

Up-regulation of surface feline CXCR4 expression following ectopic expression of CCR5: implications for studies on the cell tropism of feline immunodeficiency virus.

BRIAN J. WILLETT*, CELIA A. CANNON & MARGARET J. HOSIE

Department of Veterinary Pathology, University of Glasgow, Bearsden Road, Glasgow G61 1QH, United Kingdom.

Running title: CXCR4 and CCR5 in FIV infection

* corresponding author

Dr. B.J. Willett
Retrovirus Research Laboratory
Department of Veterinary Pathology
University of Glasgow
Bearsden Road
Glasgow G61 1QH
United Kingdom

E-mail: b.willett@vet.gla.ac.uk

Tel: +44 141 330 3274, Fax: +44 141 330 5602

SUMMARY

Feline CXCR4 and CCR5 were expressed in feline cells as fusion proteins with enhanced green fluorescent protein (EGFP). Expression of the EGFP fusion proteins was localized to the cell membrane and surface expression of CXCR4 was confirmed using a cross-species reactive anti-CXCR4 monoclonal antibody. Ectopic expression of feline CCR5 enhanced expression of either endogenous feline CXCR4, or exogenous feline or human CXCR4 expressed from a retroviral vector, indicating that experiments investigating the effect of CCR5 expression on FIV infection must be interpreted with caution. Susceptibility to infection with cell culture adapted strains of FIV, or to syncytium formation following transfection with a eukaryotic vector expressing an *env* gene from a cell culture-adapted strain of virus, correlated with expression of either human or feline CXCR4 whereas feline CCR5 had no effect. In contrast, neither CXCR4 nor CCR5 rendered cells permissive to either productive infection with primary strains of FIV or syncytium formation following transfection with primary *env* gene expression vectors. Screening a panel of GHOST cell lines expressing diverse human chemokine receptors confirmed that CXCR4 alone supported fusion mediated by the FIV Env from cell culture-adapted viruses. CXCR4-expression was up-regulated in GHOST cells co-expressing CXCR4 and CCR5, or CXCR4, CCR5 and CCR3 and susceptibility to FIV infection could be correlated with the level of CXCR4 expression. The data suggest that β -chemokine receptors may influence FIV infection by modulating the expression of CXCR4.

INTRODUCTION

Infection of the domestic cat with feline immunodeficiency virus induces an illness similar to AIDS in HIV-infected human beings (39, 48). Although infection with FIV is accompanied by a gradual decline in the number of CD4⁺ lymphocytes (2), the feline homologue of CD4 does not appear to act as a primary binding receptor for infection with the virus and ectopic expression of feline CD4 on feline cells does not confer susceptibility to infection with FIV (36). Further, the expression of CD4 in the domestic cat is restricted to helper T cells and their thymic precursors and, unlike human CD4, feline CD4 is not expressed on cells of the monocyte / macrophage lineage (1). Previous studies have demonstrated that feline monocyte/macrophage lineage cells, and a range of other CD4-negative cells (CD8⁺ lymphocytes, B lymphocytes, astrocytes and Schwann cells) are susceptible to infection with FIV (6, 14). If the primate lentiviruses evolved the use of CD4 as high affinity binding receptor in order to target the viruses more efficiently to T helper lymphocytes and monocyte/ macrophages, it is possible that FIV represents a more primitive ancestor of HIV, encoding fewer regulatory genes (the FIV genome lacks *nef*, *vpu* and *vpr* open reading frames) and lacking the specific targeting of CD4⁺ cells. As such, by studying FIV, it may be possible to identify the viral determinants that render the feline and primate lentiviruses immunodeficiency-causing rather than inducing chronic inflammatory conditions as typified by caprine arthritis encephalitis virus (CAEV) and (Maedi-Visna virus) MVV. With the discovery of the role of seven transmembrane domain superfamily (7TM) molecules in infection with the primate lentiviruses, a possible link between the feline and primate lentiviruses was uncovered. Subsequently, it was revealed that FIV uses the chemokine receptor CXCR4

as a receptor for infection (53); ectopic expression of CXCR4 confers susceptibility to infection with FIV (50, 55) and the FIV envelope glycoprotein binds CXCR4 with a high affinity (19). Further, FIV infection is inhibited by the natural ligand for CXCR4 (19) (SDF-1, CXCL12) and CXCR4 antagonists such as met-SDF, AMD3100 and ALX-404C (16, 43, 51). Given the importance of the virus-receptor interaction in determining the cell tropism of a virus, the shared usage of CXCR4 as a cellular receptor by HIV and FIV represents a potential means by which the viruses may induce similar pathologies.

The principal chemokine receptors utilized by HIV as co-receptors for infection are CXCR4 and CCR5 (a receptor for the β -chemokines RANTES, MIP-1 α and MIP-1 β) (3, 12, 15, 18). While a diverse range of other 7TM molecules has been shown to act as co-receptors for the primate lentiviruses (reviewed in (8)) the role of these additional molecules in the pathogenesis of AIDS remains unclear. While CCR5 appears to be the co-receptor utilized by the majority of strains HIV early in infection, usage of CXCR4 as a co-receptor is more frequent with disease progression (45). The shift in co-receptor usage from CCR5 to CXCR4 (formerly identified as non-syncytium-inducing (NSI) and syncytium-inducing (SI) respectively) with disease progression raises the question as to whether usage of CXCR4 as a viral receptor arises a result of disease progression or whether it hastens disease progression.

Previous studies have demonstrated that during the early phase of infection with FIV, the major reservoir of infected cells in peripheral blood is CD4⁺ lymphocytes. In contrast, in chronic infection both CD8⁺ lymphocytes and B lymphocytes are infected suggesting a shift in viral tropism with prolonged infection (10, 11, 17). These data provide compelling evidence for the existence of viruses with distinct cell tropisms in

infected cats, analogous to CCR5 and CXCR4-dependent viruses in HIV-infected individuals. Conflicting data have been presented regarding the usage of CCR5 as a co-receptor by FIV. Early studies demonstrated that ectopic expression of CCR5 did not confer susceptibility to infection with cell culture adapted strains of FIV (50) while studies on the inhibition of FIV infection with β -chemokines have provided little data to support a role for CCR5 in FIV infection, since RANTES, MIP-1 α and MIP-1 β failed to inhibit FIV_{GL8} infection of Mya-1 cells (19) and displayed only a 20-40% reduction in FIV_{PPR} infection of T cells (24). In contrast, a separate study showed that anti-human CCR3 and CCR5 could inhibit infection of human PBMC with FIV, suggesting that not only could FIV utilize CCR5 for infection, but that it could use human CCR3 and CCR5 as co-receptors for infection (22). Moreover, recent studies have suggested that the V1-CSF isolate of FIV requires co-expression of human CCR5 and CCR3 for infection of cells expressing human CXCR4 and have proposed that human CCR3 and CCR5 act as co-receptors for FIV infection (23). The aim of this study was to define further the role of CXCR4 and CCR5 in FIV infection. We demonstrate that ectopic expression of CCR5 enhances cell surface expression of CXCR4, and in doing so, may enhance susceptibility of CCR5-expressing cells to infection with CXCR4-dependent strains of FIV.

Materials and Methods

Plasmids and cDNA cloning. Feline CXCR4 (U63558) has been described previously. Feline CCR5 cDNAs (U92796) was obtained from J. Elder (Scripps Research Institute, La Jolla, CA). pCI-VSV-G was obtained from G. Nolan, Stanford University, USA. pHIT60 was obtained from A. Kingsman, Oxford Biomedica, Oxford, UK. cDNAs were sub-cloned into the EGFPN1 vector (Clontech Laboratories Inc., Palo Alto, USA.) as EcoRI/BamHI fragments creating an N-terminal fusion with EGFP. Sub-cloning of the cDNAs were performed using the polymerase chain reaction (PCR, Hi-Fidelity PCR system, Roche Diagnostics Ltd., Lewes, UK) and a GeneAmp PCR system 9700 thermal cycler (PE Applied Biosystems, Warrington, UK), with oligonucleotide primers carrying the appropriate restriction sites; amplification of CXCR4 used primers 5'-GCGAATTCACCATGGACGGGTTTCGTATATAC-3' and 5'-CGGTGGATCCGAGGAGTGAAAACCTTGAAGA-3' while CCR5 was amplified with primers 5'-GCGAATTCACCATGGATTATCAAGCCACGAG-3' and 5'-CGGTGGATCCAAGCCGACAGAGATTTCTG-3'. The CXCR4 and CCR5/EGFP fusion products were then sub-cloned as Sall/HpaI fragments into the pDONAI retroviral vector (Takara Shuzo Co. Ltd., Shiga, Japan) by re-amplification using the primers 5'-GCGTCGACGCTAGCGCTACCGGACTCAGATCT-3' and 5'-TTGTTAACGCGGCCGCTTTACTTGTACAGCTC-3'.

FIV env genes were amplified from the GL8₄₁₄ (21), PPR (40), PET_{F14} (37), and the GL8_{EK} (21) molecular clones by PCR as above using the oligonucleotide primers 5'-GGGTCGACACCATGGCAGAAGGGTTTGCAGCA-3' and 5'-GGGCGGCCCGCCATCATTCTCCTCTTTTTTCAGAC-3', incorporating Sall and NotI

restriction sites, and cloned into the eukaryotic expression vector VR1012 (Vical Incorporated, San Diego, USA). The nucleic acid sequences of all cDNAs subcloned by HiFidelity PCR were determined using Big Dye Terminator Cycle Sequencing kit Version 2 (ABI Prism, Applied Biosystems) and an Applied Biosystems 3700 capillary sequencer.

Viruses and cell lines. All cell culture media and supplements were obtained from Invitrogen Life Technologies Ltd. (Paisley, UK). Adherent cell lines were maintained in Dulbecco's modification of minimum essential medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 0.11mg/ml sodium pyruvate, 100 IU/ml penicillin and 100 µg/ml streptomycin (DMEM). GHOST(3) cell lines (34) were obtained from the MRC AIDS directed programme. AH927 and Ghost cells expressing feline CXCR4 and CCR5, or human CXCR4, were generated by transduction with the retroviral vectors pDONAI (Takara Shuzo Co. Ltd.) or pBabePuro (33) bearing the appropriate cDNA. Murine leukaemia virus (MLV) pseudotypes carrying the pDONAI or pBabePuro retroviral vectors were prepared by transfection of HEK 293T cells with the retroviral vector in conjunction with pHIT60 (47) (encoding the MLV *gagpol*) and pCI-VSV-G (encoding the vesicular stomatitis G protein) at a 1:1:1 ratio using Superfect transfection reagent (QIAGEN Ltd., Crawley, UK). Supernatants were collect 48 hours post-transfection, filtered at 0.45µm, and used to transduce the target cell lines at an approximate multiplicity of infection of 1.0 (assessed by transducing a parallel culture with pseudotypes bearing a retroviral vector encoding a *lacZ* reporter gene). Two days post-transduction the target cells were sub-cultured and re-seeded in culture medium supplemented with 800µg/ml G418 (Geneticin, Life

Technologies) or 2.5µg/ml puromycin (Sigma). Cells were maintained in the selective antibiotic until stably transduced populations outgrow the cultures. GHOST-FX4 and FR5 were generated by transduction of GHOST cells with feline CXCR4 or CCR5 in the retroviral vector pBabepuro (33).

The IL2-dependent feline T cell lines Mya-1 (32), Q201 (52) and peripheral blood mononuclear cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5×10^{-5} M 2-mercaptoethanol and 100 IU/ml recombinant human interleukin 2 (RPMI). Virus stocks were prepared from molecular clones of FIV (GL8₄₁₄ (21), PPR (40), PET_{F14} (37), TM2 (31) and the GL8_{EK} (21)). FIV-B-2542 was obtained from S. Vandewoude, Colorado State University, Fort Collins, CO, USA) Molecular clones were transfected into the human epithelioid cell line HEK 293T using Superfect transfection reagent (QIAGEN Ltd.). 48 hours post transfection, supernatants were harvested, 0.45µm filtered and used to infect the IL2-dependent feline T cell line Mya-1 (32). The infected cultures were monitored visually for cytopathicity and for the production of FIV p24 by enzyme-linked immunosorbent assay (ELISA, PetCheck FIV antigen ELISA, IDEXX Corp., Portland, Maine, USA). Supernatants were collected at peak cytopathicity / p24 production, 0.45µm filtered, dispensed into 1ml aliquots and stored at -70°C.

The growth of FIV strains *in vitro* was assessed by infection of the target cell line with virus stocks prepared in IL2-dependent T cells (Mya-1 cells). Cells were incubated with a matched tissue culture infective dose of virus for one hour at 37°C, washed twice with phosphate buffered saline, fed with fresh culture medium and maintained in culture for 7-10 days. Supernatants were collected every three days and assayed for the

production of p24 (PetCheck FIV antigen ELISA, IDEXX Corp.) or reverse transcriptase (RT) using Lenti-RT non-isotopic RT assay kit (Cavidi Tech., Uppsala, Sweden).

Antibodies and flow cytometry. Antibodies were used either un-conjugated or conjugated to either phycoerythrin (PE) or fluorescein isothiocyanate (FITC). Anti-CXCR4 (human-specific) antibody 12G5 was obtained from James Hoxie, University of Pennsylvania, USA. Anti-CXCR4 (human/feline cross-reactive) #44717 and #44708 (human CXCR4-specific) and anti-human CCR5 #45519 were obtained from Dr. Monica Tsang (R&D systems, Minneapolis, USA). Un-conjugated primary antibodies (IgG isotype) were detected using FITC or PE-coupled F(ab')₂ fragment of sheep anti-mouse IgG whole molecule (Sigma, Poole, UK). Samples were analysed on Beckman Coulter Epics Elite and EPICS XL flow cytometers and 10,000 events were collected for each sample. Data were analysed using Expo32 ADC software (Applied Cytometry Systems, Sheffield, U.K.). In the analysis of GHOST cells expressing CXCR4 or CCR5, percentages were calculated relative to the GHOST cells parent cell lines by overlaying histograms and applying Overton analysis (38) using the Expo32 ADC software package.

In vitro expression of *env* genes. The expression of functional Env proteins from the VR1012 expression constructs was confirmed by immunofluorescence using anti-FIV Env monoclonal antibody (vpg71.2). Immunofluorescence was performed on transfected HEK 293T cells at 72 hours post-transfection following fixation with ice-cold methanol. Fixed cells were re-hydrated using phosphate buffered saline containing 1.0% bovine serum albumin and 0.1% azide (PBA). The cells were then incubated with either

1µg of vpg71.2 or an isotype-matched control for 30 minutes on ice, washed twice with PBA by centrifugation, and then incubated with FITC-coupled F(ab')₂ fragment of sheep anti-mouse IgG whole molecule (Sigma Ltd.) on ice for a further 30 minutes. Finally, the cells were washed twice with PBA and then examined on a Leica UV microscope or by flow cytometry on an EPICS Elite flow cytometers (10,000 events collected in Listmode). To assess the fusogenicity of the FIV Env proteins, AH927 or Ghost cells were transfected with the VR1012-Env constructs using Superfect (QIAGEN) and incubated for 48 hours at 37°C. The cells were then fixed and stained with 1% methylene blue/ 0.2% basic fuchsin in methanol. Syncytia were enumerated by light microscopy using a x12.5 Leitz periplan eyepiece with a 6.5x9 graticule, three separate fields being counted per well, each well in duplicate. Syncytia were scored as cells with five or more nuclei.

Detection of viral entry using PCR. Ghost or AH927 cells were seeded in 6-well culture plates at 1.5×10^5 cells per well and incubated overnight at 37°C. Cells were infected with PET_{F14} or GL8₄₁₄ for 1 hour at 37°C, rinsed twice with phosphate buffered saline and then fed with fresh culture medium. Following overnight incubation at 37°C the cells were removed from the culture plates using trypsin-EDTA, pelleted and DNA prepared using a QIAamp DNA blood kit (QIAGEN). 0.5mg of DNA was then used in semi-quantitative polymerase chain reactions (PCR) in which either an 871bp FIV *gag* gene product was amplified using the primers 5'-GGG ATT AGA CAC TAG GCC ATC TA-3' and 5'-GAC CAG GTT TTC CAC ATT TAT TA-3' or a control cellular DNA for β-actin was amplified using the primers 5'-ATC TGG CAC CAC ACC TTC TAC AAT

GAG CTG CG-3' and 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'.

Reactions were denatured at 94°C for 3 min. followed by 30 cycles of denaturation at 94°C for 30sec., annealing at 50°C for 1 min. and extension at 72°C for 1min., with a final extension of 10min. at 72°C. All reactions were performed using HiFidelity PCR reaction mix (Roche) as per manufacturer's instructions on a GeneAmp PCR system 9700 (Perkin Elmer).

RESULTS

Ectopic expression of feline CXCR4 and CCR5. In order to examine further the role of CXCR4 and CCR5 in FIV infection, we developed cell lines that would stably express either molecule as C-terminal fusion proteins with enhanced green fluorescent protein (EGFP), enabling the expression of CXCR4 and CCR5 to be evaluated by flow cytometry and UV microscopy. The feline CXCR4-EGFP and CCR5-EGFP genes were subcloned into the pDONAI retroviral vector, packaged into murine leukaemia virus (MuLV) particles bearing the vesicular stomatitis virus (VSV) G-protein and used to transduce the feline cell lines AH927, CrFK and 3201, and the murine cell line 3T3. EGFP alone was included as the control. Figure 1 illustrates the results of flow cytometric analyses on the transduced AH927 cells (Fig. 1 a to c). EGFP expression was detected on each of the transduced cell lines, the highest mean fluorescence intensity being observed in the cells transduced with the retroviral vector bearing EGFP alone (mean fluorescence = 101.7, 99.2% positive). In contrast, while the majority of the cells transduced with CXCR4-EGFP or CCR5-EGFP expressed EGFP (97.3% and 91.1%), the mean fluorescence intensity was significantly lower (CXCR4-EGFP mean fluorescence = 6.1, CCR5-EGFP mean fluorescence = 3.7). UV microscopy revealed that while the fluorescence in the EGFP control was diffuse and cytoplasmic (Fig. 1d), fluorescence in the cells transduced with CXCR4-EGFP or CCR5-EGFP was largely peri-nuclear and membrane associated (Fig 1e and 1f), consistent with the predicted localisation of the EGFP-tagged chemokine receptors. Similar results were obtained with the 3T3, CrFK and 3201 cell lines (not shown).

We next examined the expression of CXCR4 at the cell surface of the transduced AH927 cells using anti-CXCR4 monoclonal antibody. Previously we identified anti-human CXCR4 monoclonal antibodies that cross-reacted with feline CXCR4 (19). Using two-colour flow cytometry we observed that a minority of the AH927 cells (4.6%) expressed CXCR4. A similar level of CXCR4 expression was detected on the cells transduced with EGFP alone (E, 4.8%, Fig. 2b). In contrast, 51.6% of the CXCR4-EGFP cells (FX4E) were CXCR4 positive (Fig. 2d) confirming that the CXCR4-EGFP fusion protein was expressed at the cell surface. 30.4% of the CXCR4-EGFP cells were EGFP positive but surface CXCR4-negative, suggesting that this represented intracellular CXCR4-EGFP. Finally, we examined at surface CXCR4 expression on the CCR5-EGFP transduced cells (FR5E). Surprisingly, transduction with the CCR5-EGFP expressing vector had increased surface expression of CXCR4 to 10.4%. Given that only 4.8% of the cells transduced with EGFP expressed surface CXCR4, these data suggested that ectopic expression of CCR5 may up-regulate endogenous CXCR4 expression on the AH927 cells.

The CCR5-EGFP cells were used to screen a range of anti-human CCR5 monoclonal antibodies (2D7, Leukosite Inc.; 45511, 45517, 45519, 45523, 45529, 45531 and 45533, R&D Systems), however, no cross-reactivity was detected (data not shown).

Up-regulation of CXCR4 following ectopic expression of CCR5. In order to investigate further the possible up-regulation of CXCR4 following CCR5 expression we examined the effects of transducing the AH927-derived cell lines expressing either EGFP (E-P) or the feCXCR4 and feCCR5-EGFP fusion proteins with a second series of

retroviral vectors expressing feline CXCR4 (FX4P), feline CCR5 (FR5P) or human CXCR4 (HX4P) from retroviral vectors carrying a selectable marker for puromycin resistance. Parallel cultures were transduced with the vector carrying puromycin resistance alone (P) as a control. CXCR4 expression was measured by flow cytometry using either an antibody recognizing a common epitope shared between feline and human CXCR4 (44718) or a human CXCR4-specific antibody (12G5). Transduction with the *puro* vector and selection in puromycin alone did not alter expression of CXCR4, 4.7% of cells in the control group “E-P” were CXCR4-positive representing basal endogenous CXCR4 expression (Fig.3a). Transduction of the EGFP control cells with FX4P (Fig. 3b) and HX4P (Fig. 3d) elevated CXCR4 expression to 26.1% and 80.1% respectively. Transduction with FR5P elevated CXCR4 (Fig. 3c) expression to 11.3%, confirming the previous observations with FR5E that ectopic CCR5 expression enhances endogenous CXCR4 expression.

We next examined CXCR4 expression on FR5E cells transduced with P (Fig. 3e), FX4P (Fig. 3f), FR5P (Fig. 3g) or HX4P (Fig. 3h). FR5E cells transduced with the *puro* vector alone continued to express enhanced levels of CXCR4 (28.7% of FR5E-P were CXCR4-positive compared with 4.7% of E-P). That 28.7% of the FR5E-P cells were CXCR4 positive (Fig. 3E) compared with 10.4% of FR5E (Fig. 2F) may reflect the higher passage number of the FR5E-P cells following selection in puromycin-containing medium and underlines the importance of the E-P control (4.7% positive) transduced and maintained in parallel. Following transduction with FX4P, 70.5% of FR5E-FX4P cells were CXCR4-positive. Compared with the E-FX4P (26.1%), the elevated expression of CXCR4 on FR5E-FX4P indicated that stable expression of feline CCR5 enhanced the

expression of both endogenous and ectopically expressed CXCR4. Transduction of FR5E with FR5P did not enhance CXCR4 expression further (FR5E-FR5P 27.8% CXCR4-positive compared with FR5E-P 28.7%). Similarly, transduction with HX4P did not enhance CXCR4-expression further (FR5E-HX4P 75.7% compared with E-HX4P 80.1%). These findings indicate that transduction with a feline CCR5 – expressing vector will a) increase surface expression of endogenous CXCR4 and b) enhance expression of ectopically expressed CXCR4 following subsequent transduction with a CXCR4-expressing vector. FX4E transduced with P (Fig. 3i), FX4P (Fig. 3j), FR5P (Fig. 3k) or HX4P (Fig. 3l) expressed similar levels of CXCR4 (86.4%, 87.2%, 88.3%, 86.7% respectively) suggesting that a maximal level of CXCR4 expression had been achieved and could not be increased further.

Using the anti-human CXCR4-specific monoclonal antibody 12G5, we examined the expression of human and feline CXCR4 on HX4P-transduced AH927- E, FR5E or FX4E cells (Figure 4). While 49.5% of control AH927-E cells transduced with HX4P were revealed as CXCR4-positive following staining with the 12G5 antibody (Fig. 4b)), 75.7% of FR5E (Fig. 4d) and 83.9% of FX4E (Fig. 4f) were positive for human CXCR4 following transduction. These findings confirm our previous findings showing enhanced human CXCR4 expression in cells expressing CCR5. Further, as prior transduction with feline CXCR4 also enhanced expression of human CXCR4, the data demonstrate that the effect is not CCR5-specific and that ectopic expression of feline CXCR4 will augment human CXCR4 expression.

Effects of CXCR4 and CCR5 expression on FIV infection. CXCR4 has been widely implicated in infection with FIV (13, 16, 41, 43, 50, 53, 55). In contrast, a single study suggested a role for human CCR5 and CCR3 in infection with FIV (22). Having generated cell lines that stably express feline CXCR4 and CCR5, we next asked whether these cells would be rendered permissive for either Env-mediated syncytium formation or cell-free virus infection with the GL8₄₁₄ and PET_{F14} clones (representing primary and CrFK-adapted strains of virus respectively). Each of the AH927 cell lines expressing feline CXCR4, feline CCR5 or human CXCR4 were either transfected with the eukaryotic expression vector VR1012 expressing the GL8₄₁₄ or PET_{F14} *env* genes, or infected with cell-free virus supernatant containing either the GL8₄₁₄ or PET_{F14} viruses (Fig. 5). Previous studies have demonstrated that infection of AH927 cells by FIV is blocked at the level of viral entry and that productive infection will occur following successful viral entry (21). Syncytium formation was monitored by light microscopy and scored + or - while productive infection was monitored by non-isotopic reverse transcriptase assay (expressed as A650nm). There was a good correlation between both syncytium formation in PET_{F14} Env transfected cells, PET_{F14} infection and ectopic expression of either feline or human CXCR4. In contrast, CXCR4 expression alone did not render AH927 cells permissive for either GL8₄₁₄ Env – mediated fusion or infection with GL8₄₁₄ virus (similar findings were obtained with the primary PPR strain of FIV, data not shown). Feline CCR5 expression alone was insufficient to render cells permissive to infection with PET_{F14} or GL8₄₁₄. Moreover, co-expression of feline CCR5 with either feline CXCR4 or human CXCR4 did not render the cells permissive to infection with GL8₄₁₄. Ectopic expression of CXCR4 or CCR5 did not render the AH927

cells permissive to infection with the primary strain clade B viruses TM2 or B₂₅₄₂ (data not shown). Thus feline CCR5 expression, either alone or co-expressed with feline CXCR4 or human CXCR4 was insufficient to render cells permissive to fusion / productive infection with four primary strains of FIV.

Feline or human CCR5 do not support fusion mediated by envelope glycoproteins from primary or cell culture adapted strains of FIV. Given that a previous study had suggested a role for human CCR5 or CCR3 in FIV infection (22), we examined the effect of transfecting chemokine receptor-expressing cells with expression vectors bearing envelope glycoproteins from primary or cell culture adapted strains of FIV. Following expression of the PET_{F14} or GL8_{EK} (a version of GL8 bearing an E₄₀₇K mutation in the V3 loop (21)) envelopes in GHOST cells expressing either feCXCR4 or feCCR5, or human CXCR4, CCR1, CCR2, CCR3, CCR4, CCR5, CCR8, Bonzo, BOB, EB-1 or V28, syncytia were observed in the cells expressing feline or human CXCR4 but in no other cell line (Figure 6.). Similarly, the primary envelopes GL8₄₁₄ or PPR did not induce syncytium formation in any of the cell lines tested. The cells co-expressing human CXCR4 and CCR5 (X4R5), or huCXCR4, CCR5 and CCR3 (X4R5R3) appeared to display enhanced syncytium formation following transfection with the PET_{F14} or GL8_{EK} Envs (Fig. 6), however they did not support syncytium formation following transfection with the GL8₄₁₄ or PPR Envs. Further, the panel of GHOST cell lines remained refractory to infection with HIV pseudotypes bearing the GL8₄₁₄ or PPR envelopes and carrying a luciferase reporter gene (9) (data not shown). Given that ectopic expression of feline CCR5 enhances cell surface expression of CXCR4, we

postulated that the enhanced syncytium formation mediated by the PET_{F14} and GL8_{EK} Envs in X4R5 and X4R5R3 cell lines may reflect enhanced CXCR4 expression in these cell lines. We therefore analysed CXCR4 and CCR5 expression on the GHOST cell lines by flow cytometry (Fig. 7). GHOST R5 (Fig. 7a) cells expressed low levels of CCR5 (6.97%). CCR5 expression was increased in GHOST X4R5 (28.27%) and more markedly in X4R5R3 (66.53%). Thus co-expression of CXCR4 or CXCR4 and CCR3 in GHOST R5 cells significantly enhanced CCR5 expression. Similar findings were observed for the expression of CXCR4 (Fig. 7b) with X4R5R3 cells expressing more CXCR4 (86.02%) than cells co-expressing CXCR4 and CCR5 (77.54%), or CXCR4 alone (73.26%). Finally, we compared the expression of CXCR4 on Ghost cells transduced with a puromycin resistance vector alone (control), CCR5 (R5) or selected for high levels of CCR5 expression (Hi5). As shown in Figure 7c, the Hi5 cells expressed more CXCR4 (33.9%) than the R5 cells (21.3%) or the control cells (19.1%), confirming that by selecting for human CCR5 expression, CXCR4 expression is also increased. The data suggest an alternative explanation for the enhanced syncytium formation in the cells co-expressing more than one chemokine receptor, this being up-regulation of surface CXCR4 expression. We next asked whether the enhanced syncytium formation in the X4R5 and X4R5R3 cells correlated with enhanced susceptibility to infection with PET_{F14} or GL8₄₁₄ (Fig. 8). Given that there is a post-entry block to the replication of FIV in Ghost cells, viral entry into the GHOST cell lines was assessed by PCR for FIV *gag* DNA at 24 hours post-infection. Infection of GHOST cells was enhanced significantly by co-expression of CXCR4 with CCR5 (X4R5) or CCR5 and CCR3 (X4R5R3) (Fig. 8a). Further, expression of CCR5 alone enhanced infection with PET_{F14} relative to the

control cells (transduced with *puro* vector alone). Similar findings were observed with GL8₄₁₄ although, as expected, infection was extremely inefficient compared to PET_{F14}. Expression of X4, X4R5, X4R53 or R5 alone increased viral entry relative to the controls. We compared (in parallel) the susceptibility of the AH927-derived cell lines FX4E, FR5E or E described above to infection with PET_{F14} or GL8₄₁₄. While a strong product was amplified from FX4E infected with PET_{F14}, extremely faint products were present in FR5E and E cells infected with PET_{F14}, or FX4E, FR5E or E infected with GL8₄₁₄. The finding that GL8₄₁₄ enters the human cell line Ghost more efficiently than the feline cell line AH927 is consistent with our previous findings which demonstrated that human CXCR4 supports fusion mediated by the FIV Env protein more efficiently than feline CXCR4 (50). Accordingly, AH927 cells expressing human CXCR4 support FIV infection more efficiently than cells expressing feline CXCR4 (data not shown).

DISCUSSION

In this study, we examined the effects of feline CXCR4 and CCR5 expression on FIV infection. In order to ensure that expression of the chemokine receptors could be monitored, they were expressed as C-terminal fusion proteins with the N-terminus of enhanced green fluorescent protein (EGFP). The CXCR4 and CCR5 EGFP fusion proteins were found to target the EGFP to the cell membrane and to be expressed at the cell surface (CXCR4). Using cells stably transduced with CXCR4-EGFP (FX4E) or CCR5-EGFP (FR5E) we demonstrated that CXCR4 expression was essential for infection of AH927 cells with the cell culture adapted strain of PET_{F14} but did not confer susceptibility to infection with the primary strain GL8₄₁₄. In contrast, CCR5 expression had no effect on susceptibility to infection with any of the four strains of FIV tested. In subsequent experiments we have found that ectopic expression of feline CCR5-EGFP on the feline lymphosarcoma cell line 3201 did not render the cells permissive to infection with primary strains of FIV (data not shown).

Previous studies have suggested that human CCR5 and CCR3 are involved in infection of human PBMC with FIV (22). These studies were based on the ability of monoclonal antibodies against human CCR5 and CCR3 to inhibit infection of human PBMC with FIV. In our studies we found no evidence for a role for feline CCR5 in FIV infection; ectopic expression of CCR5 does not confer susceptibility to infection with primary strains of FIV and infection with primary strains of FIV is not inhibited by β -chemokines (19) or viral chemokine homologues (v-MIP, data not shown). A number of studies have failed to show significant inhibition of FIV infection by β -chemokines (13,

19). RANTES may either enhance the binding of FIV SU to feline cells (13) or have partial inhibitory activity on FIV infection (24). Given that feline CCR5 and human CCR5 share only 82.6% amino acid similarity (76.6% identity), this difference being borne out by the failure of numerous anti-human CCR5 antibodies to recognize feline CCR5, the data would not predict a direct role for human CCR5 in FIV infection. Indeed, infection with the majority of FIV isolates studied to date can be blocked by the CXCR4 antagonist AMD3100 ((16, 43) and unpublished observations). In this study, we have demonstrated that ectopic expression of feline or human CCR5 leads to up-regulation of cell surface CXCR4 expression. Thus it is possible that modulating CCR3 or CCR5 expression may affect the expression of a known receptor for FIV, namely CXCR4.

The up-regulation of CXCR4 expression following ectopic expression of CCR5 has implications for experiments in which chemokine receptor usage by lentiviruses is evaluated *in vitro*. For example, a virus that is capable of using CXCR4 efficiently may infect cells transfected or transduced with a CCR5-expressing construct if CXCR4 is up-regulated to a sufficient degree. Accordingly, we found that GHOST cells expressing β -chemokine receptors expressed enhanced levels of CXCR4 and were more susceptible to fusion mediated by PET_{F14} or GL8_{EK} Envs, and supported viral entry more efficiently following challenge with either the PET_{F14} or GL8₄₁₄ strains of FIV. Our findings indicate that the ectopic expression of CCR3 and CCR5 may modulate CXCR4 expression, analogous to the modulation of feline CXCR4 expression by phorbol myristate acetate (PMA) or stromal derived factor-1 (SDF-1) (19). Overnight incubation with SDF-1 or PMA resulted in up-regulation of CXCR4 expression, enhancing

susceptibility to FIV infection (19). Previous studies have indicated that chemokine receptors may form homodimers and heterodimers and that dimerisation of chemokine receptors is a critical step in the signaling process (27, 30, 44). Indeed, the formation of heterodimers between CXCR4 or CCR5 and CCR2V64I (a mutant of CCR2 associated with a delay in progression to AIDS (46)) has been proposed as a mechanism by which CCR2V64I prevents HIV-1 infection (28). Moreover, HIV-1 infection is blocked by dimerisation of CCR5 (49) and the formation of heterodimers between CCR2 and CCR5 triggers distinct signaling pathways from either CCR2 or CCR5 expressed as homodimers (29, 44). Recently, CCR5 was found to exist in several active states and oligomerisation of CCR5 resulted in internalization of the receptor via a distinct pathway to that induced by the receptor's agonist (5), suggesting that the regulation of chemokine receptor expression is complex and dependent on many variables. Thus, the up-regulation of endogenous feline CXCR4 or exogenous human CXCR4 following ectopic expression of feline CCR5 may affect the sensitivity of the target cells to infection with FIV, or to the antagonistic effects of chemokines on FIV infection.

Previously, it was observed that human CCR3 or CCR5-expressing cells supported infection with the V1CSF strain of FIV, and yet there was an absolute requirement for CXCR4 expression for infection to occur and anti-CXCR4 antibody completely ablated infection (23). Further, although ectopic expression of human CCR3 and CCR5 on feline cells (CrFK) enhanced infection with FIV strain V1CSF, the parent cell line (CrFK) also supported infection at a lower level in the absence of human CCR3 or CCR5 (23). Either CrFK cells express a chemokine receptor in addition to CXCR4 that substitutes for human CCR3/CCR5, or V1CSF is capable of using CXCR4 alone as a

receptor inefficiently. That the V1CSF strain infects GHOST cells expressing CCR3 and CCR5 but not CXCR4 alone, and yet infection is blocked completely by anti-CXCR4 antibody, is intriguing and may suggest the use of a CXCR4-containing receptor complex for infection as has been suggested (23). We have found that ectopic expression of human β -chemokine receptors in human or feline cells may increase the expression of endogenous CXCR4, increasing the susceptibility of the cells to infection with X4-dependent viruses. Infection of GHOST cells with FIV_{PET} or FIV_{GL8} correlates with the expression of human CXCR4 within these cells and is consistent with the preference for human CXCR4 over feline CXCR4 as a co-factor for Env-mediated fusion by cell culture adapted strains of FIV (50). The contribution of CXCR4 up-regulation to infection with the V1CSF strain will require further investigation, however, it is possible that the V1CSF isolate reflects a novel strain of FIV with a broad preference for chemokine receptor usage, analogous to dual-tropic strains of HIV. Determination of the prevalence of such strains of virus in the cat population clearly merits further investigation and comparison with established, highly characterized, strains of FIV.

The mechanism by which co-expression of one chemokine receptor up-regulates the surface expression of another chemokine receptor remains to be established. Previous studies have demonstrated that 7TM molecules are capable of forming both homodimers and heterodimers on the cell surface. Further, engagement of chemokine receptors by either natural or synthetic ligands can induce receptor down-regulation. Chemokine receptors such as CCR5 distribute asymmetrically in polarized cells, associating with membrane microdomains (26), structures of importance in membrane trafficking and signal transduction. HIV infection is thought to proceed following an interaction with

chemokine receptors clustered within these regions (25) and the co-localisation of CD4 with CXCR4 and CCR5 within these regions is required for productive infection of PM1 T cells (42). If CXCR4 and CCR5 or CCR3 exist as heterodimers, or are localized to microdomains on the cell surface, it is conceivable that anti-CCR3 and CCR5 antibodies may interfere with viral access to CXCR4 or disrupt the membrane microdomains essential for viral entry. Indeed, monoclonal antibodies recognizing molecules associated with lipid rafts inhibit syncytium formation mediated by HTLV-1 (35). Moreover, since CD9 (a TM4 superfamily molecule) is also associated with lipid rafts (7), the disruption of membrane microdomains by anti-CD9 antibodies may account for previous conflicting results in which anti-CD9 antibodies blocked infection with FIV and ectopic expression of feline CD9 enhanced susceptibility to FIV infection, and yet, ectopic expression of CD9 was insufficient to render non-susceptible cells permissive for FIV infection (20, 54).

The results of this study demonstrate that while FIV infection of the domestic cat provides a unique opportunity to study an immunodeficiency-causing lentivirus in its natural host, resolution of the role of β -chemokine receptors in FIV infection will be of importance to our understanding of the evolution of virulence in lentiviruses. In this way the cat model will be valuable for the study of β -chemokine receptor antagonists as potential therapeutics for the treatment of AIDS.

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FIGURE LEGENDS

Figure 1. Expression of feline CXCR4 and CCR5 as EGFP fusion proteins. AH927 cells were transduced with retroviral vectors carrying feline CXCR4.EGFP (b., e.), CCR5.EGFP (c., f.) or EGFP (a., d.) alone and selected in G418 (800µg/ml). EGFP expression was quantified by flow cytometry (a., b., c.) while sub-cellular localization was analysed by UV microscopy (d., e., f.). Histograms represent plots of fluorescence intensity (x-axis) vs. relative cell number (y-axis).

Figure 2. Estimation of surface CXCR4 expression in transduced cells by flow cytometry. AH927 cells were transduced with vectors carrying feline CXCR4-EGFP (c.,d.), feline CCR5-EGFP (e.,f.) or EGFP alone (a.,b.). Two colour dot plots of EGFP expression (x-axis) vs. CXCR4 expression (y-axis) (b.,d.,f.) compared with isotype-matched control (A,C,E). Each dot plot represents 10,000 events.

Figure 3. Estimation of total surface CXCR4 expression in transduced AH927 cells by flow cytometry. AH927 cells stably transduced with pDONAI-based retroviral vectors bearing EGFP (E), feCXCR4.EGFP (FX4E) or feCCR5.EGFP (FR5E) were transduced again with a second series of pBabePuro vectors bearing feCXCR4 (FX4P), feCCR5 (FR5P), huCXCR4 (HX4P) or vector only (P) and selected in puromycin. Two colour dot plots of EGFP expression (x-axis) vs. surface CXCR4 expression (y-axis). Each dot plot represents 10,000 events.

Figure 4. Enhanced expression of human CXCR4 in cells transduced previously with feline CXCR4-EGFP (FX4E, (e., f.)) or CCR5-EGFP (FR5E, (c., d.)) or EGFP alone (E, (a., b.)). E, FX4E or FR5E cells were transduced with feline CXCR4 (FX4P, (a., c., e.) or human CXCR4 (HX4P, (b., d., f.)) in the vector pbabepuro. Human CXCR4-specific monoclonal antibody 12G5 was used to differentiate human and feline CXCR4. Two colour dot plots of EGFP expression (*x*-axis) vs. surface CXCR4 expression (*y*-axis). Each dot plot represents 10,000 events.

Figure 5. Susceptibility of AH927 cells transduced with CXCR4 or CCR5 to cell-free virus infection with cell culture adapted (PET_{F14}) or primary strains of FIV (GL8₄₁₄) and to syncytium formation following transfection with expression vectors carrying the PET_{F14} or GL8₄₁₄ *env* genes. Productive infection was measured by non-isotopic reverse transcriptase assay (absorbance 405nm), syncytium formation was scored + or -. Feline T cell line Mya-1 was included as a control for infection with GL8₄₁₄.

Figure 6. Syncytium formation in GHOST cells expressing feline or human chemokine receptors. Expression vectors carrying primary (GL8₄₁₄ or PPR) or cell culture adapted (PET_{F14} or GL8_{EK}) *env* genes were transfected into GHOST cells expressing a range of feline or human chemokine receptors. Syncytium formation was assessed by light microscopy at 48 hours post transfection. Typical results for PET_{F14} are shown with scoring + or - (left panel), a summary of the results for all four *env* genes are shown (right panel).

Figure 7. Flow cytometric analysis of CCR5 and CXCR4 expression on Ghost cell lines. Ghost cell lines X4, R5, X4R5 and X4R5R3 were stained with either anti-huCCR5 (R&D systems #45519) or anti-huCXCR4 (R&D systems #44708). Bound antibody was detected using PE-conjugated anti-mouse IgG. 10,000 events were collected for each sample. Histograms represent plots of fluorescence intensity (x-axis) vs. relative cell number (y-axis).

Figure 8. Detection of viral entry into chemokine receptor-expressing cell lines. A. Ghost X4, R5, X4R5, X4R5R3 or vector-only control cells, or (B) AH927 FX4E, FR5E or E cells, were infected with PET_{F14} or GL8₄₁₄ for 24 hours and the polymerase chain reaction was then used to detect viral (*gag*) or cellular (β -actin) DNA. Products were visualized by agarose gel electrophoresis and ethidium bromide staining.

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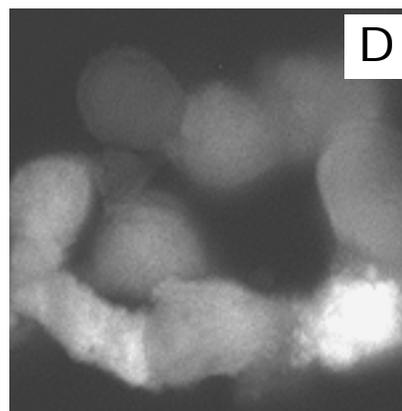
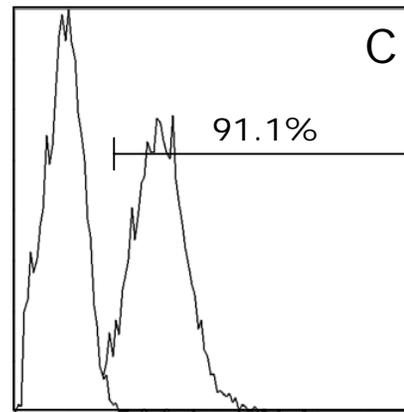
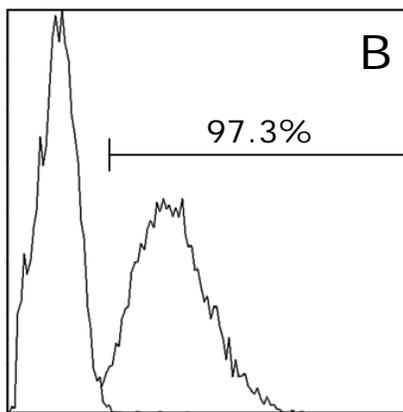
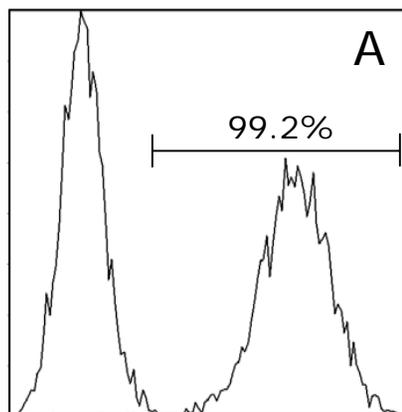
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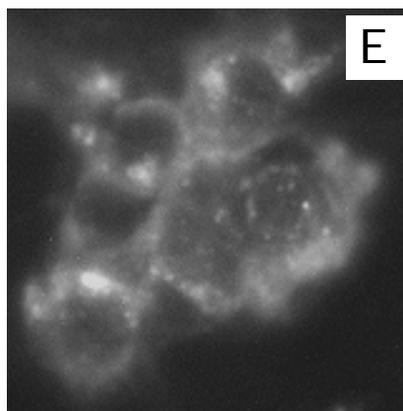
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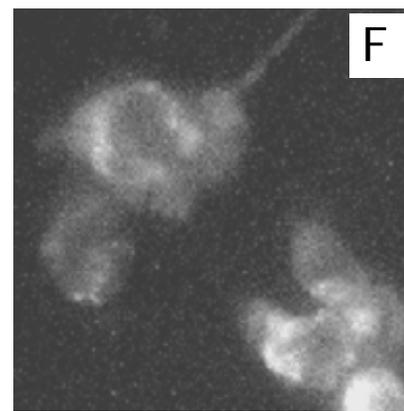
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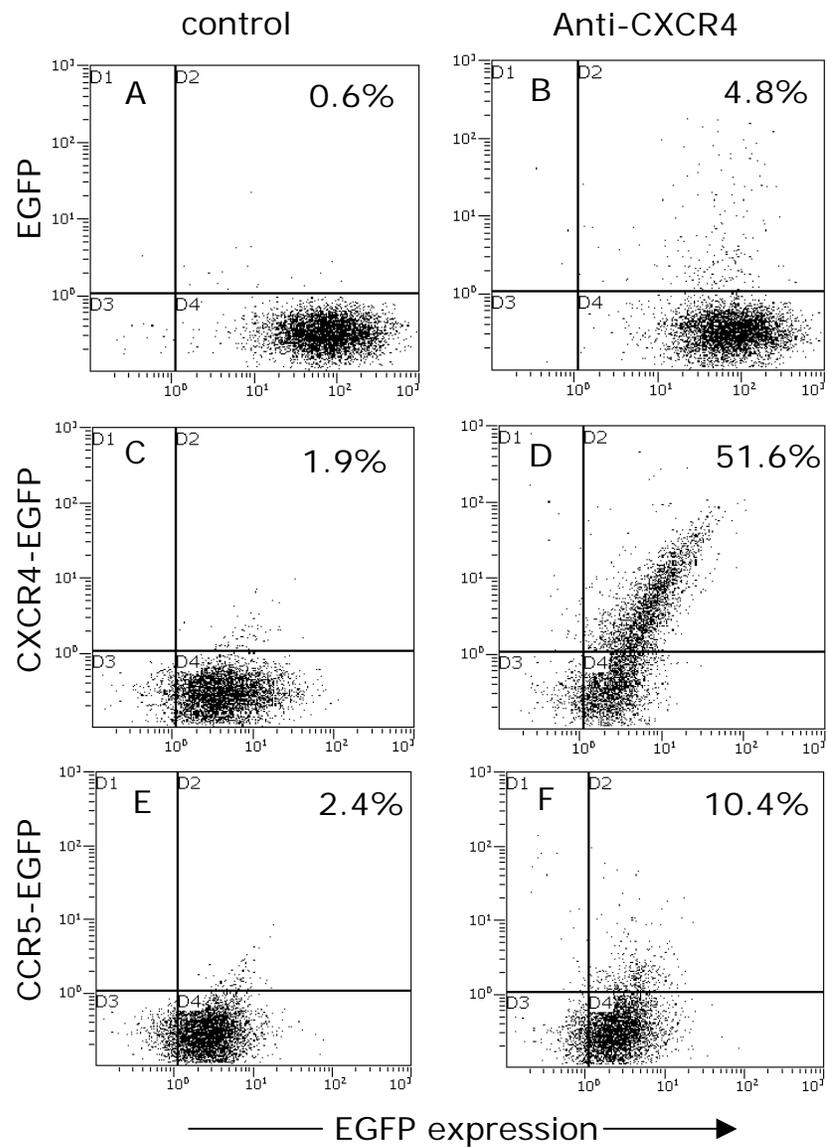
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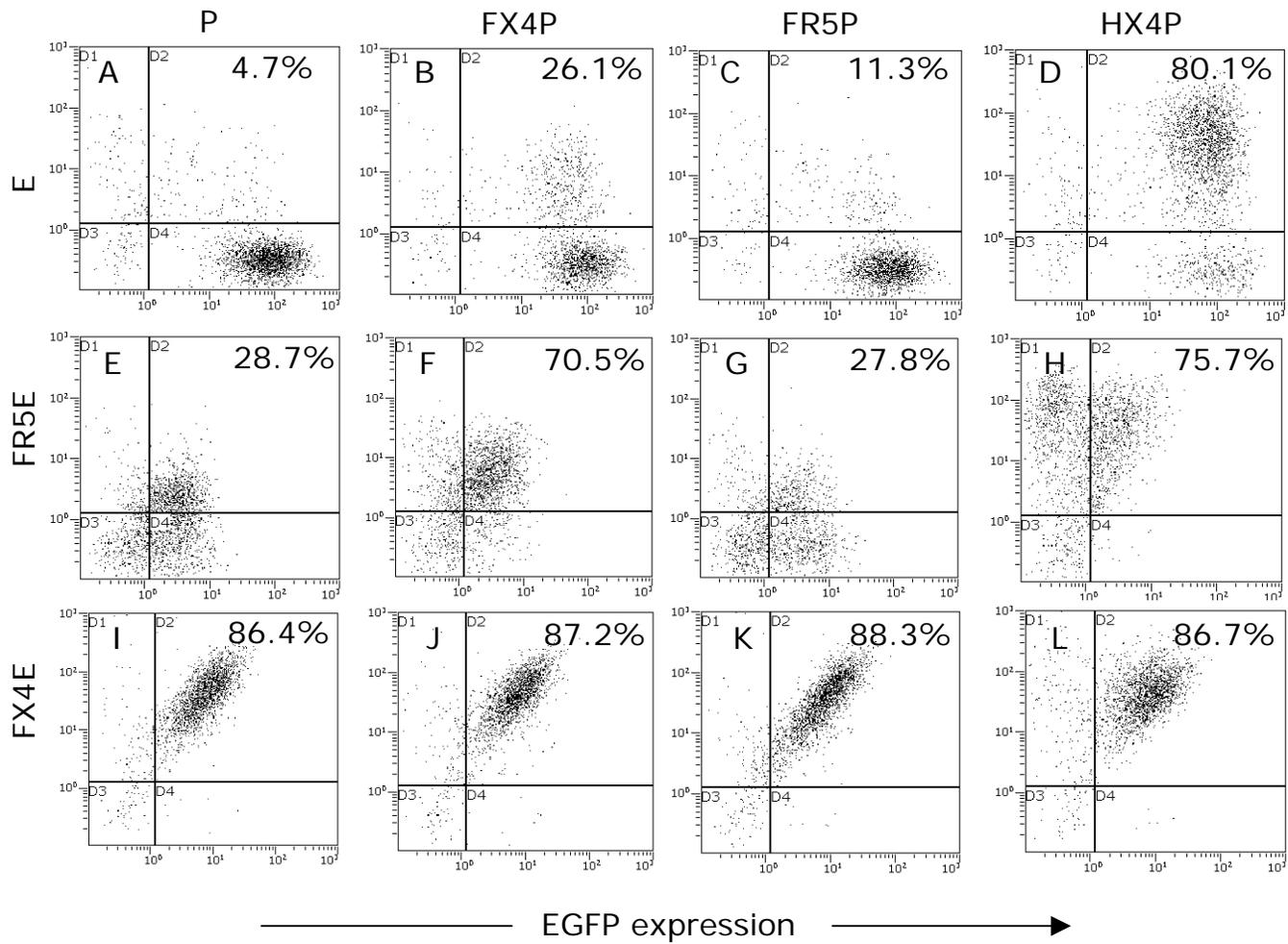


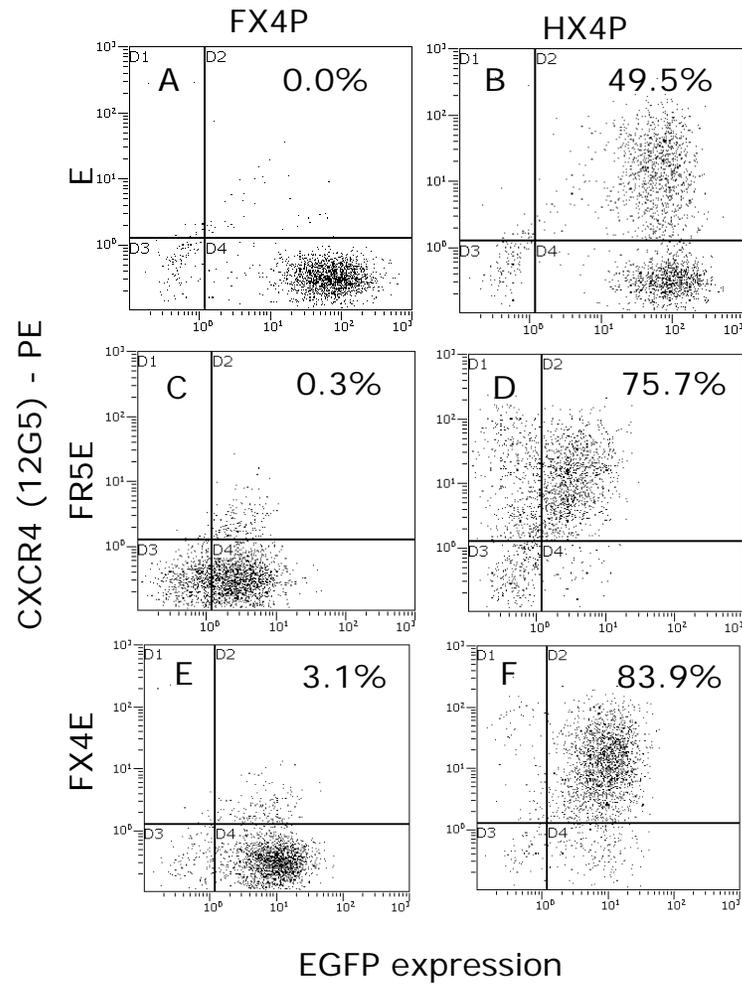
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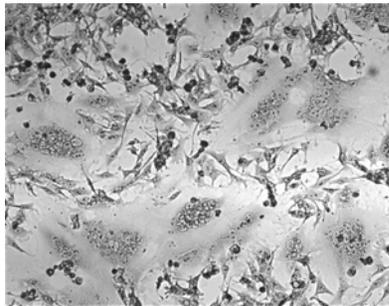
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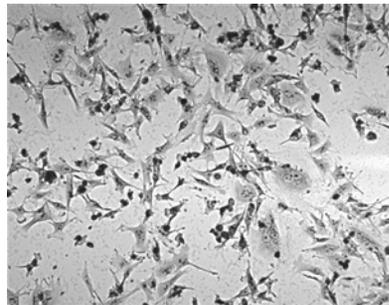


PET



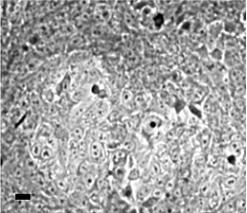
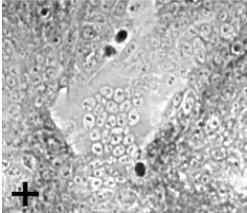
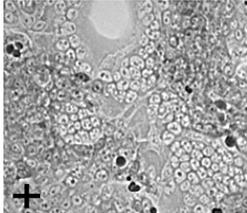
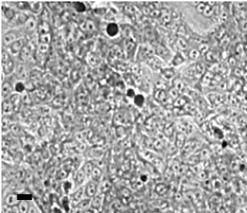
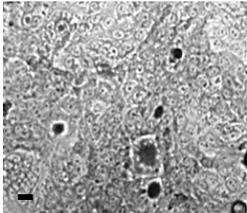
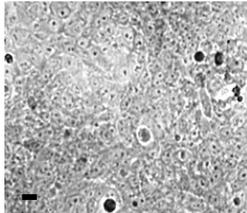
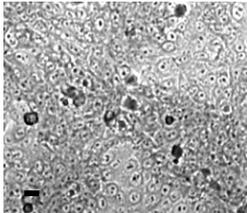
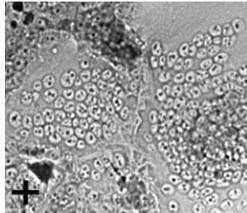
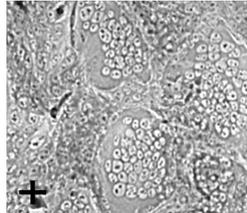
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GL8



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Receptor construct		PET _{F14}		GL8 ₄₁₄	
Vector 1 (G418)	Vector 2 (puro)	fusion	infection	fusion	infection
FX4E	P	+	5.28	-	0.18
FR5E	P	-	0.18	-	0.19
E	HX4P	+	4.49	-	0.13
E	FX4P	+	6.00	-	0.19
E	FR5P	-	0.17	-	0.13
FX4E	FR5P	+	5.09	-	0.14
FX4E	HX4P	+	2.03	-	0.14
FX4E	FX4P	+	3.77	-	0.23
FR5E	FR5P	-	0.15	-	0.13
FR5E	FX4P	+	6.00	-	0.23
FR5E	HX4P	+	4.57	-	0.15
E	P	-	0.21	-	0.20
Mya-1			4.43		6.00

			Env-mediated fusion				
			receptor	PET _{F14}	PPR	GL8 ₄₁₄	GL8 _{EK}
feCCR5	feCXCR4	huCXCR4	CCR1	-	-	-	-
			CCR2	-	-	-	-
CCR1	CCR2	CCR3	CCR3	-	-	-	-
			CCR4	-	-	-	-
CCR5	X4R5	X4R5R3	CCR5	-	-	-	-
			CCR8	-	-	-	-
			CXCR4	+	-	-	+
			V28	-	-	-	-
			EB1	-	-	-	-
			Bonzo	-	-	-	-
			BOB	-	-	-	-
			feCXCR4	+	-	-	+
			feCCR5	-	-	-	-
			control	-	-	-	-

