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Differential utilization of CD134 as a functional receptor by diverse strains of feline immunodeficiency virus (FIV).

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ABSTRACT

The feline homologue of CD134 (fCD134) is the primary binding receptor for feline immunodeficiency virus (FIV), targeting the virus preferentially to activated CD4+ helper T cells. However, with disease progression, the cell tropism of FIV broadens such that B cells and monocyte/macrophages become significant reservoirs of proviral DNA, suggesting that receptor utilisation may alter with disease progression. We examined the receptor utilisation of diverse strains of FIV and found that all strains tested utilised CD134 as primary receptor. Using chimaeric feline x human CD134 receptors, the primary determinant of receptor function was mapped to the first cysteine rich domain (CRD1) of fCD134. For the PPR and B2542 strains, substitution of CDR1 of fCD134 (amino acids 1 to 64) into human CD134 (hCD134) alone was sufficient to confer near optimal receptor function. However, evidence of differential utilisation of CD134 was revealed since GL8, CPGammer (CPG41), TM2, 0827 and NCSU1 required determinants in the region spanning amino acids 65-85, indicating that these strains may require a more stringent interaction for infection to proceed.

INTRODUCTION

The initial event in the process of viral entry into a target cell is the interaction between the virus and its cellular receptor and the specificity of this interaction determines both the cell tropism and the pathogenicity of the virus. The primary receptor for HIV and SIV is CD4, targeting the virus to helper T cells and resulting in progressive depletion of these cells and the eventual development of AIDS[46]. However, CD4 expression alone is insufficient to confer susceptibility to infection with HIV, which also depends on expression of co-receptors, principally the chemokine receptors CXCR4 and CCR5[3]. The virus attaches via a high affinity interaction with CD4, resulting in a conformational change in the envelope glycoprotein (Env) and exposing the binding site for the chemokine receptor[30]. This then triggers a further conformational change that exposes the fusion domain of the viral transmembrane protein gp41 and enables fusion of the viral and cellular membranes[17].

The feline immunodeficiency virus (FIV) is unique among the non-primate lentiviruses because in its natural host species, the domestic cat, it induces a disease similar to AIDS, characterised by a progressive depletion of CD4+ T lymphocytes[39]. In chronic infection, an immunodeficiency syndrome develops that is characterised by wasting, neurological manifestations, chronic stomatitis and gingivitis and an increased incidence of lymphoma[38]. In contrast, the ungulate lentiviruses induce diseases reminiscent of chronic inflammatory conditions. However, CD4 is not the primary receptor for FIV, the primary receptor was revealed recently to be CD134 (OX40)[48], a member of the tumour necrosis factor receptor/nerve growth factor receptor superfamily. Primary isolates of FIV use CD134 as the binding receptor in conjunction with the chemokine receptor CXCR4 as a co-factor for infection[45,48,60].

FIV binds specifically to CD134-expressing cells[48], the FIV Env interacts directly with CD134[12] and pre-treatment of virus with sCD134 facilitates infection of CD134-ve / CXCR4+ve cells[11].

CCR5 appears to be the co-receptor utilized by the majority of HIV strains early in infection, usage of CXCR4 as a co-receptor is more frequent with disease progression[10,47]. In contrast, FIV appears to use CXCR4 alone as its sole co-receptor for infection[19,45,61] and yet with disease progression the viral cell tropism expands[14,20] suggesting an alteration in receptor utilisation. Previous studies have demonstrated that upregulation of endogenous CXCR4 can increase susceptibility to infection with FIV[59] and that over-expression of exogenous human CXCR4 on a target cell may overcome the requirement for the expression of CD134[13]. Thus it is possible that with disease progression, FIV may either lose its dependence on an interaction with CD134, or interact more efficiently with CXCR4.

In this study we investigate the use of CD134 as a functional receptor by diverse strains of FIV. As a first step towards understanding the role of CD134 in the pathogenesis of FIV infection, we examine the receptor utilisation of a panel of FIV isolates of diverse subtypes and geographical origins. We define the region of CD134 that confers functional FIV receptor activity and present evidence for differential usage of CD134 as a viral receptor by diverse strains of FIV.

MATERIALS AND METHODS

Cells and viruses. MYA-1[33] , MCC[7] and NSO were cultured in RPMI 1640 medium. 293T and HeLa were maintained in Dulbeccos modification of Eagle's medium (DMEM). All media were supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 0.11mg/ml sodium pyruvate 100 IU/ml penicillin, 100 μg/ml streptomycin. The medium for MYA-1 cells was supplemented with conditioned medium from a murine cell line (L2.3) transfected with a human IL-2 expression construct (equivalent to 100 U/ml of recombinant human IL-2) and 50μM 2-mercaptoethanol. All media and supplements were obtained from InVitrogen Life Technologies Ltd. (Paisley, UK). Cell lines expressing CD134 and the chimaeric constructs were maintained in G418 (InVitrogen, Paisley, UK). The canine lymphocytic leukemia (CLL) cell line was generated by prolonged *in vitro* culture of peripheral blood mononuclear cells, the established line is CD3+ve/CD4-ve/CD8-ve.

The B2542[15] (subtype B) and CPGammer[16] isolates of FIV (subtype C) were obtained from E. Hoover and S. Vandewoude. Molecular clones of TM219[32] and NCSU1 (JSY3)[63] were from T. Miyazawa and W. Tompkins respectively. PPR[41] Env was (plasmid pΔ11[37]) was obtained from G. Pancino. pFIV-PPR was obtained from the NIH AIDS Research & Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr John Elder. GL8[24], 0827, 0425 and 1419 are subtype A field isolates of FIV from the United Kingdom.

Antibodies and flow cytometry. Anti-human CD134 (BerACT35) was obtained from Alexis Corporation. Anti-human CXCR4 (#44701) was obtained from R&D Systems, Abingdon, Oxford, United Kingdom. Cells to be processed for flow cytometry were resuspended in phosphate buffered saline (PBS) supplemented with 1.0% (w/v) bovine serum albumin, 0.1% (w/v) sodium azide (PBA). Cells were incubated with 1μg of primary antibody for 30 minutes at 4°C and then washed twice with PBA by centrifugation at 1000 rpm for 5 minutes. Bound primary antibody was detected with the appropriate anti-mouse IgG secondary antibody (Serotec, Oxford, United Kingdom) corresponding to the isotype of the primary antibody and conjugated to either fluorescein isothiocyanate (FITC) or R-phycoerythrin (RPE). Cells were incubated with secondary antibody for 30 minutes at 4°C and then washed

twice with PBA by centrifugation at 1000rpm for 5 minutes and resuspended in 1ml of PBA for analysis. All samples were analysed on a Beckman Coulter EPICS MCS-XL flow cytometer, 10,000 events being collected for each sample in LIST mode. Data were analysed using EXPO 32 ADC Analysis (Advanced Cytometry Systems).

Construction of feline x human CD134 chimaeras. Previously, we demonstrated that feline, but not human CD134, supported infection with FIV[48]. The amino acid sequences of feline and human CD134 were aligned using the Clustal W algorithm[55], enabling the identification of non-conserved amino acids, many of which were localised to the N-terminal (extracellular) region of the molecule. The predicted three dimensional structure of feline CD134 was generated using Swiss-Model[40] in first approach mode and images were then manipulated using Swiss-PdbViewer v3.7b2 (Glaxo Wellcome Experimental Research). The 3D structure prediction enabled the identification of non-conserved amino acids that were either within, or in close proximity to the N-terminal cysteine-rich domains (CRDs). A strategy was therefore devised that would enable the generation of feline x human chimaeric CD134 molecules creating chimaera junctions in conserved regions of amino acid sequence, preserving the sequence and thus being less likely to affect the tertiary structure of the chimaeric protein. To facilitate the construction of these chimaeras, NruI and BsrGI restriction sites were introduced into the feline and human CD134 cDNA clones by using the polymerase chain reaction (PCR) and the primers 5- CCAATA CCCTACCTCAGCGCTACGTCG-3’ (NruI) and 5’-GGCACGTTCGGGACATGT-3’ (BsrGI). These restriction sites created chimaera junctions between residues 56 and 57, and 85 and 86 of feline CD134. Thus chimaera “FHF” represents “feline $CD134_{1-56}$ x human $CD134_{57-85}$ x feline $CD134_{86-270}$”. Additional chimaeras were prepared in which amino acids 47 to 56 of human CD134 were introduced into chimaera FFH, generating (FH)FH, and in which amino acids 65 to 85 of feline CD134 were introduced into chimaera FHH, generating F(FH)H. The nucleic acid sequence of each of the chimaeric CD134s was confirmed by cycle sequencing on an Applied Biosystems 9700 thermal cycler using a BigDye® Terminator v1.1 cycle sequencing kit (Applied Biosystems) followed by analysis on an Applied Biosystems 3700 genetic analyser. cDNAs were subcloned into the retroviral vectors pDONAI (Takara, Tokyo, Japan) and pCNC-MCS[25].

In order to generate CD134-EGFP gene fusions, the coding sequences for the CD134s and CD134 chimaeras were re-amplified with the primers 5'-TT GGTACCATGAGGGTGGTTGTGGGGCT-3' (KpnI) and 5'-ATCCCGGGCGA TCTTGGCCAGGGTGGAGTT-3' (SmaI) and cloned into pEGFPN1 (BD Biosciences Clontech, Cowley, Oxford, UK). The resulting gene fusion was then subcloned into pDONAI or pCNC-MCS[25]. The nucleic acid sequence of all gene fusions was confirmed as above.

HIV pseudotype assays. FIV *env* gene expression constructs GL8, 0827, 0425, 1419, PPR and TM2 have been described previously[48]. B2542 and CPGammer *envs* were amplified by PCR from DNA prepared from infected MYA-1 cells and cloned directly into VR1012 (Vical Inc., San Diego, CA). 5μg of each VR1012-*env*, NCSU1*env*-cDNA3[21] or pCI-VSV-G, and 7.5μg of pNL4-3-Luc-$E^{-}R^{-}$ were co-transfected into HEK-293T cells using the Calcium-Phosphate co-precipitation technique essentially as described previously[23]. Culture supernatants were collected at 48 hours post-transfection, filtered at 0.45μm and frozen at –70°C until required. Adherent target cell lines were seeded at $1x10^4$ cells per well of a CulturPlate™-96 assay plate (Perkin Elmer, Life and Analytical Sciences, Beaconsfield, UK) and cultured overnight, while suspension target cell lines were seeded at $5x10^4$ cells per well and used immediately. The cells were then infected with 50μl HIV (FIV) luciferase pseudotypes, cultured for 72 hours and then luciferase activity quantified by the addition of 50μl of Steadylite HTS™ (Perkin Elmer) luciferase substrate and measurement by single photon counting on a MicroBeta luminometer (Perkin Elmer).

Growth of FIV *in vitro*. The growth of FIV *in vitro* was assessed in MCC cells transduced with each of the chimaeric CD134 constructs. $1x10^6$ cells were infected with 0.45μm-filtered culture supernatant from FIV infected MYA-1 cells containing approximately 10,000 TCID50 (titred on MYA-1 cells) of virus for 2 hours at 37°C. The cells were then pelleted by centrifugation at 1000rpm, the medium aspirated and the cells washed twice with PBS. The cells were then resuspended in culture medium and plated in 12 well culture plates. Supernatants were collected every three days and assayed for reverse transcriptase (RT) activity using Lenti-RT non-isotopic RT assay

kit (Cavidi Tech., Uppsala, Sweden). RT values were then calculated relative to purified HIV-1 RT standard.

RESULTS

BerACT35 recognises feline CD134.

Previous studies have used the anti-human CD134 monoclonal antibody BerACT35 to monitor CD134 expression on feline cells[22,26,48], demonstrating that fCD134 is expressed on IL2-dependent T cells in vitro[48] and on CD4+/CD25+ T_{reg} cells *in vivo*[26]. However recent studies have asserted that the BerACT35 antibody is human CD134-specific and does not recognise feline CD134[11,12]. Given that the ability to monitor surface expression of CD134 is critical to the interpretation for assays of receptor function, and in order to resolve these discordant findings, we examined the binding of BerACT35 to feline, human and murine cells stably transduced with retroviral vectors bearing feline CD134 (Fig.1). Feline CD134-expressing feline cells (MCC, Fig. 1a), human cells (NP2, Fig.1b) and murine cells (NSO, Fig. 1c) cells displayed strong, specific binding of BerACT35 following stable transduction with the feline CD134 expression vector. To further confirm the specificity of the interaction, MCC cells were stably transduced with a retroviral vector bearing a feline CD134/C-terminal EGFP fusion (Fig. 1d). The majority of BerACT35 reactivity (CD134-PE, 74.3%) was directly proportional to intensity of CD134-EGFP expression. Finally, BerACT35 reacted with CrFK cells following either transient transfection (Fig. 1e, 5.7% double positive for CD134-EGFP/BerACT35 PE) or stable transduction (Fig. 1f, 55.6% double positive for CD134-EGFP/BerACT35 PE) with feline CD134-EGFP expression vectors. These data are consistent with BerACT35 recognising feline CD134 irrespective of the cellular target and means of ectopic expression.

Given that BerACT35 binds specifically to feline CD134, we examined the effect of infection with subtype A, B and C viruses on CD134 expression on IL2-dependent feline T cells (MYA-1[33]). MYA-1 cells were infected with the GL8[24], B2542[15] and CPGammer[16] strains of FIV (subtypes A, B and C respectively) and monitored by flow cytometry for expression of CD134 and CXCR4. In parallel, supernatants were collected to measure reverse transcriptase (RT) activity. FIV infection resulted in a sharp decline in CD134 expression within 2 days post-infection (Fig. 2b., c. and d.) irrespective of the viral subtype. The reduction in CD134 expression mirrored a rise in RT production and persisted for the duration of the time course. In contrast, CD134 expression remained relatively stable on the uninfected

control cells (Fig. 2a.). Similar findings were observed with CXCR4 expression, a sharp decline in CXCR4 expression (Fig. 2g., h. and i.) mirroring the concomitant rise in RT production. However, the reduction in CXCR4 expression did not persist, expression levels recovering to control levels by the end of the time course. These results would be consistent with the expansion of a CD134-negative and FIV-resistant population of cells. To further address the specificity of the fall in CD134-expression in FIV-infected cells, MYA-1 cells were infected with the non-domestic cat lentiviruses PLV (puma lentivirus) and LLV (lion lentivirus). There was no reduction in CD134 expression in either PLV (Fig. 2c.) or LLV (Fig. 2f.) infected cells, indeed PLV infection resulted in an increase in expression of both CXCR4 (Fig. 2i.) and CD134 suggesting an increase in the activation state of the T cells (both CXCR4 and CD134 are up-regulated on activated T cells). The data suggest that the reduction in CD134 expression following FIV infection is specific to the domestic cat virus.

Expression of CD134 chimaeras.

In order to discern whether diverse isolates of FIV interacted with CD134 in a similar way, we mapped the functional determinants of receptor activity on feline CD134 using series of chimaeras generated between human and feline CD134 (previously, we had demonstrated that human CD134 did not support infection with FIV[48]). Chimaeras were designed based on the predicted three-dimensional structure of feline CD134 generated by submitting the feline CD134 amino acid sequence translation to the SWISS-PROT server in First Approach mode. The predicted structure of fCD134 was compared with that of the TNFR[2] and herpes simplex virus entry mediator (HveA)[6,8,9]. Given the distribution of divergent amino acid residues between feline and human CD134, the most likely site for interaction between FIV Env and CD134 was predicted to lie in the first cysteine-rich domain (CRD1), analogous to the binding site for HSV gD on HveA[6,8,9]. Chimaeras were therefore designed such that the junctions were located in regions of sequence conservation between the human and feline CD134 translations and focused primarily on the first and second CRDs (Fig.3). The CRD definitions are as described previously[31] and correspond to the molecular architecture A1B2 (CRD1 and CRD2) and A1B1 (CRD3) as detailed previously[5]. The nucleic acid sequence of each chimaera in the retroviral vector was confirmed prior to stable transduction of the target cell line (MCC).

Surface expression of each chimaera was assessed using BerACT35 antibody. Of the seven constructs, five were stably expressed on the cell surface in a form that was recognised by BerACT35; namely FFF, FFH, FHF, FHH and HHF (Fig. 4). Two chimaeras (HFF and HFH) appeared to express poorly at the cell surface and moreover the cell lines bearing these molecules took longer to expand following G418 selection, perhaps indicating an inhibitory effect on cell growth. To ascertain whether the HFF and HFH chimaeras were being expressed, we generated a second series of cell lines in which the C-termini of the CD134 constructs were tagged with EGFP. Transduction of MCC cells with the GFP-tagged CD134 constructs indicated that the constructs were indeed being expressed, but that expression was not being detected at the cell surface by BerACT35 (data not shown), indicating that either the molecule did not reach surface of the cells or the BerACT35 epitope had been disrupted in these cells.

MCC cells transduced with the CD134 chimaeras were infected with HIV (FIV) pseudotypes carrying a luciferase marker gene (HIV(FIV)-luc) and bearing Envs from the GL8, PPR and TM2 strains of FIV (Fig. 5). Viral entry was detected in two of the eight cell lines; FFF (the reconstructed full length CD134) and FFH (feline $CD134_{1-85}$ x human $CD134_{86-270}$). All chimaeras bearing amino acids 57 to 85 of human CD134 were rendered non-functional as viral receptors, despite encoding CD134 molecules that were stably expressed on the surface of the transduced cells that could be detected with BerACT35. Thus chimaera FFH was a functional receptor whereas FHH was not. These data indicated that the region spanning amino acids 1 to 86 conferred FIV receptor activity to feline CD134. Amino acids 1 to 86 comprise CRD1 and part of CRD2 and encompass the region known to form the binding domain for gD on HveA. To further define the region that conferred functional receptor activity on CD134, we prepared two additional chimaeras exchanging amino acids 47 to 56 and 65 to 85 between chimaeras FFH and FHH, generating chimaeras F(FH)H and (FH)FH (see Fig.3). MCC cells were transduced with retroviral vectors bearing the novel chimaeras, stably selected (cell surface expression of the chimaeras was confirmed using BerACT35, not shown) and challenged with HIV (FIV) luc pseudotypes bearing the GL8, PPR and TM2 Envs (Fig. 6). Chimaera F(FH)H, which effectively contains CRD1 of feline CD134 in the context of human CD134, almost completely lost the ability to act as a functional receptor for infection mediated by the GL8 or TM2 Envs (Fig. 6a. and c., 0.0% and 0.4% of FFF control). In contrast, infection mediated by

PPR Env was supported with reduced efficiency (Fig. 6b, 3.8% of FFF control). Chimaera (FH)FH was more effective as a functional receptor supporting viral entry mediated by the GL8, PPR and TM2 strains (16.5, 39.3 and 21.4% of FFF control). It has been reported that substitution of the region defined as CRD1 from feline CD134 into human CD134 (equivalent to chimaera F(FH)H) is sufficient to confer full receptor function for a single isolate of FIV[11]. The data with chimaeras F(FH)H and (FH)FH are consistent with CRD1 containing a major component of the receptor binding domain. However, for reconstitution of a functional receptor, additional determinants outwith CRD1 are clearly required. Given that F(FH)H did not support infection with the GL8 and TM2 pseudotypes while showing reduced activity with PPR pseudotypes, a broader range of pseudotypes was investigated for usage of the chimaeric receptors. Pseudotypes were prepared bearing Envs prepared from the highly pathogenic subtype C virus FIV-CPGammer[16] (CPG41); the subtype A isolates 1419 and 0827[48]; and subtype B NCSU1[63] and B2542[15] strains of FIV. The CPG41 and NCSU1 (Fig. 6d. and h.) strains yielded similar results to the GL8 and TM2 strains; chimaera (FH)FH supporting viral entry mediated by CPG41, 1419, 0827 and NCSU1 Envs (32%, 21, 21 and 38% of FFF control respectively) while F(FH)H had no or low activity (0.0%, 3.6%, 0.0% and 0.9% of FFF control). In contrast, the subtype B isolate, B2542 (Fig. 6e.) yielded findings to PPR, utilizing both the (FH)FH and F(FH)H chimaeras efficiently (76% and 24% of FFF control) . The data suggest that determinants outwith CRD1 in the region spanning amino acids 65 to 85 are required to form a functional receptor for the majority strains of virus. That chimaera F(FH)H showed activity with the B2542 and PPR strains may provide evidence for the utilisation of distinct regions of CD134 by diverse strains of FIV.

Additional cell lines stably expressing the (FH)FH and F(FH)H constructs and based on the cell line HeLa were generated in order to assess the reproducibility of the findings with MCC - (FH)FH and F(FH)H (MCC cells are feline lymphoid cells while HeLa cells are human epithelioid cells). The cells were then challenged with HIV (FIV) luc pseudotypes bearing a range of Envs, the results are summarised in table 1 with receptor function expressed as "–fold increase" in susceptibility relative to cells transduced with vector only. Strains GL8, CPG41, TM2, 0827 and NCSU1 showed a preference for chimaera (FH)FH over F(FH)H, most marked with the FIV-CPGammer[16] (CPG41) Env which used (FH)FH 59-fold and 269-fold more efficiently than F(FH)H in MCC and HeLa cells respectively. The data confirm that

determinants in the region spanning amino acids 65 to 85 of CD134 are required for to viral entry to proceed.

We next asked whether cells expressing the (FH)FH and F(FH)H chimaeras showed differences in their ability to support productive infection (Fig. 7). CLL cells expressing the chimaeric receptor molecules were infected with matched doses of the cloned viruses GL8 and GL8.CPG41 (preference for FHFH) and GL8.B2542 and PPR (no preference or weak preference). Cells were infected in parallel with HIV(FIV) pseudotypes bearing the equivalent Envs. Whereas assays using viral pseudotypes measure viral entry alone, cell-free virus infection represents the sum of entry, replication, egress and cell-to-cell spread. Therefore we predicted that the efficiency of the chimaeras as viral receptors might be less evident in an assay for productive virus infection. HIV (FIV) pseudotypes bearing the GL8 and CPG41 Envs used chimaera FHFH with greater efficiency than FFHH in CLL cells while PPR or B2542 pseudotypes used both FHFH and FFHH efficiently (Fig. 7a.), consistent with previous findings using MCC and HeLa cells transduced with the FFHH and FHFH expression vectors (Table 1). It was notable that viral entry into the vector only control CLL cells was ~10-fold higher than for GL8, PPR and B2542 than for CPG41. These data may suggest upregulation of endogenous canine CD134 during the selection process; studies are currently underway to address whether this is indeed the case.

Productive infection experiments recapitulated these findings in that GL8 and GL8.CPG41 replicated more efficiently in CLL-FHFH cells than CLL-FFHH. GL8-B2542 and PPR did not display a preference for CLL-FHFH over CLL-FFHH, indeed B2542 replicated more efficiently in CLL-FFHH than in CLL-FHFH (Fig.7b.) suggesting that in this system, the selectivity for the two chimaeras extended into replicative capacity.

DISCUSSION

The virus-receptor interaction is the primary event in the viral replicative cycle, a major determinant of cell tropism and a target for therapeutic intervention. Antibodies that block the HIV-CD4 interaction have been shown to have broad neutralising activity (anti-CD4 binding site antibodies); neutralising strains of virus from diverse clades and geographical origins. In this study we investigated the binding site for FIV Env on CD134, the cellular receptor for FIV. We show primary isolates of FIV can be classified into at least two groupings based on their interaction with CD134. Expression of feline CD134 CRD1 alone in the context of human CD134 is sufficient to confer near optimal receptor function for infection with strains such as PPR, in agreement with recent findings[11]. However, pathogenic primary strains of virus such as GL8, CPGammer and NCSU1 require additional determinants in CRD2 for restoration of receptor function. These data provide the first evidence for differential utilisation of feline CD134 by FIV. The extent of the binding site utilised by strains such as GL8, CPGammer and NCSU1 remains to be established; substitution of amino acids S60D, N62D, H45S, R59G and V64K in human CD134 recreated the binding site for FIV PPR Env and restored infectivity for CrFK cells, in contrast chimaera F(FH)H in this study (containing all the above amino acids) had near negligible activity as a receptor for Envs GL8 and CPG41. The PPR strain infects CrFK cells with a low efficiency in the absence of CD134[11], and over-expression of human CXCR4 alone is sufficient to render (CD134-negative) G355 cells permissive for productive infection with FIV PPR[13]. These data may indicate either a reduced requirement for CD134 or a reduced threshold for induction of the conformational change in the PPR Env that permits a direct interaction with CXCR4. Consistent with this hypothesis, an FIV PPR SU-Fc fusion protein bound to 60% of purified resting feline PBMC and the majority of binding was via CXCR4 alone[12] suggesting that viruses such as PPR may have a low requirement for CD134 for binding/infection. A reduced activation threshold for envelope-mediated fusion has been implicated in the propensity for CD4-independent infection by HIV-2[44]. Given that the interaction between the GL8, CPG41 and NCSU1 Envs would appear to be more complex than that of PPR, extending outwith CRD1, our studies raise the possibility that infection by such viruses may well be modulated by the natural ligand for feline CD134 (CD134L or OX40 ligand).

The interaction between HIV and CD4 induces a conformational change in gp120 that results in the exposure of the chemokine receptor binding site, this conformational change being marked by the exposure of epitopes for CD4i (CD4-induced) antibodies such as 17b and 48d[30,50,51,53,62]. Primary strains of HIV become more sensitive to sCD4 with prolonged culture[1,34,35]. With the discovery of the co-receptors CXCR4 and CCR5, it became apparent that X4 variants of HIV were more sensitive to surface CD4 concentration than R5 strains of virus[29]. Subsequently, the affinity of the interaction between Env and CD4 was shown to be critical to the process of adaptation to cell culture[42] and primary and laboratory-adapted strains of HIV were shown to display differential requirements for levels of both CD4 and CXCR4[56]. By analogy with HIV, our data may indicate that some primary strains of FIV may require a more complex interaction with CD134 than others for infection to proceed. Although at present the reagents with the interaction between these primary Envs and CD134 may be dissected are not currently available, future studies should investigate whether the complexity of the interaction between Env and CD134 for strains such as GL8 is reflected in an increased (or decreased) binding affinity for CD134. An increased affinity for CD4 has been shown to accompany laboratory adaptation for HIV[42]. One of the FIV strains (PPR) able to utilise the fCD134 CDR1-only chimaera has been shown previously to infect CD134-negative feline cells in the following over-expression of human CXCR4[13]. Although human CXCR4 does have an intrinsically higher activity as a co-receptor for FIV than feline CXCR4[58], these data may indicate that strains such as PPR, with a less stringent interaction with CD134, may be more readily adapted for culture in CD134-negative cell lines. Moreover, the emergence of such strains *in vivo* may accompany a broadening of the viral cell tropism as has been observed with the shift from acute to chronic FIV infection[14,20]. The link between the nature of the Env-CD134 interaction and the broadening of viral cell tropism with disease progression will an important avenue of future research.

The surface expression of feline CD134 and the feline x human CD134 chimaeras was monitored using the anti-human CD134 antibody BerACT35 and yet previous studies[11,12] have suggested that this antibody is human-specific. Given the importance of this reagent to the study of the virus-receptor interaction it is crucial that these discordant observations be reconciled. BerACT35 has been used to monitor feline CD134 expression *in vitro* and *in vivo*[22,26,48]. Further, we have shown that surface

CD134-expression can be evaluated using BerACT35 following stable transduction of the adherent cell lines HeLa, NP2 and AH927, or suspension cells NSO, MCC or 3201 with retroviral vectors bearing feline CD134, suggesting that using this system feline CD134 is expressed in the same conformation as the endogenously expressed molecule. The studies in which BerACT35 failed to recognise feline CD134[11,12] were performed using transient retroviral transduction of CrFK cells; it is possible that in such an expression system feline CD134 may be expressed in a distinct antigenic conformation, a conformation distinct from that present *in vivo*[26]. The significance of these distinct antigenic conformations to FIV receptor function remains to be established however given that BerACT35 recognises feline CD134 *in vivo*, this antigenic conformation is likely to be biologically relevant.

CD4-independent strains of HIV are particularly sensitive to neutralizing antibodies[18,27,28,43] and thus may be more likely to arise where the host humoral immune response is failing, or in immunoprivileged sites (reviewed in ref. 4). CD134-independent strains of FIV are readily neutralised by sera from infected animals, most markedly when the assays are performed on CD134-negative/CXCR4-positive cells[36]. Similarly, neutralising antibodies against CD4-independent strains of HIV/SIV are detected more readily when the assays are based on CD4-negative cells[43,54]. Many of the prototypic strains of FIV were either isolated from animals with clinical signs of AIDS[39,41] or have subsequently undergone prolonged culture *in vitro*[49,52,57], with the consequence that a degree of selection for neutralisation-sensitive strains of virus with a propensity for adaptation to CD134-independent infection. Given that the *in vivo* cell tropism of FIV would appear to expand with disease progression[14,20], it is likely that the strains of virus present in animals displaying clinical signs may differ from those present in the early/acute phase of infection, analogous to the shift in cell tropism observed with the emergence of X4 strains of HIV with the progression to AIDS[10]. Future studies should address whether strains of FIV present in the early acute phase of infection differ in *in vitro* cell tropism and receptor usage from those isolated from animals displaying clinical signs. Vaccine efforts may then be targeted against the strains of virus that are most likely to be transmitted between animals.

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Figure legends

Figure 1. BerACT35 recognises feline CD134. BerACT35 reactivity was assessed by flow cytometry on a) MCC (feline), b) NP2 (human) and c) murine (NSO) cells stably transduced with either fCD134.pDONAI (filled histogram) or pDONAI vector only (open histograms). Reactivity was also assessed on MCC (D) and CrFK (F) cells stably transduced, and CrFK cells (E) transiently transfected with fCD134.EGFP.pDONAI.

Figure 2. Reduced surface expression of CD134 and CXCR4 in FIV infected cells. Surface expression of CD134 (a.-f.) and CXCR4 (g.-l.) expression was monitored by flow cytometry using anti-CD134 (BerACT35) and anti-CXCR4 (RND44701) in IL2-dependent T cells infected with FIV strains GL8 (b.,h.), B2542 (d.,j.) and CPGammer (e.,k.), the puma lentivirus PLV (c.,i), the lion lentivirus LLV (f.,l) or mock-infected (a.,g.). Percentage positive was calculated relative to isotype-matched control. Supernatants were collected in parallel and reverse transcriptase (RT) activity estimated by non-isotopic assay, results are expressed as absorbance 405nm.

Figure 3. Construction of feline x human CD134 chimaeras. a.) Predicted 3D structure of feline CD134, comparison with TNFR and HveA, location of CRD1 and CRD2. b.) Schematic of feline x human CD134 chimaeras showing the location of the junctions between the feline (grey) and human (white) regions. Numbered arrows indicate amino acids flanking the junctions.

Figure 4. Surface expression of CD134 chimaeras. MCC cells were stably transduced with a retroviral vector bearing each chimaera. Cell surface expression of CD134 was analysed by flow cytometry using BerACT35. Each histogram represents 10,000 events and is representative of two analyses.

Figure 5. Ability of CD134 chimaeras to act as functional receptors for FIV. MCC cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimaeras, or vector only (CON), were infected with HIV (FIV) luciferase pseudotypes bearing the GL8, PPR or TM2 Envs. Luciferase activity was assayed at

72 hours post-infection and is expressed as mean (n=3) counts per minute (CPM) +/- SE.

Figure 6. Differential utilisation of CD134 chimaeras by FIV. MCC cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimaeras (FFF, (FH)FH, F(FH)H, HHH or vector only (CON), were infected with HIV (FIV) luciferase pseudotypes bearing the GL8 (a), PPR (b), TM2 (c), CPG41 (d), B2542 (e), 1419 (f), 0827 (g) and NCSU1 (h) Envs. Luciferase activity was assayed at 72 hours post-infection and is expressed as mean (n=3) counts per minute (CPM) +/- SE.

Figure 7. Productive infection of cells expressing CD134 chimaeras. a). CLL cells stably transduced with a retroviral vector (pDONAI) bearing each of the chimaeras (FFF, (FH)FH, F(FH)H or vector only (CON), were infected with HIV (FIV) pseudotypes bearing the GL8, B2542, CPG41 and PPR Envs and assayed for viral entry by luciferase assay. Luciferase activity was assayed at 72 hours post-infection and is expressed as mean (n=3) counts per minute (CPM) +/- SE. B.) Cells were infected in parallel with matched titres of replication competent virus from the molecular clones GL8, GL8.2542, GL8.CPG41 and PPR; supernatants were collected at days 0, 1, 4 and 6 post-infection and assayed for virus production by non-isotopic reverse transcriptase activity (absorbance 405nm). Results represent the mean of duplicate experiments (each time point the mean of two replicates).

Fig. 1

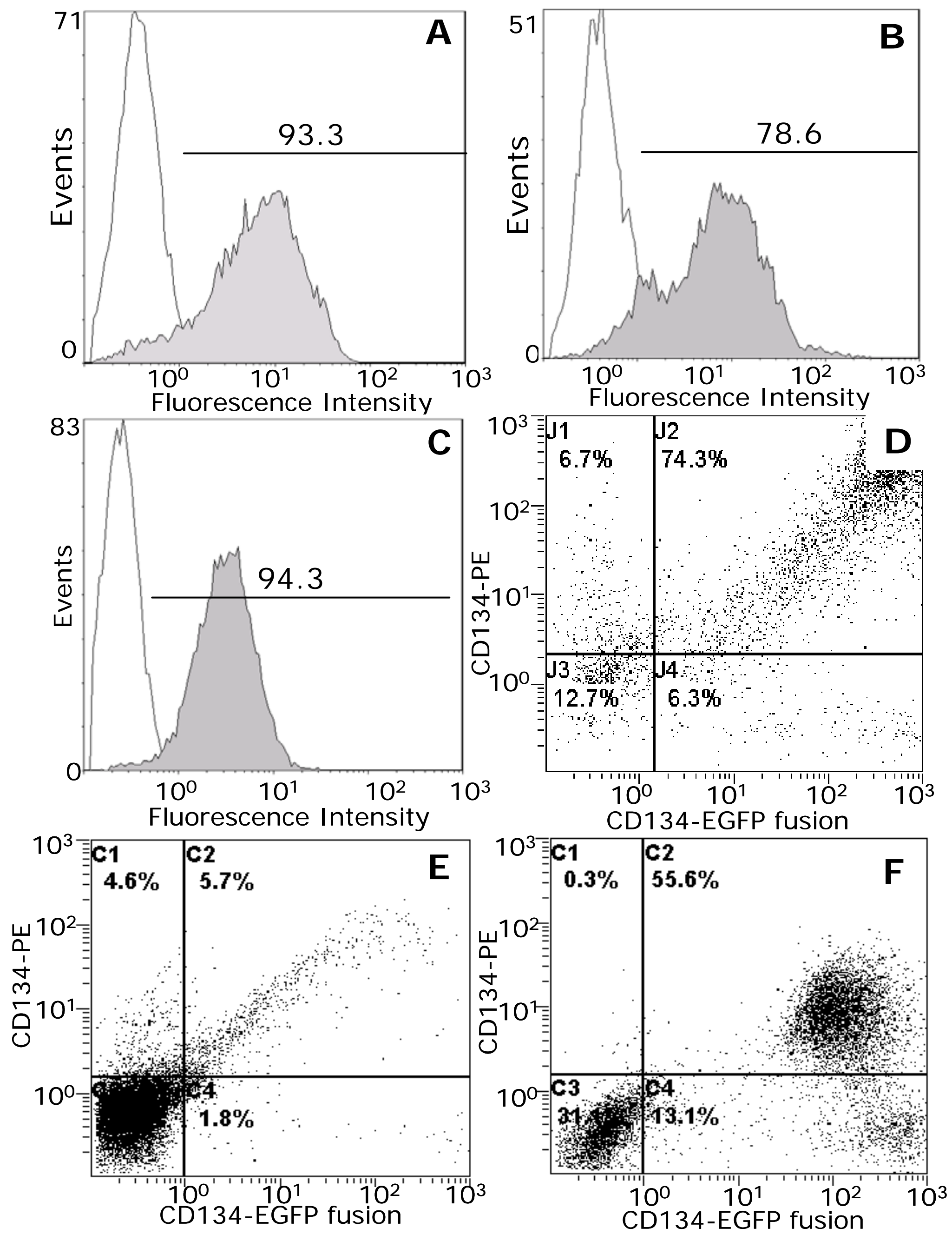
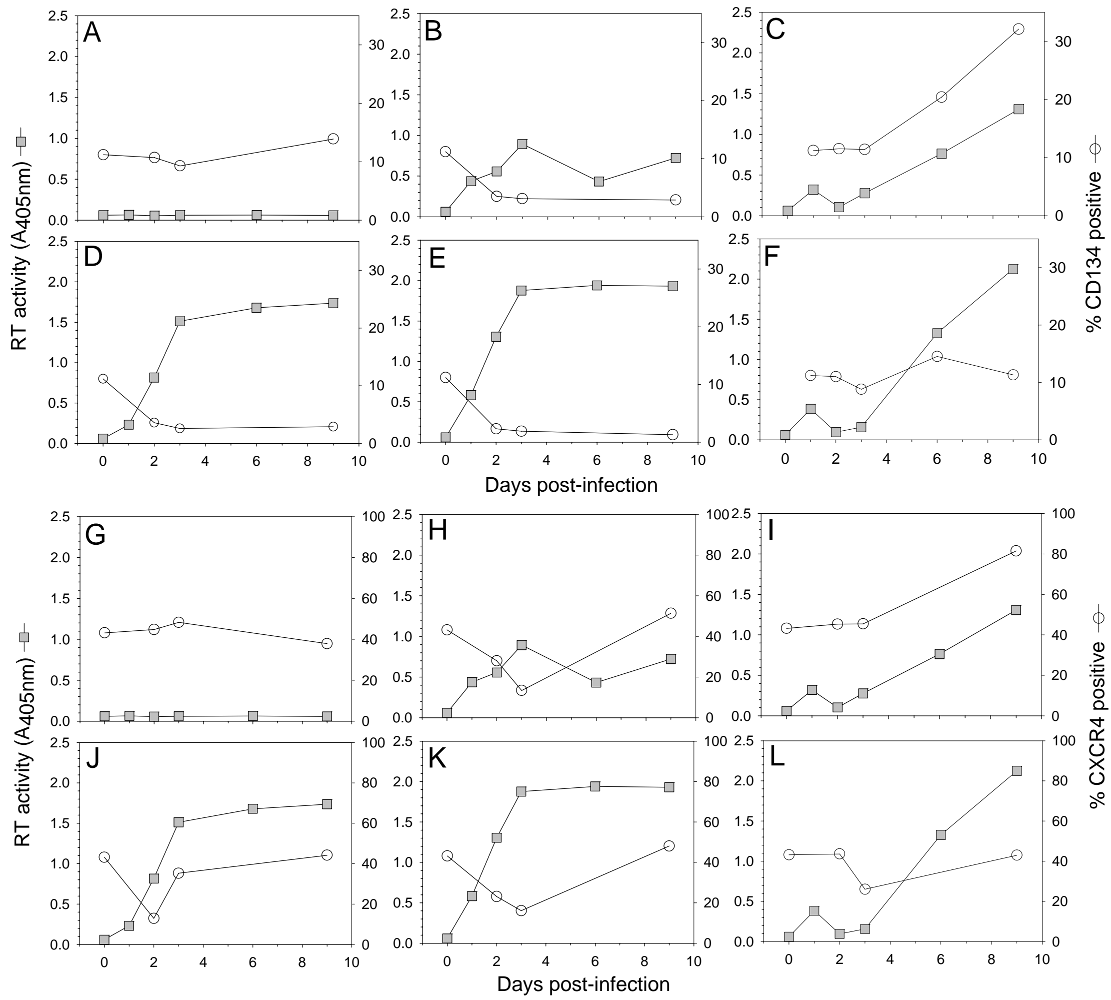


Fig. 2.



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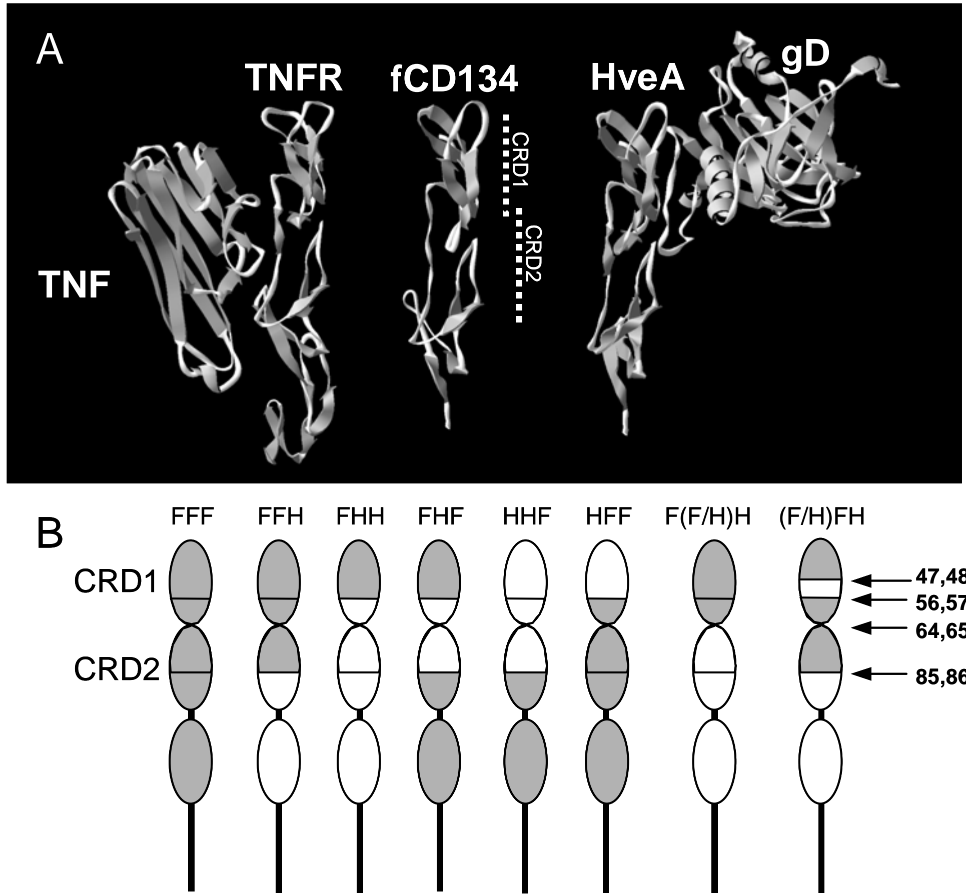

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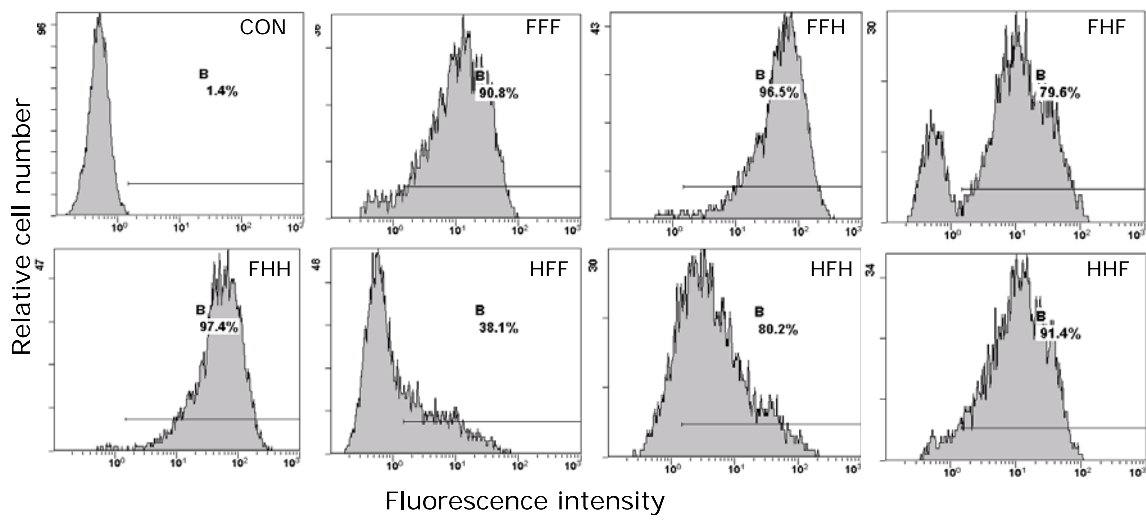

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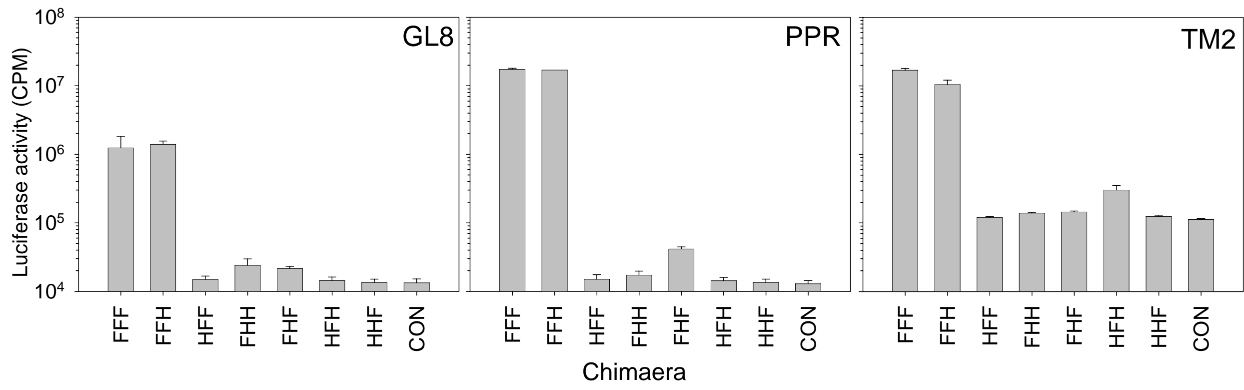

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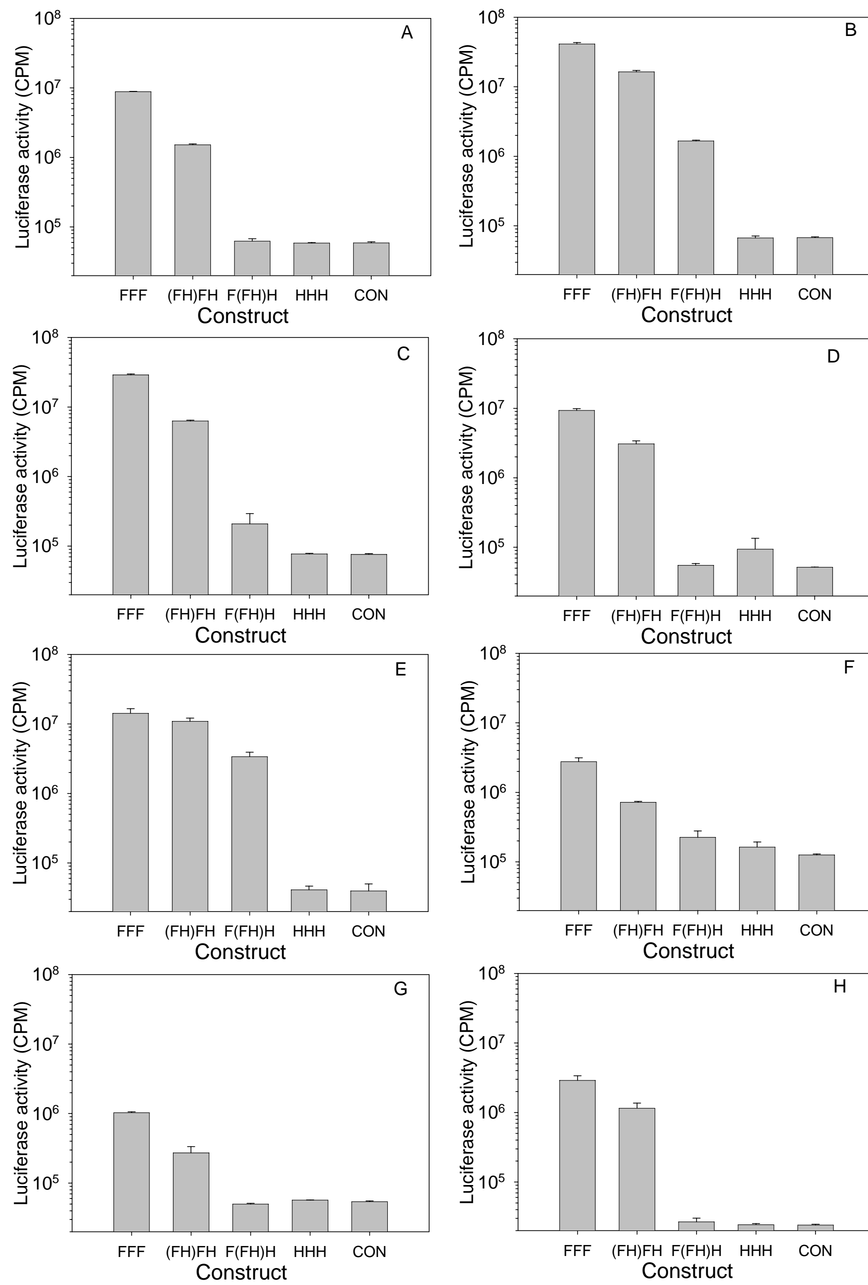

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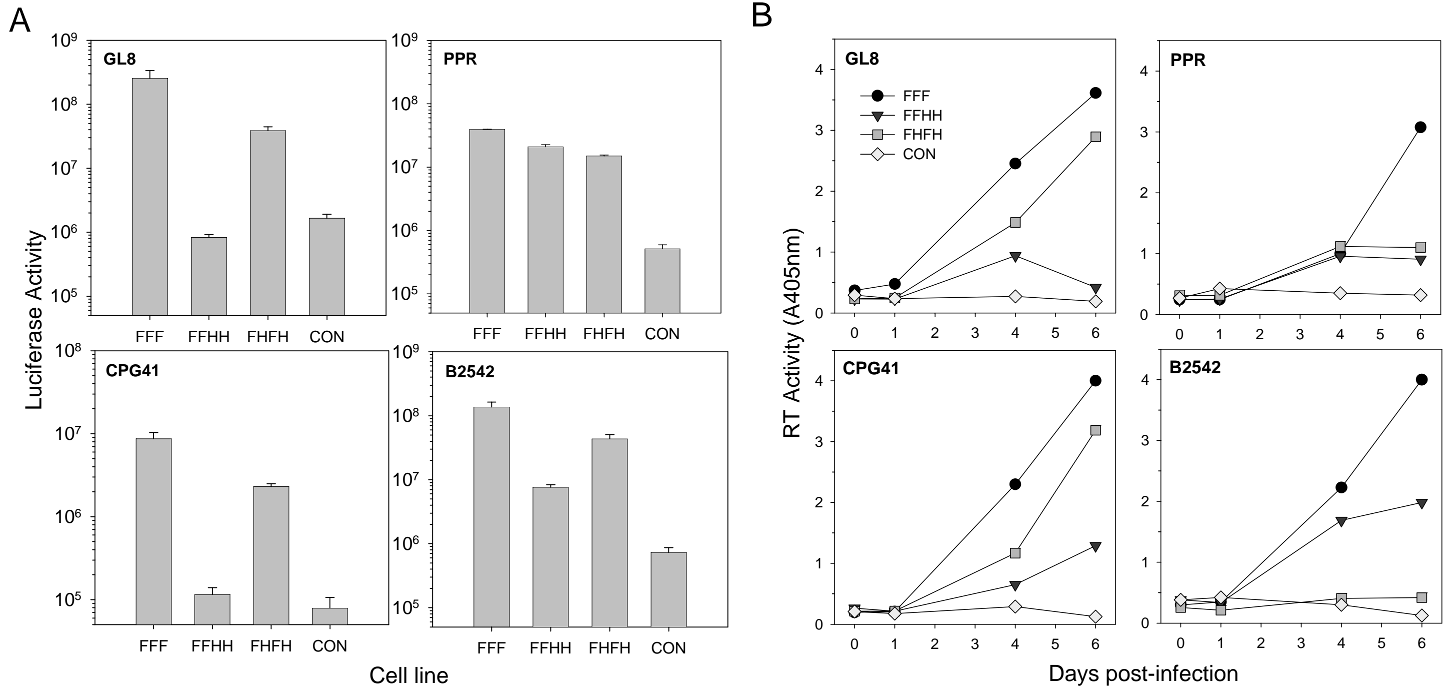

Table 1. Comparison of CD134 chimaera utilisation by diverse strains of FIV.

	Cell line					
	MCC			HeLa		
Virus	(FH)FH	F(FH)H	ratio	(FH)FH	FFHH	ratio
GL8	25	1	25*	1720	10	172*
PPR	609	241	2.5	4919	2608	1.9
TM2	82	2	41*	1878	199	9.4*
CPG41	59	1	59*	2690	10	269*
0425	3	5	0.6	107	95	1.1
1419	5	1	5	577	720	0.8
0827	5	1	5	459	13	35.3*
B2542	275	85	3.2	302	220	1.3
NCSU1	49	1	49*	2802	798	3.5

*FIV Env pseudotypes displaying a preference for (FH)FH over F(FH)H. Results are typical of two separate experiments.