing Ponasterone A concentration. However, the saturated level of wild-type CCR5 was not enhanced under PHA treatment. Thus, inducement by PHA recovered trafficking of CCR5-893(–) rather than enhancing the accessibility of the HA-tag to its detecting antibody. These observations also strongly suggested the existence of other cell factors that regulate membrane expression of CCR5. The role of the C tail of CCR5 in its cellular surface expression was also stressed in the CCR5-CXCR4 chimera experiment [24], in which the C tail of CCR5 was replaced by that of CXCR4 and expression of the chimera was seen to be substantially reduced on the membrane.

In addition to the impaired membrane expression of CCR5-893(–), the mutant was also shown to be defective in intracellular signaling transduction [25]. The present study demonstrated the significant effect of PHA inducement in recovering impaired membrane expression. However, we were able to show that the treatment did not result in repairing the defective intracellular signaling transduction (data not presented but available on request). Among conditions proposed to influence HIV-1 coreceptor activity of CCR5 are its association with CD4 [42, 43], its functional interaction with the virus envelope glycoprotein that is mediated by the extracellular domain of CCR5 [44–46], and its local density at the cell surface [47, 48]. A high local density of HIV coreceptor could fa-

vor the clustering of activated viral fusiogenic proteins that seems important for the formation of fusion pores [49–51]. Entry of HIV-1 into its target cells does not need intracellular signaling or internalization mediated by the C tail domain of CCR5 [52–54]. Thus, inhibition of virus entry can be effectively realized by invoking CCR5 dysfunction, including down-regulating of cell membrane expression of CCR5 or directly blocking the virus/coreceptor interaction. Our experiment demonstrated recovery of impaired membrane expression of CCR5-893(–) in the presence of PHA. This means that PHA inducement may maintain a required level of CCR5 membrane density for the deadly virus to enter the target cells. Further studies focusing on screening for the factors that directly affliate the defective trafficking of the mutant CCR5, could potentially identify selective targets for anti-HIV drugs.

Acknowledgments. We thank the family members who participated in the study. We are grateful for the comments and criticisms of two anonymous reviewers, which were very helpful in improving the presentation of and clarifying several ambiguities in the present paper. The research was financially supported by the China Basic Research Project (G1998051020), Shanghai Committee of Science and Technology (01JC14012), the State Education Ministry, the National Natural Science Foundation of China (39993420), MTIC and Shanghai Committee of Education. Z. W. L. is also supported by research grants from BBRSC and NERC.

1 Boulay F., Naik N., Giannini E., Tardif M. and Brouchon L. (1997) Phagocytes. Ann. N. Y. Acad. Sci. **832:** 69–84
2 Combadiere C., Ahuja S. K., Tiffany H. L. and Murphy P. M. (1996) Cloning and functional expression of CC CKR5, a human monocyte CC chemokine receptor selective for MIP-1 alpha, MIP-1 beta, and RANTES. J. Leukoc. Biol. **60:** 147–152
3 Choe H., Farzan M., Sun Y., Sullivan N., Rollins B., Ponath P. D. et al. (1996) The β-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:** 1135–1148
4 Deng H., Liu R., Ellmeier W., Choe S., Unutmaz D., Burkhart M. et al. (1996) Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:** 661–666
5 Doranz B. J., Rucker J., Yi Y., Smyth R. J., Samson M., Peiper S. C. et al. (1996) A dual-tropic primary HIV-1 isolate that uses fusion and the beta-chemokine receptors CKR-5, CKR-3 and CKR-2b as fusion cofactors. Cell **85:** 1149–1158
6 Dragic T., Litwin V., Allaway G. P., Martin S. R., Huang Y., Nagashima K. A. et al. (1996) HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:** 667–673
7 Berger E. A., Murphy P. M. and Farber J. M. (1999) Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:** 657–700
8 Berger E. A., Doms R. W., Fenyo E. M., Korber B. T. M., Littman D. R., Moore J. P. et al. (1998) A new classification for HIV-1. Nature **391:** 240
9 Zhang Y. J. and Moore J. P. (1999) Will multiple coreceptors need to be targeted by inhibitors of human immunodeficiency virus type 1 entry? J. Virol. **73:** 3443–3448
10 Littman D. R. (1998) Chemokine receptors: key to AIDS pathogenesis? Cell **93:** 677–680
11 Cocchi F., DeVico A. L., Garzino-Demo A., Arya S. K., Gallo R. C. and Lusso P. (1995) Identification of RANTES, MIP-1α, and MIP-1β as the major HIV-suppressive factors produced by CD8+ T cells. Science **270:** 1811–1815
12 Arenzana-Seisdedos F., Virelizier J. L., Rousset D., Clark-Lewis I., Loetscher P., Moser B. et al. (1996) HIV blocked by chemokine antagonist. Nature **383:** 400
13 Miedema F., Meyaard L., Koot M., Klein M. R., Roos M. T. L., Groenink M. et al. (1994) Changing virus-host interactions in the course of HIV-1 infection. Immunol. Rev. **140:** 35–72
14 Scarlatti G., Tresoldi E., Bjorndal A., Fredriksson R., Colognesi C., Deng H. K. et al. (1997) In vivo evolution of HIV-1 coreceptor usage and sensitivity to chemokine-mediated suppression. Nat. Med. **3:** 1259–1265
15 McDermott D. H., Zimmerman P. A., Guignard F., Kleeberger C. A., Leitman S. F. and Murphy P. M. (1998) CCR5 promoter polymorphism and HIV-1 disease progression. Lancet **352:** 866–870
16 Dean M., Carrington M., Winkler C., Huttley G. A., Smith M. W., Allikmets R. et al. (1996) Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CCR5 structural gene. Science **273:** 1856–1862
17 Michael N. L., Louie L. G., Rohrbaugh A. L., Schultz K. A., Dayhoff D. E., Wang C. E. et al. (1997) The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and diseases progression. Nat. Med. **3:** 1160–1162
18 AnsariLari M. A., Liu X. M., Metzker M. L., Rut A. R. and Gibbs R. A. (1997) The extent of genetic variation in the CCR5 gene. Nat. Genet. **16:** 221–222
19 Shioda T., Nakayama E. E., Tanaka Y., Xin X. M., Liu H. L., Kawana-Tachikawa A. et al. (2001) Naturally occurring deletional mutation in the C-terminal cytoplasmic tail of CCR5 affects surface trafficking of CCR5. J. Virol. **75:** 3462–3468
20 Chen Z. W., Kwon D., Jin Z. Q., Monard S., Telfer P., Jones M. S. et al. (1998) Natural infection of a homozygous delta 24 CCR5 red-capped mangabey with an R2b-tropic simian immunodeficiency virus. J. Exp. Med. **188:** 2057–2065
21 Palacios E., Digilio L., McClure H. M., Chen Z. W., Marx P. A. and Goldsmith M. A. (1998) Parallel evolution of CCR5-null phenotypes in humans and in a natural host of simian immunodeficiency virus. Curr. Biol. **8:** 943–946
22 Bockaert J. and Pin J. P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J. **18:** 1723–1729
23 Mellado M., Rodriguez-Frade J. M., Manes S. and Martinez C. (2001) Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. Annu. Rev. Immunol. **19:** 397–421
24 Venkatesan S., Petrovic A., Locati M., Kim Y. O., Weissman D. and Murphy P. M. (2001) A membrane-proximal basic domain and cysteine cluster in the C-terminal tail of CCR5 constitute a bipartite motif critical for cell surface expression. J. Biol. Chem. **276:** 40133–40145
25 Percherancier Y., Planchenault T., Velenzuela-Fernandez A., Virelizier J. L., Arenzana-Seisdedos F. and Bacheleies F. (2001) Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. J. Biol. Chem. **276:** 31936–31944
26 Steinberger P., Andris-Widhopf J., Buhler B., Torbett B. E. and Barbas C. F. (1999) Functional deletion of the CCR5 receptor by intracellular immunization produces cells that are refractory to CCR5-dependent HIV-1 infection and cell fusion. Proc. Natl. Acad. Sci. USA **97:** 805–810
27 Balashov K. E., Rottman J. B., Weiner H. L. and Hancock W. W. (1999) CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. Proc. Natl. Acad. Sci. USA **96:** 6873–6878
28 Gomez-Reino J. J., Pablos J. L., Carreira P. E., Santiago B., Serrano L., Vicario J. L. et al. (1999) Association of rheumatoid arthritis with a functional chemokine receptor, CCR5. Arthritis Rheum. **42:** 989–992

29 Mack M., Bruhl H., Gruber R., Jaeger C., Cihak J., Eiter V. et al. (1999) Predominance of mononuclear cells expressing the chemokine receptor CCR5 in synovial effusions of patients with different forms of arthritis. Arthritis Rheum. **42:** 981–988
30 Hall I. P., Wheatley A., Christie G., McDougall R. E., Arai H., Tsou C.-L. et al. (1999) Association of CCR5 delta32 with reduced risk of asthma. Lancet **354:** 1264–1265
31 Stellebjerg F., Madsen H. O., Jensen C. V., Jensen J. and Garred P. (2000) CCR5 delta32, matrix metalloproteinase-9 and disease activity of multiple sclerosis. J. Neuroimmunol. **102:** 98–106
32 Ensoli B., Lusso P., Schachter F., Josephs S. F., Rappaport J., Negro F. et al. (1989) Human herpes virus-6 increases HIV-1 expression in co-infected T cells via nuclear factors binding to the HIV-1 enhancer. EMBO J. **8:** 3019–3027
33 Gendelman H. E., Phelps W., Feigenbaum L., Ostrove J. M., Adachi A., Howley P. M. et al. (1986) Trans-activation of the human immunodeficiency virus long terminal repeat sequence by DNA viruses. Proc. Natl. Acad. Sci. USA **83:** 9759–9763
34 Scala G., Quinto I., Ruocco M. R., Mallardo M., Ambrosino C., Squitieri B. et al. (1993) Epstein-Barr virus nuclear antigen 2 transactivates the long terminal repeat of human immunodeficiency virus type 1. J. Virol. **67:** 2853–2861
35 Moriuchi M., Moriuchi H., Turner W. and Fauci A. S. (1998) Exposure to bacterial products renders macrophages highly susceptible to T-tropic HIV-1. J. Clin. Invest. **102:** 1540–1550
36 Leonard E. J. and Yoshimura T. (1990) Human monocyte chemoattractant protein-1 (MCP-1). Immunol. Today **11:** 97–101
37 Reale M., Barbacane R. C., DiGioacchino M., Felaco M., Croce A., Ferro F. M. et al. (2001) Differential expression and secretion of RANTES and MCP-1 in activated peripheral blood mononuclear cell cultures of atopic subjects. Immunol. Lett. **76:** 7–14
38 Casanova J. L. and Abel L. (2002) Genetic dissection of immunity to mycobacteria: the human model. Annu. Rev. Immunol. **20:** 581–620
39 Wahl S. M. and Orenstein J. M. (1997) Immune stimulation and HIV-1 viral replication. J. Leukoc. Biol. **62:** 67–71
40 Donovan R. M., Bush C. E., Markowitz N. P., Baxa D. M. and Saravolatz L. D. (1996) Changes in virus load markers during AIDS-associated opportunistic diseases in human immunodeficiency virus-infected persons. J. Infect. Dis. **174:** 401–403
41 Denis M. and Ghadirian E. (1994) *Mycobacterium avium* infection in HIV-1-infected subjects increases monokine secretion and is associated with enhanced viral load and diminished immune response to viral antigens. Clin. Exp. Immunol. **97:** 76–82
42 Lapham C. K., Ouyang J., Chandrasekhar B., Nguyen N. Y., Dimitrov D. S. and Golding H. (1996) Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. Science **274:** 602–605
43 Xiao X. D., Wu L. J., Stantchev T. S., Feng Y. R., Ugolini S., Chen H. et al. (1999) Constitutive cell surface association between CD4 and CCR5. Proc. Natl. Acad. Sci. USA **96:** 7496–7501
44 Wu L. J., LaRosa G., Kassam N., Gordon C. J., Heath H., Ruffing N. et al. (1997) Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp-120 binding and a single domain for chemokine binding. J. Exp. Med. **186:** 1373–1381
45 Picard L., Simmons G., Power C. A., Meyer A., Weiss R. A., Clapham P. R. et al. (1997) Multiple extracellular domains of CCR5 contribute to human immunodeficiency virus type 1 entry and fusion. J. Virol. **71:** 5003–5011
46 Bieniasz P. D., Fridell R. A., Aramori I., Ferguson S. S., Caron M. G. and Cullen B. R. (1997) HIV-1-induced cell fusion is mediated by multiple regions within both the viral envelope and CCR5 coreceptor. EMBO J. **16:** 2599–2609
47 Platt E. J., Wehrly K., Kuhmann S. E., Chesebro B. and Kabat D. (1998) Effects of CCR5 and CD4 cell surface concentrations on infections by macrophage tropic isolates of human immunodeficiency virus type 1. J. Virol. **72:** 2855–2864
48 Kuhmann S. E., Platt E. J., Kozak S. L. and Kabat D. (2000) Cooperation of multiple CCR5 coreceptors is required for infections by human immunodeficiency virus type 1. J. Virol. **74:** 7005–7015
49 Bentz J. (2000) Minimal aggregate size and minimal fusion unit for the first fusion pore of influenza hemagglutinin-mediated membrane fusion. Biophys. J. **78:** 227–245
50 Chernomordik L. V., Frolov V. A., Leikina E., Bronk P. and Zimmerberg J. (1998) The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. J. Cell Biol. **140:** 1369–1382
51 Monck J. R. and Fernandez J. M. (1996) The fusion pore and mechanisms of biological membrane fusion. Curr. Opin. Cell Biol. **8:** 524–533
52 Farzan M., Choe H., Martin K. A., Sun Y., Sidelko M., Mackay C. R. et al. (1997) HIV-1 entry and macrophage inflammatory protein-1β-mediated signaling are independent functions of the chemokine receptor CCR5. J. Biol. Chem. **272:** 6854–6857
53 Atchison R. E., Gosling J., Monteclaro F. S., Franci C., Digilio L., Charo I. F. et al. (1996) Multiple extracellular elements of CCR5 and HIV-1 entry: dissociation from response to chemokines. Science **274:** 1924–1926
54 Doranz B. J., Lu Z. H., Rucker J., Zhang T. Y., Sharron M., Cen Y. H. et al. (1997) Two distinct CCR5 domains can mediate coreceptor usage by human immunodeficiency virus type 1. J. Virol. **71:** 6305–6314