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Complexity in human immunodeficiency virus type 1 (HIV-1) co-receptor usage: roles of CCR3 and CCR5 in HIV-1 infection of monocyte-derived macrophages and brain microglia

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CCR3 has been implicated as a co-receptor for human immunodeficiency virus type 1 (HIV-1), particularly in brain microglia cells. We sought to clarify the comparative roles of CCR3 and CCR5 in the central nervous system (CNS) HIV-1 infection and the potential utility of CCR3 as a target for manipulation via gene transfer. To target CCR3, we developed a single-chain antibody (SFv) and an interfering RNA (RNAi), R3-526. Coding sequences for both were cloned into *Tag*-deleted SV40-dervied vectors, as these vectors transduce brain microglia and monocyte-derived macrophages (MDM) highly efficiently. These anti-CCR3 transgenes were compared to SFv-CCR5, an SFv against CCR5, and RNAi-R5, an RNAi that targets CCR5, for the ability to protect primary human brain microglia and MDM from infection with peripheral and neurotropic strains of HIV-1. Downregulation of CCR3 and CCR5 by these transgenes was independent from one another. Confocal microscopy showed that CCR3 and CCR5 co-localized at the plasma membrane with each other and with CD4. Targeting either CCR5 or CCR3 largely protected both microglia and MDM from infection by many strains of HIV-1. That is, some HIV-1 strains, isolated from either the CNS or periphery, required both CCR3 and CCR5 for optimal productive infection of microglia and MDM. Some HIV-1 strains were relatively purely CCR5-tropic. None was purely CCR3-tropic. Thus, some CNS-tropic strains of HIV-1 utilize CCR5 as a co-receptor but do not need CCR3, while for other isolates both CCR3 and CCR5 may be required.

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INTRODUCTION

Several chemokine receptors are used, together with CD4, for human immunodeficiency virus type 1 (HIV-1) entry into cells (Deng *et al.*, 1996; Feng *et al.*, 1996). T-cell tropic (T-tropic) viruses use CXCR4 as a co-receptor (Feng *et al.*, 1996), whereas macrophage-tropic (M-tropic) HIV-1 uses CCR5 (Choe *et al.*, 1998). Some strains of HIV-1 may bind CCR3 and other chemokine receptors and use them to enter cells that express them following stable transfection (Doranz *et al.*, 1996), but the role of CCR3 in primary cells in HIV-1 infection is unclear.

CCR5 is the major co-receptor for M-tropic HIV-1. It is utilized for HIV-1 entry into macrophages in the peripheral immune system and into brain microglia cells (Albright *et al.*, 1999; Gorry *et al.*, 2001; Shieh *et al.*, 1998; Smit *et al.*, 2001). It is mainly expressed on eosinophils, microglial cells, basophils and some T lymphocytes (Berger *et al.*, 1999). The extent to which it functions as a co-receptor for HIV-1 entry into central nervous system (CNS) cells is conjectural, but considerable data suggest that some forms of HIV-1 envelope may bind to CCR3, so it could act as a minor co-receptor for HIV-1 entry (Alkhatib *et al.*, 1997; Choe *et al.*, 1996; Ghorpade *et al.*, 1998; He *et al.*, 1997; Ho *et al.*, 2004; Martin-Garcia *et al.*, 2002; Peters *et al.*, 2004; Shieh *et al.*, 1998). The cloned envelopes of many primary HIV-1 isolates show comparable tropism for CCR3 and CCR5 (Aasa-Chapman *et al.*, 2006).

We approached the study of the roles of CCR3 and CCR5 in CNS HIV-1 infection from the perspective of the potential therapeutic applicability of targeting CCR3 and CCR5 to protect CNS and monocyte-derived macrophages (MDM)

A supplementary table is available with the online version of this paper.

from HIV-1. We used viral vector-delivered transgenes that specifically target CCR3 and CCR5 to decrease their concentrations at the plasma membrane: interfering RNA (RNAi) and single-chain Fv antibodies (SFv).

Post-transcriptional gene silencing by small double-stranded RNAs (siRNA) is highly sequence specific and leads to the degradation of the targeted mRNA (Yu *et al.*, 2002). On the other hand, SFv are the smallest antibody fragments that retain antigen-binding configurations of parent antibodies.

We used primary cells to study co-receptor usage by both CNS-derived and peripheral strains of HIV-1 in infection of microglia and MDM. We compared the effects of downregulating CCR3 and CCR5 using rSV40-delivered RNAi and SFv against these co-receptors, as well as inhibition by ligands (eotaxin for CCR3) or ligand antagonists (TAK-779 for CCR5). SV40-derived vectors were used because they transduce brain microglia and macrophages very efficiently.

We found that some strains of HIV-1 utilize CCR5 exclusively, while for other strains inhibiting either CCR3 or CCR5 alone decreased HIV-1 replication. These data suggest that for some strains of HIV-1, including primary isolates from both the CNS and the periphery, CCR3 may be an important partner co-receptor that is used together with CCR5 in HIV-1 entry into macrophages and brain microglia. Therapeutic strategies targeting both CCR3 and CCR5 may thus be helpful in inhibiting HIV-1 infection.

METHODS

Cell lines and transfection assays. U87-CD4-CCR3 and HOS-CD4-CCR3 cells (Bjorndal *et al.*, 1997; Deng *et al.*, 1996) were obtained from NIH-AIDS Research and Reference Reagent Program (AIDS-RRRP). U87-CD4-CCR3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 15 % new born calf serum (NCS) (Hyclone), 1 μg puromycin ml^{-1}, 300 μg G418 ml^{-1}, 2 mM glutamine, 200 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. HOS-CD4-CCR3 cells were cultured in DMEM containing 10 % NCS and 1 μg puromycin ml^{-1}. COS-7 cells (ATCC) were maintained in DMEM, 10 % NCS, 2 mM L-glutamine, 1.5 g $NaHCO_3$ l^{-1}, 4.5 g glucose l^{-1}, 1 mM sodium pyruvate, 200 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. U87-CD4-CCR3 or microglial cells were transfected with either plasmids (5 μg) or soluble siRNAs (0–10 nm) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Cell surface expression of CCR3 and AU1 expression were analysed after 48 h.

Antibodies and proteins. Antibody to CCR3 (clone 5E8-G9-B4) was developed in the laboratory of Dr Charles Mackay (Heath *et al.*, 1997). Monoclonal anti-CCR5 (clone 2D7), anti-CD68 and anti-CD11B were purchased from BD Biosciences. Polyclonal anti-CCR5 was purchased from Abcam and anti-CD4 (clone RPAT4) from BD Biosciences. Anti-AU1-fluorescein isothiocyanate (FITC) was purchased from Covance. Eotaxin was purchased from Peprotech Inc. TAK-779 was obtained from AIDS-RRRP.

Primary microglial cells. Microglial cells were isolated from human fetal brain as described previously (Cordelier & Strayer, 2006). Briefly, fetal brain (Human Fetal Tissue Bank, Albert Einstein College of Medicine, Bronx, NY, USA) was homogenized in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 0.05 % trypsin and 100 U DNase, passed through 170 μm nylon mesh, then plated at 50×10^6 cells per T-150 flask. Non-adherent cells were removed by washing with DMEM/F-12 (Invitrogen). Microglia were harvested as clarified supernatants after 2 weeks and collected on alternate 2 days for 4 weeks. Cell purity was >95 %, as verified by flow cytometry analysis using anti-CD68 or anti-CD11B antibodies.

Preparation of macrophages. Macrophages were prepared from fresh buffy coat (TJU Blood Center) as described previously (Simmons *et al.*, 1995). Briefly, cells were plated at a density of 10^8 cells in DMEM with 10 % human serum for 2 h at 37 °C. Adherent cells were incubated in DMEM containing macrophage colony stimulating factor and granulocyte macrophage colony stimulating factor (50 U ml^{-1}; Sigma Chemicals). The nature and purity of these cells were verified by staining with anti-CD68.

SFv against CCR3. An SFv against CCR3 was constructed using the mouse SFv module according to the manufacturer's instructions (Amersham Biosciences). Briefly, total RNA from CCR3 hybridoma was used with 10 different sets of primers (in the module) for amplification of variable heavy (V_H) and variable light (V_L) domains using RT-PCR. V_H- and V_L-coding fragments were cloned into pGEM-T (Promega). V_H and V_L domains were amplified by PCR to generate an SFv construct containing a $(Gly_4\ Ser)_3$ spacer, plus a C-terminal AU1 epitope (DTYRYI).

FP_H, forward primer (heavy chain): 5′-GCCACCATGGTGCA GCTGCAGCAGTCA-3′; RP_H, reverse primer (heavy chain): 5′-AGATCCGCCACCACCGGATCCGCCTCCGCCTGAGGAGACGGT GAC-3′; FP_L, forward primer (light chain): 5′-GGTGGTGG CGGATCTGGAGGTGGCGGAAGCGACATCCAGATGACA-3′; RP_L, reverse primer (light chain): 5′-CTATCAGATGTATCGGTACGTG TCTTTGATTTCCAGCTTGGTG-3′.

SFv-CCR3-AU1-coding sequences were subcloned into pcDNA3.1(+) mammalian expression vector (Clontech) and also into SV40 vector, pT7A5. SFv-CCR3-AU1 structure has been reported previously to GenBank (accession no. EU019106).

siRNA against CCR3. Synthetic siRNA duplexes were chemically synthesized by Integrated DNA technologies. They were approximately 21 nt in length, with 2 nt deoxythymidine 3′-overhangs.

A 63 nt R3-526-siRNA was synthesized containing both sense and antisense strands for R3(526)-siRNA and cloned into pT7VA. This plasmid contains the adenovirus VA1 promoter (pol III) (Cordelier *et al.*, 2003). All constructs were sequenced to confirm structure.

The sequence below represents the synthetic (R3-526)-siRNA construct that was chemically synthesized for cloning into SV40 vector pT7VA. Restriction site *Bam*HI (ggatcc) with three 'a' overhangs was synthesized with the sense sequence of (R3-526)-siRNA followed by the introduction of an *Eco*RI site (gaattc). This was followed by the antisense sequence of (R3-526)-siRNA and a *Sac*II site (ccgcgg) and two 'a' overhangs. *Bam*HI and *Sac*II were used for directional cloning in pT7VA vector and *Eco*RI was introduced for orientation screening purposes.

AaaggatccGCCTTGTACAGCGAGATCT-gaattc-*AGATCTCGCTGTAC AAGGCT*ccgcgggaa

Plasmids and viral expression constructs. SFv-CCR3-AU1 cDNA was subcloned into pT7A5, with expression driven by cytomegalovirus (CMV) immediate-early promoter (CMV-IEP) (Cordelier *et al.*, 2004; Cordelier *et al.*, 2003). pT7A5 and pT7VA lack the large T-antigen gene (Tag), but carry SV40 capsid genes, ori, packaging and polyadenylation signals. SV(RNAi-R5) is an rSV40 that encodes an

siRNA targeting CCR5 mRNA. SV(2C7) contains an SFv versus human CCR5. The specificity of this SFv and its parent hybridoma have been reported previously (Lee *et al.*, 1999; Strayer *et al.*, 2001). Control vectors were SV(HBS), which encodes hepatitis B surface antigen (HBsAg) (Kondo *et al.*, 1998), and SV(Lamin-A/C)-siRNA, which encodes siRNA targeting lamin-A/C (Cordelier *et al.*, 2003, 2004).

Procedures for generating rSV40s have been reported in detail previously (Strayer *et al.*, 2001). Briefly, recombinant viral genomes were transfected into COS-7 cells, which supply all SV40 genes needed for virus packaging. Recombinant viral stocks were prepared as cell lysates, band purified by sucrose cushion (Strayer *et al.*, 2001) and titrated by quantitative PCR (Q-PCR; Stratagene) (Strayer *et al.*, 2006).

Transduction with SV40 vectors. U87-CD4-CCR3 or primary microglial cells and macrophages were transduced with rSV40s on days 0, 3 and 5 at virus : cell ratios (m.o.i.) of 10, 3 and 3, respectively. Cells were tested for transgene expression by immunostaining for SFv-CCR3-AU1, using anti-AU1, which showed >90 % transduction efficiency (data not shown), and by assessing downmodulation of CCR3 by both the constructs. Cells were maintained for 5 days in DMEM with 2 % fetal bovine serum. All transduced cell groups, whether receiving test or control vectors [SV(HBS) and SV(Lamin-A/C-siRNA)], showed >95 % viability throughout.

Immunostaining and FACS analysis. For immunostaining and FACS, cells were grown on four-chamber slides and in six-well plates coated with poly-D-lysine, respectively. At the indicated times, cells were fixed with 1 % paraformaldehyde for 30 min on ice and permeabilized with 0.1 % Triton X-100 in sodium citrate buffer. Non-specific binding was blocked using normal serum from the animal species in which the secondary antibody was raised. Cells were immunostained with anti-CCR3 (1 : 50), anti-CCR5 (1 : 100), anti-CD4 (1 : 100) or anti-AU1-FITC (1 : 100) for 1 h on ice. After extensive washing with PBS containing 1 % BSA, secondary antibodies conjugated with rhodamine (Sigma Chemicals) for CCR3, were added. Cells were washed and analysed on a fluorescence imaging microscope (Olympus IX70) or analysed by FACS (FACSort; BD Biosciences) using CellQuest software.

HIV-1 stocks. HIV-1 ADA (R5) was described previously (Gendelman *et al.*, 1992). Briefly 30×10^6 monocytes were infected with the original virus preparation. Supernatants were collected every 2–3 days, fresh phytohaemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) were added and p24 concentration was measured. For HIV-1 JR-FL(R5), 10^7 PHA-stimulated PBMCs were infected with viral inoculum containing 10 μg polybrene ml^{-1}. Supernatants were harvested daily for 3 days (O'Brien *et al.*, 1990). Preparation of HIV-1 SF-2(R5/X4) has been described previously (Levy *et al.*, 1984).

Generation of primary HIV-1 isolates. Primary isolates, 92US657(R5/X4), 92US723(R5/X4), 96USHIPS7(R5) and ASJM108-(R5) were propagated *in vitro* as described in protocols through UNAIDS network http://www.aidsreagent.org/support_docs/virus.pdf. HIV-1 96USSN20(R5/X4) and ASM108(R5) were prepared by co-culture of patient PBMCs with PHA-stimulated PBMCs in RPMI 1640 containing 10 % fetal bovine serum with 100 U interleukin-2 ml^{-1} (Sullivan *et al.*, 2000). Viruses were used at 10 ng p24 equivalents in infecting both microglia and macrophages.

Full-length envelope genes of primary brain isolates NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5) have been previously amplified from frontal lobes of patients by using PCR (Peters *et al.*, 2004) and full-length replication competent clones were constructed by subcloning brain-derived envelopes into pNL4.3. p24 protein concentrations were measured using an ELISA kit (Zeptometrix).

HIV-1 challenge studies. After gene transfer, primary microglia or macrophages were cultured at 1×10^6 cells per well in DMEM containing 10 % NCS. The next day the cells were infected with 10 ng p24 equivalents of cell-free ADA(R5), JR-FL(R5) or SF-2(R5/X4). Primary peripheral isolates 92US657(R5/X4), 92US723(R5/X4), 96USHIPS7(R5), ASJM108(R5) and primary brain isolates NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5) were used at 1 ng p24 equivalents. After 2 h at 37 °C, cells were extensively washed and maintained in growth medium containing 10 % (v/v) NCS. Supernatants were collected every 3 days and p24 antigen levels were determined by ELISA (Zeptometrix).

Microglia and macrophages were also pre-treated with anti-CCR3 (1 μg ml^{-1}), anti-CCR5 (1 μg ml^{-1}), eotaxin (500 ng ml^{-1}) or TAK-779 (25 μM), and challenged with input doses of virus similar to those described above.

Confocal microscopy. For co-localization, U87-CD4-CCR3 or microglia cells were grown in poly-D-lysine-coated confocal glass bottom dishes (MatTeK) at 0.5×10^6 cells per dish. At the indicated times post-transduction, cells were fixed with 1 % paraformaldehyde for 30 min on ice, permeabilized with 0.1 % Triton X-100 in sodium citrate buffer, then immunostained with anti-CCR3 (1 : 50), anti-CCR5 (1 : 100), anti-CD4 (1 : 200) or purified anti-AU1 (1 : 100) for 1 h on ice. After washing in PBS containing 1 % BSA, secondary antibodies conjugated with Alexa-Fluor (Molecular Probes) for CCR3 and CD4 were added. Secondary antibodies conjugated with rhodamine (Sigma) were used to stain AU1. The cells were washed and confocal analysis performed using an imaging system (Radience 2100; Bio-Rad) with Kr/Ar-ion laser source (488 and 568 nm excitation) on an inverted microscope (× 40, UApo 340, NA 1.35).

Statistical analysis. One way ANOVA was used to compute differences between groups (Instat). Significant effects were further analysed using Bonferroni *post hoc* comparisons.

RESULTS

rSV40s encoding R3-526-siRNA and SFv-CCR3-AU1 decrease CCR3

Initially, we screened the SFv-CCR3-AU1 construct in pcDNA3.1 and a panel of soluble siRNAs targeting CCR3, using transient transfection in U87-CD4-CCR3 cells (Supplementary Table S1, available in JGV Online). R3-526-siRNA at 10 nM was found to be most effective in downregulating CCR3. R3-526-siRNA and SFv-CCR3-AU1 were cloned into SV40 vector backbones and were designated SV(R3-526)-siRNA and SV(SFv-CCR3-AU1), respectively.

We then characterized and tested the ability of rSV40 vectors carrying these anti-CCR3 transgenes to lower CCR3 in U87-CD4-CCR3 cells, primary brain microglia and macrophages (Figs 2 and 3). Transduction with SV(R3-526)-siRNA and SV(SFv-CCR3-AU1) decreased membrane CCR3 by 76 and 68 %, respectively, compared with their respective control vectors in U87-CD4-CCR3 cells ($P<0.001$) (Fig. 2). Most cells (80–90 %) were transduced as determined by AU1 immunopositivity (data not shown).

Expression levels of CD4 were unchanged with the different transductions (Fig. 2). We then analysed downmodulation of CCR3 and CCR5 in brain microglial cells and macrophages (Fig. 3). CCR3 in microglial cells transduced with SV(R3-526)-siRNA and SV(SFv-CCR3-AU1) was specifically decreased by 60 and 36 %, respectively, ($P<0.001$ and $P<0.01$) with no change in CCR5 surface expression when analysed by FACS (Fig. 3). Fig. 3 represents average percentages of positive cells from three different experiments. A representative panel of FACS histograms is shown. Transduction with SV(RNAi-R5) in microglial cells led to decreased CCR5 expression by 89 %, without affecting CCR3 expression. These manipulations did not alter expression of cell surface CD4 (Fig. 3). Transduction of macrophages with SV(R3-526)-siRNA and SV(SFv-CCR3-AU1) decreased surface CCR3 by 73 and 64 % when analysed by FACS. SV(RNAi-CCR5) decreased cell surface CCR5 by approximately 77 % when analysed by FACS in macrophages. Both RNAi- and SFv-bearing constructs were specific for their targets: RNAi-R5 did not decrease cell membrane CCR3; neither SV(SFv-CCR3-AU1) nor SV(R3-526)-siRNA altered CCR5 (Figs 2 and 3).

Several HIV-1 isolates use CCR3 as a co-receptor

We studied HIV-1 replication in microglia following transduction with anti-CCR3 transgenes, as a measure of potential CCR3 co-receptor activity. HIV-1 isolates, with co-receptor usage shown in parentheses after the strain designation, ADA(R5), 96USSN20(R5/X4), JR-FL(R5) and SF-2(R5/X4) were used. Transduction using SV(SFv-CCR3-AU1) or SV(R3-526)-siRNA significantly decreased replication of HIV-1 strains ADA(R5) and 96USSN20(R5/X4) ($P<0.01$), but had little effect on JR-FL(R5) or SF-2(R5/X4) replication, when compared with control transductions using SV(HBS) and SV(Lamin-A/C)-siRNA, respectively (Fig. 1a, b). SV(RNAi-R5), targeting CCR5, decreased p24 levels comparably for HIV-1 ADA(R5) and 96USSN20(R5/X4) ($P<0.01$) (Fig. 1b). SV(RNAi-R5) also significantly inhibited JR-FL(R5) and SF-2(R5/X4) ($P<0.01$) (Fig. 1b). When analysed by FACS histograms, both RNAi- and SFv-bearing constructs were specific for their targets (Fig. 3). HIV-1 ADA(R5) and 96USSN20(R5/X4) were more sensitive to decreases in CCR3 in microglia than other HIV-1 isolates tested. HIV-1 JR-FL(R5) and SF-2(R5/X4) were sensitive to decreases in CCR5 but not

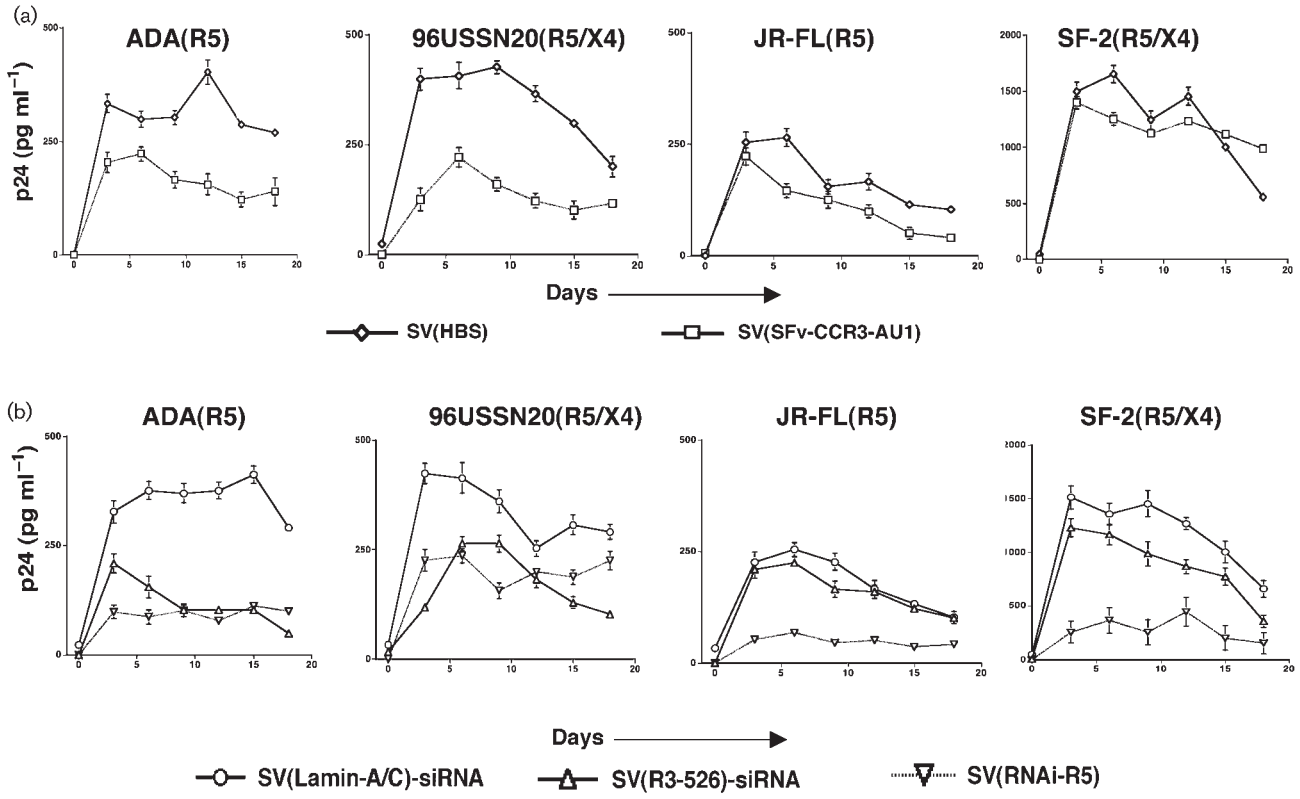

Fig. 1. Transduction with SV(SFv-CCR3-AU1)- and SV(R3-526)-siRNA protects microglia from some HIV-1 isolates. Microglial cells were transduced at an m.o.i. of 10 with either rSV40s carrying anti-CCR3 SFv SV(SFv-CCR3) (a) or SV(R3-526)-siRNA, SV(RNAi-R5) (b) or SV(HBS)- or SV(Lamin-A/C)-siRNA as respective control vectors. Transduced cells were challenged using 10 ng p24 equivalents of HIV-1 strains ADA(R5), 96USSN20(R5/X4), JR-FL(R5) or SF-2(R5/X4) isolates. Supernatants were collected every 3 days and HIV-1 replication was quantified by p24 ELISA.

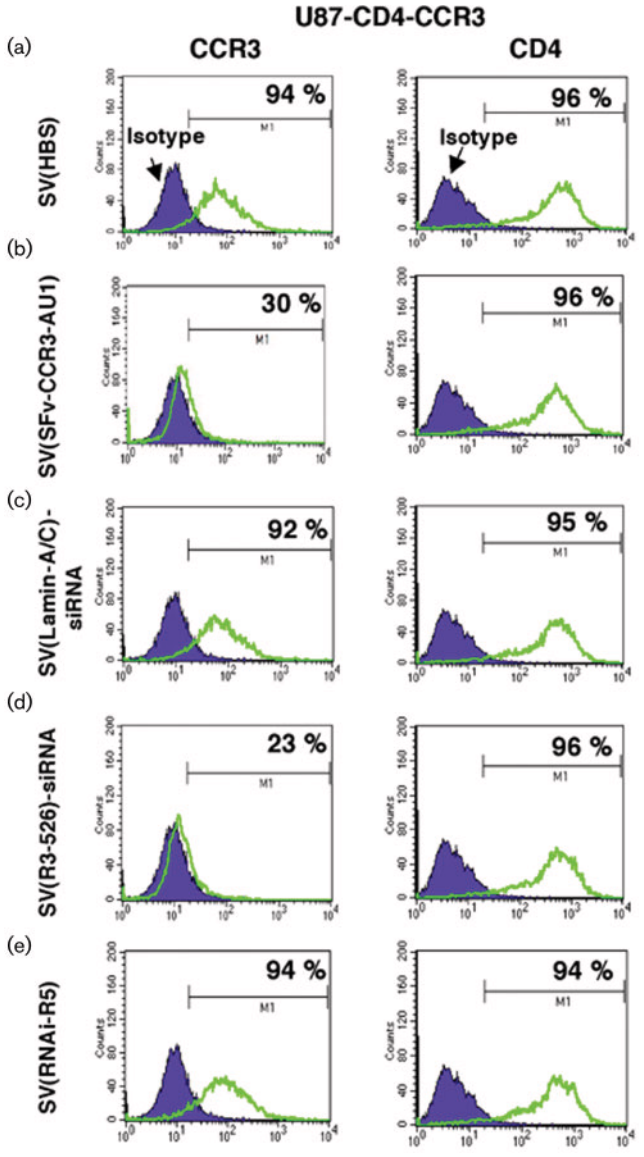

Fig. 2. Specific downregulation of CCR3 in U87-CD4-CCR3 cells. Cells were transduced with rSV40 vectors carrying control transgenes SV(HBS) and SV(Lamin-A/C-siRNA) or with vectors carrying transgenes SFv-CCR3-AU1, R3-526-siRNA or RNAi-R5. FACS analysis was performed as described in Methods. Data are shown as percentages of cells positive for CCR3 averaged from three independent experiments. Decrease in CCR3 expression was significant ($P<0.001$) as compared with their respective controls (b vs a, d vs c and e vs d). FACS histograms show data from a single, representative experiment. Filled purple histograms on the left represent isotype controls, and staining of CCR3 and CD4 is represented by open green histograms.

CCR3 (Fig. 1a). Productive infection of LAV and HxB2, which are X4-tropic isolates, was not affected by transductions targeting CCR5 or CCR3 (data not shown). These data suggest that R5-tropic strains of HIV-1 may fall into two groups: some strains may require CCR3 in addition to CCR5 for entry into microglial cells, while others require CCR5 but not CCR3.

Replication of some primary CNS and peripheral HIV-1 isolates may be inhibited by downregulating either CCR3 or CCR5

We then studied co-receptor usage for microglial cell infection by primary CNS isolates NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5), and peripheral isolates, including 92US657(R5/X4), 92US723(R5/X4), 96USHIPS7(R5) and ASJM108(R5). Initially, titration was done with primary brain isolates NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5) in microglial cells. A challenge dose of 1 ng p24 showed optimal p24 values to compare the entry efficiency and was used for other primary isolates as well.

All viruses replicated in microglia, with varying efficiency. Both CCR3 and CCR5 were efficiently utilized as co-receptors by NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5). Transduction with SV(SFv-CCR3-AU1), SV(R3-526)-siRNA, SV(SFv-CCR5) or SV(RNAi-R5) significantly inhibited ($P<0.05$) replication of NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5): HIV-1 replication was decreased by 80 % (Fig. 4) compared with controls, SV(HBS) for the SFvs and SV(Lamin-A/C-siRNA) for the RNAis.

The conclusion that some strains of HIV-1 may utilize both CCR3 and CCR5 was further tested by adding anti-CCR3 or anti-CCR5 monoclonal antibodies (5E8-G9-B4 and 2D7, respectively, see Methods), or soluble inhibitors of CCR5 and CCR3, TAK-779 or eotaxin, respectively, to the culture medium (Fig. 5). Generally, the results of these studies paralleled those observed with gene delivery of RNAi and SFv targeting CCR3 and CCR5. That is, some strains of HIV-1, in particular NA176 (B93)(R3/R5), 96USHIPS7(R5) and ASJM108(R5), were largely inhibited by these external agents blocking either CCR3 or CCR5 (P at least <0.05). Infection with NA353 (B27)(R3/R5) was more strongly inhibited by TAK-779, compared with controls ($P<0.001$), than by any of the other inhibitors, although inhibition by the latter was also statistically significant ($P<0.05$). The two strains of HIV-1 that were only inhibited by targeting CCR5 in transduction studies [92US657(R5/X4) and 92US723(R5/X4)] were, similarly, only inhibited by TAK-779 (CCR5-blocking agent) and, to a lesser extent with the 2D7 anti-CCR5 antibody (Fig. 5).

CCR3 is used efficiently by several HIV-1 isolates in MDM

The observation that some strains of HIV-1 utilize CCR3 and CCR5 in infecting brain microglia raised the question of whether a similar situation may be obtained for HIV-1 infection of phagocytes outside of the CNS. Therefore, we tested inhibition of HIV-1 replication in primary MDM by anti-CCR3 gene delivery in addition to anti-CCR5 gene transfer. MDM were transduced with SV(HBS), SV(Lamin-A/C-siRNA), SV(SFv-CCR3-AU1), SV(R3-526)-siRNA or

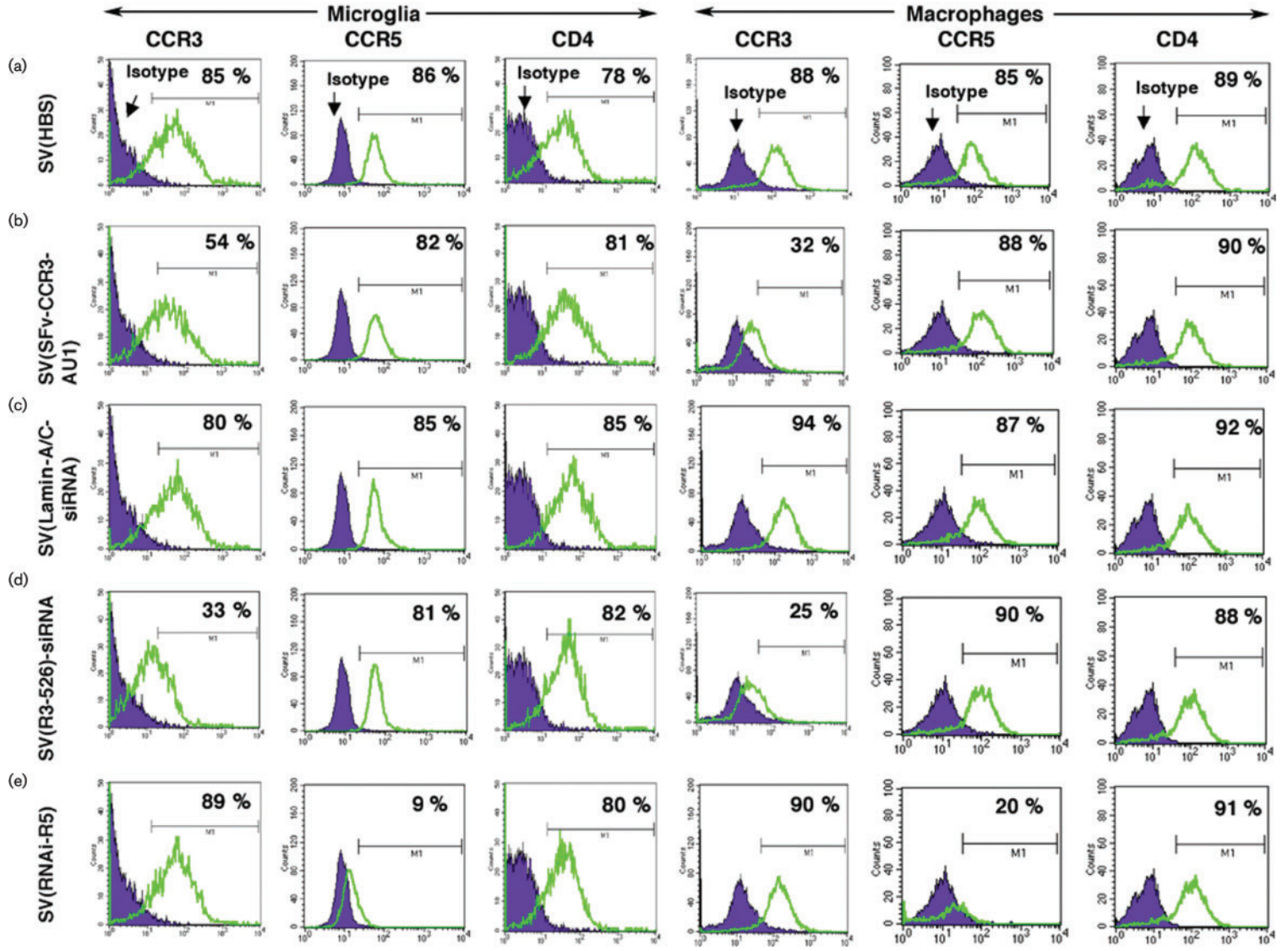

Fig. 3. Downregulation of CCR3 and CCR5 in microglia and macrophages. Microglia and macrophages were transduced with respective rSV40 vectors as described in Methods. FACS data represent individual histograms from experiments in Fig. 1, before HIV-1 challenge. Data are shown as percentages of cells positive for CCR3, CCR5 and CD4 averaged from three different experiments. In microglia, the decrease in CCR3 was significant as compared with their respective controls (b vs a, $P<0.01$), (d vs c, $P<0.001$), (e vs d, $P<0.001$). In macrophages, CCR3 was also significantly downregulated (b vs a, $P<0.001$), (d vs c, $P<0.001$), (e vs d, $P<0.001$). The decrease in CCR5 was also significant in both microglia and macrophages (e vs d, $P<0.001$; or e vs c, $P<0.001$). Filled purple histograms on the left represent isotype controls, and staining of CCR3, CCR5 and CD4 is represented by open green histograms.

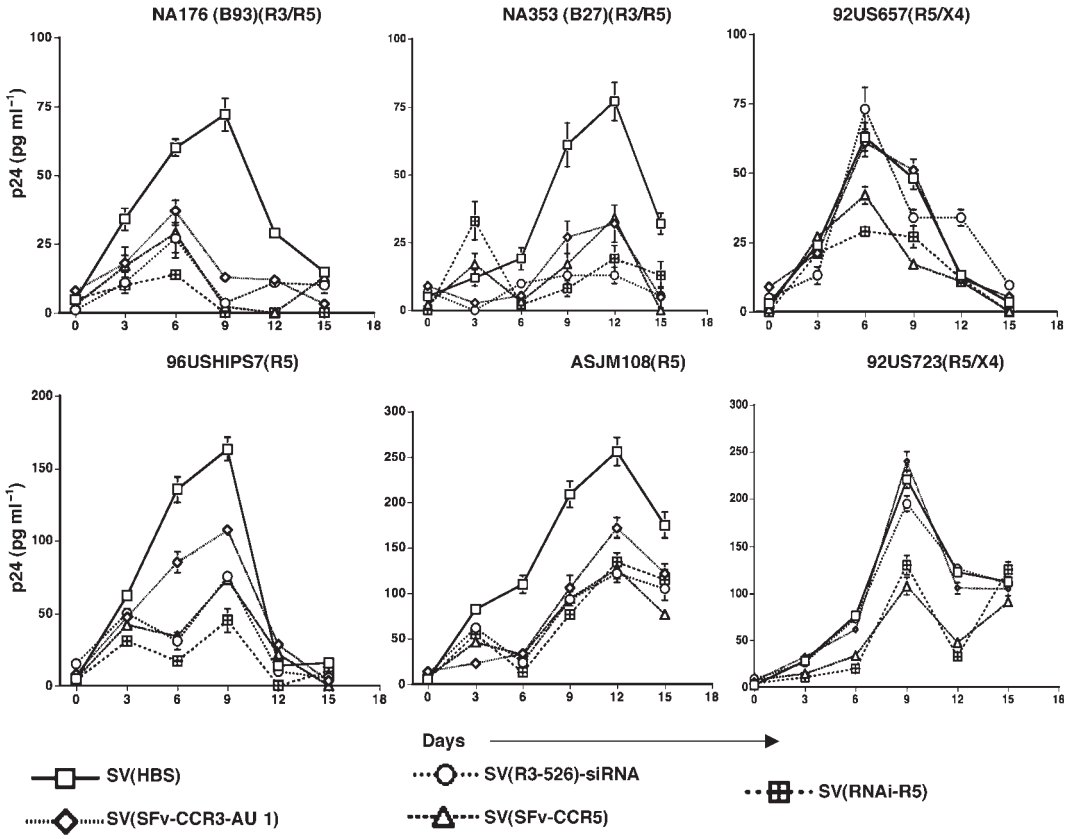

Fig. 4. HIV-1 strain differences in CCR3 and CCR5 usage as detected using rSV40-delivered RNAi and SFv against CCR3 and CCR5 in protection from CCR3/CCR5-tropic HIV-1 (primary brain isolates) and peripheral HIV-1 isolates in microglial cells. Microglia were transduced at an m.o.i. of 10 with respective anti-CCR3 or anti-CCR5 rSV40s (or control vectors) as described in Methods. Cultures were challenged with HIV-1 strains NA176 (B93)(R3/R5), NA353 (B27)(R3/R5), 92US657(R5/X4), 92US723(R5/X4), 96USHIPS7(R5) or ASJM108(R5). Supernatants were collected every 3 days and HIV-1 replication was quantified by p24 ELISA. Data represent averages of two independent experiments.

SV(RNAi-R5), then challenged with ADA(R5), NA176 (B93)(R3/R5), NA353 (B27)(R3/R5), 96USHIPS7(R5), ASJM108(R5) or 92US723(R5/X4). FACS analysis confirmed decreased plasma membrane CCR3 and CCR5 in MDM after transduction with their respective rSV40s (Fig. 3). Control transductions using both SV(HBS) and SV(Lamin-A/C-siRNA) also showed similar productive infection profiles in MDM.

HIV-1 strain ADA(R5) infection of MDM was comparably inhibited by targeting either CCR3 or CCR5 (Fig. 6) ($P<0.05$). Similar results were obtained when cultures were treated with either of the hybridoma antibodies to CCR3 or CCR5, or the respective inhibitors, eotaxin and TAK-779 (Fig. 7).

Transduction with either anti-CCR3 or anti-CCR5 transgene carrying constructs significantly reduced HIV-1 replication in MDM for HIV-1 isolates NA176 (B93)(R3/R5) and NA353 (B27)(R3/R5) ($P<0.05$) (Fig. 7). In parallel these isolates were inhibited in MDM after treatment with anti-CCR3 or anti-CCR5 antibodies, and TAK-779 or eotaxin ($P<0.01$) (Fig. 7).

Significant protection from infection with 96USHIPS7(R5) and ASJM108(R5) was seen in macrophages transduced with either of the anti-CCR3 constructs ($P<0.05$) (Fig. 6) and especially with SV(RNAi-R5) ($P<0.01$). Both sets of hybridoma antibodies and chemokine receptor ligands also blocked MDM infection by 96USHIPS7(R5) significantly ($P<0.01$) and ASJM108(R5) ($P<0.05$ for either of the hybridoma antibodies and eotaxin, and $P<0.01$ for TAK-779) (Figs 6 and 7).

None of the anti-CCR3 or anti-CCR5 transgenes, hybridoma antibodies or chemokine receptor ligands significantly reduced replication of 92US723(R5/X4) HIV-1 consistently (Figs 6 and 7).

Taken together, the data suggest that all R5-tropic strains of HIV-1 tested utilize CCR5 as a co-receptor for productive infection of primary microglia and MDM, but that some R5-tropic HIV-1 isolates also utilized CCR3 for viral entry into both of these primary cell types. The fact that inhibition of either CCR3 or CCR5 diminished productive infection by

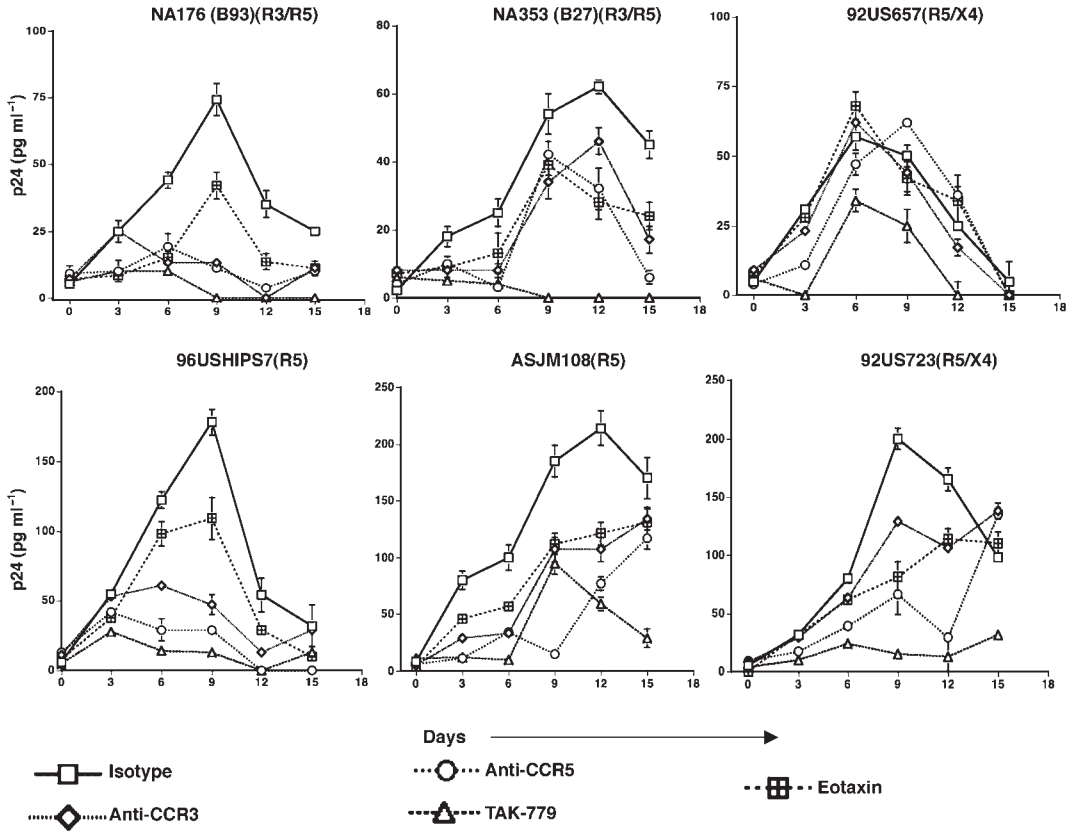

Fig. 5. HIV-1 strain differences in CCR3 and CCR5 usage as detected using anti-CCR3 or anti-CCR5 hybridoma antibodies, eotaxin or TAK-779 in protection from HIV-1 (primary brain and peripheral isolates) in microglia cells. Microglial cells were treated with 1 μg ml^{-1} of either anti-CCR3 or anti-CCR5. Parallel cultures were incubated with 500 ng eotaxin ml^{-1} or 25 μM TAK-779. As a control, unrelated IgG of the same isotype was used. Antibodies, eotaxin and TAK-779 were replenished every 3 days, after collection of supernatants. HIV-1 replication was quantified by p24 ELISA. Data represent averages of two independent experiments.

these latter HIV-1 isolates suggests that both chemokine receptors may act together as HIV-1 co-receptors.

CCR3 co-localizes with CD4 and CCR5

The possibility that some strains of HIV-1 may require both CCR3 and CCR5 co-receptors suggests that these two chemokine receptors may co-localize at the cell membrane, with each other and with CD4. We therefore studied the subcellular localization of CD4 and CCR3 on U87-CD4-CCR3 cells and of CCR3, CD4 and CCR5 on microglia using confocal microscopy. CCR3 co-localized with CD4 in both cell types (Fig. 8). CCR3 was more diffusely distributed on U87-CD4-CCR3 cells than was CD4 (Fig. 8a). The latter was more localized at the cell membrane and >90 % of CD4 co-localized with CCR3. In microglia, however, CD4 and CCR3 were distributed similarly at the cell surface and approximately 50 % of CD4 co-localized with CCR3 (Fig. 8c). In microglia and U87-CD4-CCR3 cells transduced with SV(SFv-CCR3), the anti-CCR3 SFv and its target, CCR3, co-localized mainly in the cytoplasm (Fig. 8d). In microglia, CCR5 co-localized virtually completely with CCR3 (Fig. 8e). The physical proximity of CCR3 and CCR5 may help explain the apparent requirement of some strains of HIV-1 for both CCR3 and CCR5 for productive infection.

DISCUSSION

In these experiments, we generated and characterized gene delivery reagents designed to target and downregulate the eotaxin receptor, CCR3, and we used these reagents to study the role of CCR3 in HIV-1 infection, in both CNS and non-CNS cells. Two types of transgenes were devised to target CCR3. An SFv was produced from a well-characterized anti-CCR3 hybridoma (Heath *et al.*, 1997) using standard techniques, and delivered effectively to both a CCR3-expressing cell line and primary human brain microglia. We also identified an RNAi species that targeted the CCR3 transcript (R3-526) and decreased cell surface CCR3. In parallel, we used two previously reported vectors, one with an SFv and one with an RNAi, that reduce CCR5 to examine co-receptor usage by a series of M-tropic and neurotropic strains of HIV-1. The specificity of each vector

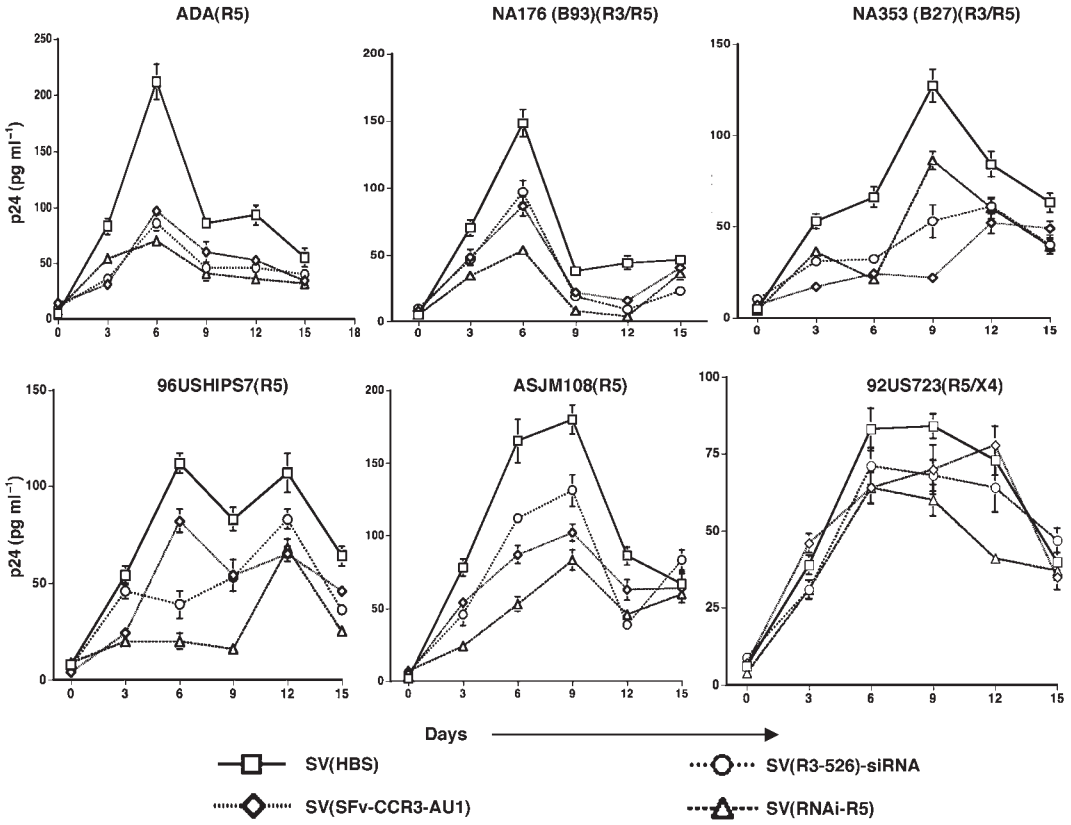

Fig. 6. HIV-1 strain differences in CCR3 and CCR5 usage as detected using rSV40-delivered RNAi and SFv against CCR3 and CCR5 in protection from CCR3/CCR5-tropic HIV-1 (primary brain isolates) and peripheral HIV-1 isolates in MDM. MDM were transduced at an m.o.i. of 10, with respective rSV40s as described in Methods. Transduced cells were challenged with HIV-1 strains ADA(R5), NA176 (B93)(R3/R5), NA353 (B27)(R3/R5), 92US723(R5/X4), 96USHIPS7(R5) or ASJM108(R5). Supernatants were collected every 3 days and were quantified by p24 ELISA. Data represent averages of two independent experiments. Cells were also analysed for surface expression of CCR3 on transduced cultures before HIV-1 infection with the isolates.

for its respective chemokine receptor target was documented: vectors targeting CCR5 did not reduce cell membrane CCR3 and vice versa.

To deliver these transgenes, we used *Tag*-deleted SV40-derived gene transfer vectors. rSV40s were used because of their high efficiency in transducing microglia, monocytes, MDM and dendritic cells (Cordelier *et al.*, 2003). A C-terminal AU1 epitope was added to the anti-CCR3 SFv cDNA to facilitate detection. It was observed by FACS analysis that the SFv and RNAi decreased cell membrane CCR3 by 40–75 %. This is comparable in magnitude to the reduction of CCR5 achieved using the RNAi versus CCR5 (Cordelier *et al.*, 2003).

Confocal microscopy showed almost complete co-localization of the anti-CCR3 SFv and cellular CCR3 mostly within the cytosol in SV(SFv-CCR3-AU1)-transduced microglia. Little CCR3 remained unbound, although some was present at the cell membrane, where it co-localized with the anti-CCR3 SFv. In U87-CD4-CCR3 cells, considerably more CCR3 did not co-localize with the SFv. This may be because these cells overexpressed CCR3 at levels exceeding the binding capacity of the SFv.

The quantitative functional effects of these transgenes on CCR3 activity exceeded their reduction in immunologically detectable cell membrane CCR3. Transduction with both transgenes completely blocked eotaxin-induced cellular calcium transients and chemotaxis (data not shown).

We then used these vectors, together with one carrying SFv against CCR5 and another with an RNAi to CCR5, to study co-receptor requirements among different strains of HIV-1. Antibodies against CCR3 and CCR5 and respective ligands, eotaxin and TAK-779, were also used to block these co-receptors. We primarily focused on brain microglia, as it has been suggested that CCR3 may play a role in CNS infection by HIV-1 (Choe *et al.*, 1996). Since MDM also carry both CCR3 and CCR5, selective downregulation of each was also used to evaluate their respective contributions to HIV-1 infectivity. Replication of some strains of HIV-1 in microglia and MDM was not impaired by decreasing CCR3, but was

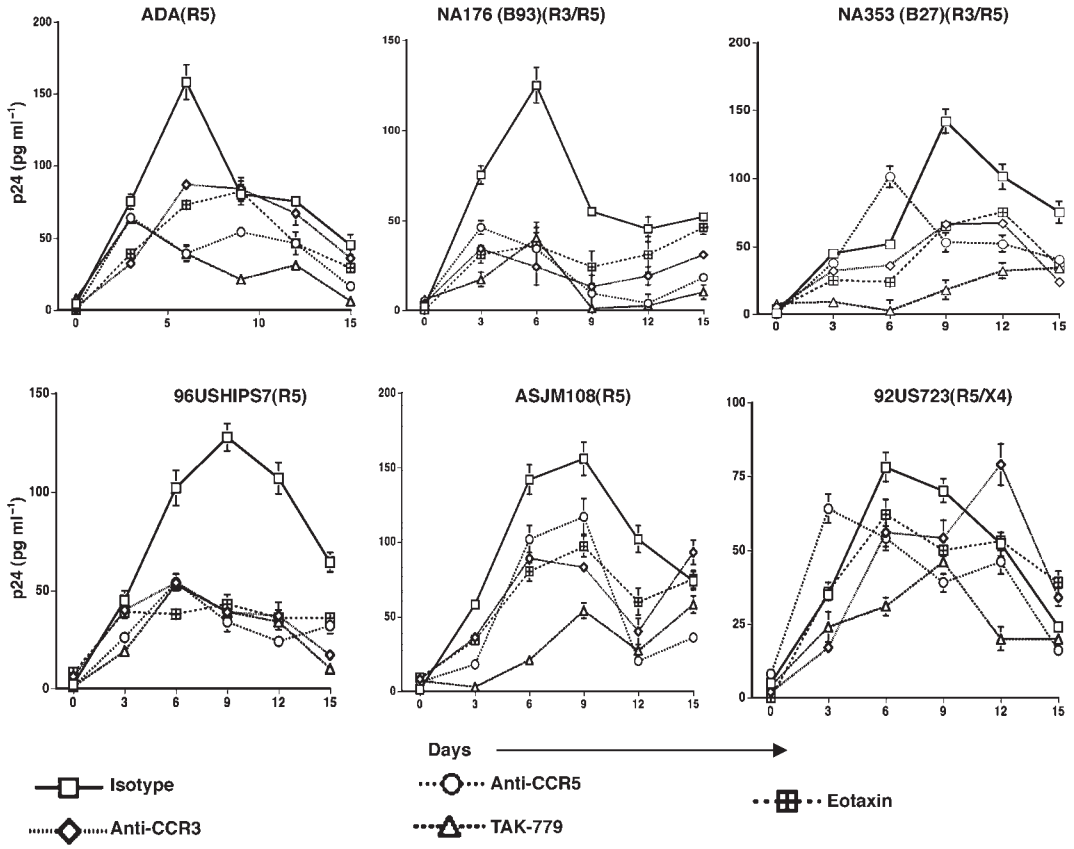

Fig. 7. HIV-1 strain differences in CCR3 and CCR5 usage as detected using anti-CCR3 or anti-CCR5 hybridoma antibodies, eotaxin or TAK-779 in protection from HIV-1 (primary brain and peripheral isolates) in MDM. MDM were prepared as described in Fig. 6 legend, and then cultured with 1 μg ml^{-1} of either anti-CCR3 or anti-CCR5 or an isotype-matched normal IgG antibody as a control. MDM were also incubated with 500 ng eotaxin ml^{-1} or 25 μM of TAK-779. Antibodies, eotaxin and TAK-779 were replenished every 3 days after collection of supernatants. HIV-1 replication was quantified by p24 ELISA. Data represent averages of two independent experiments.

inhibited by decreasing CCR5. These strains included HIV-1 SF-2(R5/X4), JR-FL and primary isolates, 92US657(R5/X4) and 92US723(R5/X4). The primary brain isolates, NA176 (B93)(R3/R5), NA353 (B27)(R3/R5) and isolates ADA(R5), 96USHIPS7(R5) and ASJM108(R5) showed a different phenotype. They were largely inhibited by prior treatment with vectors targeting either CCR3 or CCR5: SV(R3-526)-siRNA, SV(SFv-CCR3-AU1), SV(SFv-CCR5) or SV(RNAi-R5), suggesting that they utilize both simultaneously.

It should be noted that SV(RNAi-R5) and SV(SFv-CCR5), which decrease CCR5 comparably to the reductions in CCR3 seen with the anti-CCR3 SFv or RNAi, inhibit productive infection of microglia and MDM by R5-tropic strains of HIV-1 out of proportion to the magnitude of their downregulation of CCR5 (Cordelier *et al.*, 2003). Also, unimpeded HIV-1 replication in the face of CCR3 or CCR5 downregulation does not necessarily imply that this strain of HIV-1 does not utilize, or even require, the chemokine receptor in question. It may mean simply that it can enter cells with lower co-receptor density.

Growth of HIV-1 strains HIV-1 ADA(R5), HIV-1 96USSN20(R5/X4), NA176 (B93)(R3/R5), NA353 (B27)-(R3/R5), 96USHIPS7(R5) and ASJM108(R5) was inhibited comparably by both the anti-CCR3 and the anti-CCR5 antibodies and eotaxin. None of these strains was completely inhibited, but residual HIV-1 replication was comparable in CCR3-depleted and CCR5-depleted cells. These data again suggest that, at least for these strains of HIV-1, both chemokine receptors were needed for optimal effective infection. TAK-779 was a more potent inhibitor than eotaxin, the anti-CCR3 hybridoma, or even the 2D7 anti-CCR5 hybridoma for some strains of HIV-1 (e.g. NA353 in MDM), suggesting a strong preference for CCR5 by those strains and/or the possibility that TAK-779 is a more avid receptor blocker. The latter conclusion appears more likely as, even in strains strongly inhibited by TAK-779, substantial inhibition by eotaxin was also seen (e.g. NA176 in MDM).

If some strains of HIV-1 require (or prefer) both CCR5 and CCR3, one would expect that CD4, CCR5 and CCR3

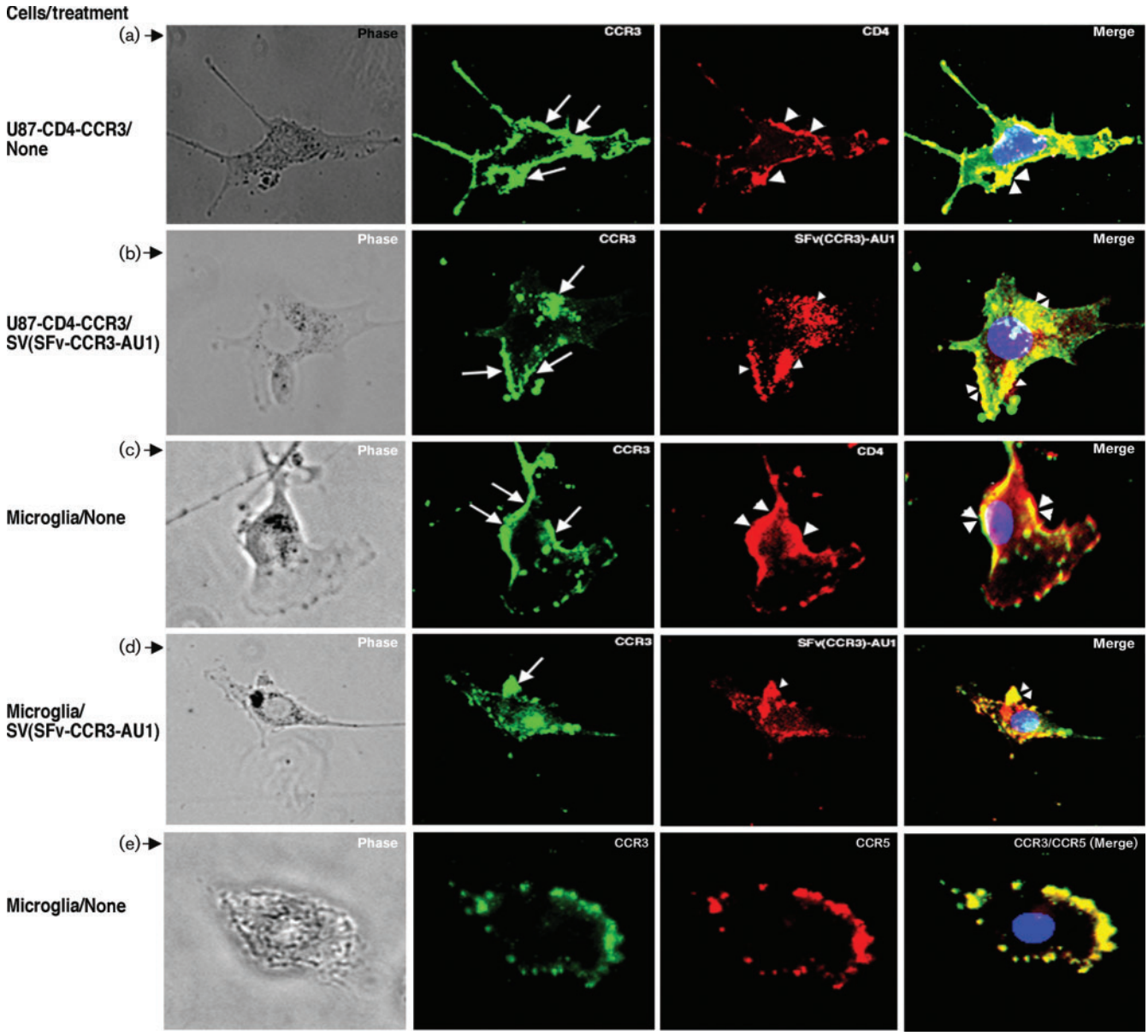

Fig. 8. Co-localization of CD4/CCR3 and CCR3/SFv(CCR3) in microglia and U87-CD4-CCR3 cells. U87-CD4-CCR3 (a and b) or primary microglia cells (c and d) were immunostained with anti-CCR3, anti-CD4 or anti-AU1 as described in Methods. CCR3 immunostaining is shown in green and representative areas are indicated by complete arrows (a–d). CD4 immunostaining (large arrow heads) is shown in red in panels (a) and (c), whereas red staining in panels (b) and (d) represents AU1 staining (small arrow heads) to detect the anti-CCR3 SFv expression delivered by transduction with SV(SFv-CCR3-AU1). Co-localization is depicted in yellow in right panels (a)–(d) (double arrowheads). CCR3/CD4 co-localization on the surface is highlighted by large double arrowheads, whereas co-localization of CCR3 and anti-CCR3 SFv in the cytoplasm is highlighted by small double arrowheads. Panel (e) depicts the co-localization of CCR3 and CCR5 in microglial cells. 4,6-diaminido-2-phenylindole (DAPI, nuclear staining) is shown in blue.

would co-localize on the cell membrane in microglia. Confocal microscopy demonstrated that these three cell membrane proteins were distributed along the cell membrane virtually identically. Therefore, it is possible that these strains of HIV-1 demonstrate a complexity of co-receptor usage, at least in microglia that differs from what has been described previously. The use of CCR3 as a co-receptor for infection of microglial cells by some strains of HIV-1 has been reported (Heath *et al.*, 1997). However, our data show that some strains of HIV-1 may be inhibited if either CCR5 or CCR3 is decreased, suggesting that both co-receptors may be needed, or preferred, by those isolates of HIV-1 in order to achieve optimal replication in microglia and macrophages. This phenotype is somewhat

different from that observed with most dual-tropic strains of HIV-1. Dual-tropic strains may generally utilize one or another co-receptor (e.g. CXCR4 or CCR5) with comparable efficiency so that the virus replication occurs if either, or neither, co-receptor is blocked. It is possible, however, to see the apparent requirement or preference for both co-receptors on the part of some HIV-1 strains as representing a special case of dual tropism. It should be emphasized that this phenotype was observed when the target cells were of peripheral origin (MDM) as well as of CNS origin (microglia), and in HIV-1 strains isolated both from the CNS and from the periphery.

These data have important implications for therapeutics. If CCR3 is commonly involved as a co-receptor for HIV-1, perhaps in preference to CCR5 in some cases, therapeutic strategies focused on inhibiting viral entry may benefit by taking such tropism into account.

In conclusion, CCR3 may play an important role in the pathogenesis of HIV-1 infection in the CNS and in the periphery. Some strains of HIV-1 may use CCR3 together with CCR5. For other strains, the presence of CCR5 may be sufficient for cell entry. Reagents described here, viral vectors carrying SFv and RNAi that are effective in downregulating CCR3, may be useful in elucidating the role of the eotaxin receptor in HIV-1 infection.

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