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Short communication

Comparison of serum sensitivities of pseudotype retroviruses produced from newly established packaging cell lines of human and feline origins

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Abstract

To apply retrovirus vectors for in vivo gene therapy in cats, it is necessary to develop vector systems that are not inactivated by cat serum. In this study, the retrovirus packaging cell lines 2SC-1 and AHCeB7 were newly established from human embryonic kidney (HEK) 293 and feline fibroblastic AH927 cells, respectively. Then the sensitivities of pseudotype viruses released from these cell lines to fresh sera from humans and cats were compared. Pseudotype viruses from the 2SC-1 cells were inactivated efficiently by cat serum but not by human serum. Pseudotype viruses from the AHCeB7 cells were also inactivated efficiently by human serum, however they were rather resistant to cat serum. When the xenoantigenicity of the cell lines was examined by flow cytometry, AH927 cells reacted with human serum, however, HEK293 cells did not react with cat serum. These results suggested that pseudotype viruses from 2SC-1 cells were inactivated by the fresh cat serum in an antibody-independent manner. Chelating experiments revealed that certain temperature-sensitive factor(s) other than complements might be involved in the inactivation. The usage of feline cells as packaging cells is suitable for in vivo gene therapy in cats.

Keywords: Cats; FeLV; Gene therapy; Packaging cells; Serum sensitivity

Viral vectors based on retroviruses, such as murine leukemia virus (MLV) and human immunodeficiency virus, are powerful tools for gene delivery in vitro and in vivo (for a review, see reference Takeuchi and Pizzato, 2000). For in vivo gene therapy, it is necessary to develop vectors resistant to serum. In terms of human in vivo gene therapy, there have been many reports on retrovirus vectors which can not be inactivated by human serum (Kafri, 2001; Quinonez and Sutton, 2002). However, retroviral vector systems effective for in vivo gene transfer in domestic cats have not been established so far.

Since retroviruses except spumaviruses bud from the cell membrane, they incorporate components expressed on the cell membrane into the virion membrane. Humans have natural antibodies against xenoantigens expressed on non-primate cells such as murine cells (Takeuchi et al., 1996, 1997). These natural antibodies can bind to the virion membrane and lyse retroviruses via activation of the classical complement pathway. Thus, human serum efficiently inactivates retroviruses produced from non-primate cells. Similar to humans, cats may have natural antibodies against xenoantigens expressed on non-feline mammalian cells, and cat serum may inactivate retrovirus particles. In addition to antibody-dependent inactivation, retroviral particles might be lysed by activation of an antibody-independent alternative or mannan-binding-lectin (MBL) complement pathway (Favoreel et al., 2003).

In this study, we newly established MLV-based packaging cell lines derived from human and feline cells. From these packaging cell lines, we generated cell lines producing pseudotype viruses which have the envelope of feline leukemia virus subgroup B (FeLV-B) and express a reporter gene. Then, we compared the sensitivities of the pseudotype

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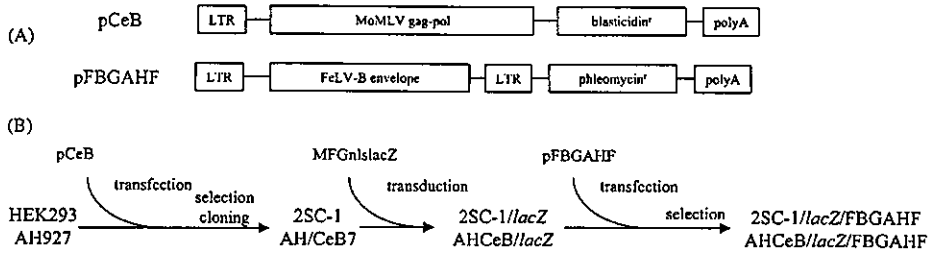

Fig. 1. Establishement of the pesudotype virus-producing cell lines. (A) Schematic representation of plasmid constructs used in this study. MoMLV *gag-pol* expression plasmid, pCeB and FeLV-B envelope expression plasmid, pFBFeLV-B are illustrated. Abbreviations: LTR, long terminal repeat; poly A, poly-adenylation signal. (B) Flow chart of procedures of establishment of pseudotype virus-producing cell lines.

viruses released from these cells to sera from humans and cats, and also examined the xenoantigenicity of the packaging cell lines by flow cytometry.

Human embryonic kidney (HEK) 293 cells, AH927 cells (feline fibroblast cell line), NIH3T3 cells (mouse fibroblast cell line) and TELCeB/SALF cells (Cosset et al., 1995) (a derivative of TE671 cells (human rhabdomyosarcoma cells) producing LacZ pseudotype viruses) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MI, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml) and streptomycin (100 μg/ml).pCeB [1], an expression vector for the Moloney MLV (MoMLV) *gag-pol* gene as well as a blasticidin resistant selectable marker (Fig. 1A), was kindly provided by Dr. Y. Takeuchi (University College London, London, UK). The construction of an *env* expression plasmid for FeLV-B, termed pFBGAHF (Fig. 1A), was described previously (Nakata et al., 2003). pCAGVSV-G, an expression plasmid for the G protein of vesicular stomatitis virus (VSV), and pMX-EGFP, a vector plasmid to express enhanced green fluorescence protein (EGFP), were described elsewhere (Misawa et al., 2000; Matsuura et al., 2001).

For establishment of Gag-Pol producing HEK293 cells (Fig. 1B), HEK293 cells were seeded at a concentration of 4.0×10^5 cells per 35 mm plate the day before transfection. Then, 1 μg of pCeB was transfected using FuGene6 (Roche, Basel, Germany). Two days after transfection, the cells were transferred to two 100 mm plates (Greiner, Frickienhausen, Germany), and selected with 4 μg/ml of Blasticidin S (Calbiochem, Schwalbach, Germany). The selection medium was replaced every 3 days until resistant cell colonies had appeared. Two weeks after the selection, the resistant colonies were picked up using penicillin cups, and further grown in 12-well plates (Greiner) for the assessment of reverse transcriptase (RT) production.

For establishment of Gag-Pol producing AH927 cells (Fig. 1B), AH927 cells were seeded in a 25 cm^2 flask the day before transfection. Then, 10 μg of pCeB was transfected by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973). Four hours after transfection, the cells were washed twice with FCS-free medium and glycerol-shocked, and then the culture medium was replaced with fresh medium. Two days after transfection, the cells were transferred to two 75 cm^2 flasks and selected with 4 μg/ml of Blasticidin S. The resistant cells were cloned by the limiting dilution method using conditioned medium. Each cell clone was expanded in 25 cm^2 flasks for the assessment of RT activity.

Mn^{2+}-dependent RT activity in the culture supernatants from blasticidin-resistant 293 clones was measured using a Reverse Transcriptase Assay, Chemiluminescent (Roche) following the manufacturer's instructions using $MnCl_2$ instead of $MgCl_2$. The Mn^{2+}-dependent RT activity in the cell culture supernatants of blasticidin-resistant AH927 clones was assayed using [α-^{32}P]dTTP as described previously (Ohki et al., 1992). Briefly, 10 μl of the culture supernatant was mixed with a reaction mixture containing poly(rA)-oligo(dT) and [α-^{32}P]dTTP. After incubation for 3 h at 37 °C, the mixture was dotted on DEAE filter paper (DE81) (Whatmann, Kent, UK). RT activity was measured using scintillation.

The Gag-Pol expressing cell clones were introduced with a vector plasmid MFGnlsLacZ (Ferry et al., 1991) that expresses the *lacZ* gene with a nuclear localization signal. Cells were seeded at a concentration of 1×10^4 cells per well of six-well plates and inoculated with a helper-free MFGnlslacZ pseudotype virus bearing an envelope of amphotropic MLV (MLV-A) in the presence of 8 μg/ml of polybrene. The MFGnlslacZ pseudotype viruses were prepared from the culture supernatant of TELCeB/SALF cells as described previously (Cosset et al., 1995). Two days after infection, some of the cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Sigma) as described previously (Sanes et al., 1986). Infection with the MFGnlslacZ pseudotype viruses and X-gal staining were repeated until more than 95% of the infected cells became *lacZ*-positive.

The *lacZ*-positive cell clones were seeded at a concentration of 4×10^5 cells per 35 mm plate (Greiner) the day before transfection. Then, 1 μg of pFBGAHF was transfected using FuGene6. Two days after transfection, the cells were transferred into 75 cm^2 flasks (Corning, NY, USA) and selected with 50 μg/ml of phleomycin (Sigma). Two weeks after selection, phleomycin resistant cell populations were obtained. The culture supernatants were harvested from the

confluent cell cultures in the presence or absence of FCS, filtrated through a 0.45 μm filter (Millipore, Bedfold, MA, USA), and used immediately for the infection assays.

For titration of LacZ pseudotype virus, target cells were seeded at a concentration of 3×10^4 cells for both AH927 and NIH3T3, and 5×10^4 cells for HEK293 in 0.25 ml per well of 48-well plates the day before infection. Cells were inoculated with 100 μl of serially diluted viruses in the presence of 8 μg/ml of polybrene. Four hours after infection, the virus was removed and the cells were cultured in the DMEM. Two days after infection, cells were stained with X-gal, and *lacZ*-positive foci were counted as described previously (Takeuchi et al., 1994).

From HEK293 cells transfected with pCeB (293/CeB), 34 blasticidin-resistant clones were obtained. Each clone was tested for the production of RT in the culture supernatants. Nine clones showed relatively high levels of RT activity (Fig. 2A). For titration of EGFP pseudotype virus, HEK293 cell clones expressing MLV Gag-Pol proteins were seeded at a concentration of 4×10^5 cells per well of six-well plates the day before transfection. Then, 1 μg of pCAG-VSVG and 1 μg of pMX-EGFP were cotransfected using FuGene6. Two days after transfection, the culture medium was replaced with fresh medium. Then the culture supernatants were harvested after incubation overnight, filtrated through a 0.45 μm filter and used for the infection assay as described previously (Takeuchi et al., 1994). In brief, target HEK293 cells were seeded at a density of 2×10^5 cells per well of 24-well plates the day before infection. Cells were inoculated with 500 μl of the serially diluted viruses in the presence of 8 μg/ml of polybrene. Four hours after infection, the inocula were removed and the cells were cultured in fresh medium. Two days after infection, EGFP-positive foci were counted.

After the transfection of pMX-EGFP and pCAG-VSVG into the clones, all clones produced EGFP pseudotype viruses bearing an envelope of VSV G proteins ranging from 1.5×10^5 to 3.0×10^6 focus forming units (ffu)/ml (Fig. 2B). Among the clones, we selected number 1 (designated 2SC-1) which showed the highest production of pseudotype viruses for further study.

From AH927 cells transfected with pCeB (AH/CeB), 24 blasticidin-resistant clones were obtained by the limiting dilution method. Because of the low transfection efficiency of the cells, AH/CeB clones were assessed only by RT production. Among the blasticidin-resistant clones, 10 showed relatively high levels of RT activity (Fig. 2A). Among these clones, we selected number 7 (designated AHCeB7) which showed the strongest RT activity for further study.

The 2SC-1 and AHCeB7 cells were introduced with a *lacZ* vector by infection. After confirming that more than 95% of the cells express the lacZ gene by X-gal staining, these cell lines were further transfected with pFB-GAHF and selected using phleomycin. Consequently, we obtained cell lines, designated 2SC-1/*lacZ*/FBGAHF and AHCeB/*lacZ*/FBGAHF, respectively, which produce LacZ pseudotype viruses. LacZ pseudotype viruses produced from 2SC-1/lacZ/FBGAHF and AHCeB/*lacZ*/FBGAHF were referred to as FeLV-B(*lacZ*)/2SC and FeLV-B(*lacZ*)/AH, respectively and were tested for transduction of the *lacZ* gene to HEK293, AH927 and NIH3T3 cells. These stable transfectants produced LacZ pseudotype viruses which can infect HEK293 and AH927 cells but not NIH3T3 cells. The titers of the pseudotype viruses were more than 10^3 ffu/ml even in the absence of FCS during virus preparation and infection (Table 1). Pseudotype viruses prepared in the absence of FCS were subjected to serum sensitivity tests.

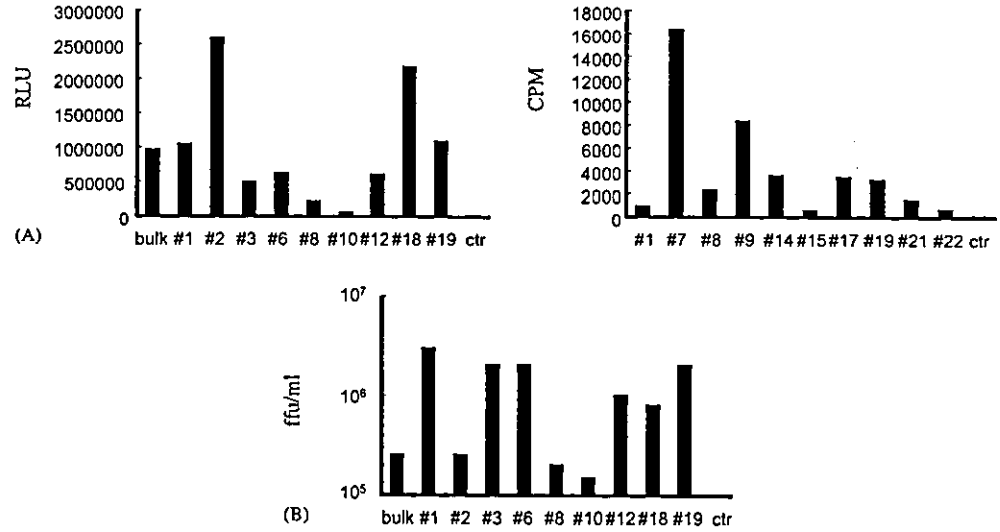

Fig. 2. (A) RT activities in the culture supernatants from 293/CeB (left) and AH927/CeB (right). RT activities are shown as the intensity of relative lights units (RLU) and counts per minute (CPM) of [α-^{32}P], respectively. (B) Infectious virus titers of EGFP-VSV G pseudotype viruses released from 293/CeB clones. EGFP-positive focus forming units (ffu) are shown.

Table 1
Titers of pseodotype viruses from producer cell lines in the absence of FCS

Target cells	Producer cell lines	
	2SC-1/*lacZ*/FBFeLV-B	AHCeB/*lacZ*/FBFeLV-B
HEK293	1.5×10^4[a]	1.0×10^4
AH927	3.0×10^3	3.5×10^3
NIH3T3	<10	<10

[a] Averages of titers (ffu/ml) of *lacZ* pseudotype viruses in three independent experiments are shown.

The sensitivity of *lacZ* pseudotype viruses to sera from humans and cats was examined by titrating the surviving viruses after incubation with 10% serum. In brief, the culture medium of each producer cell line was changed with FCS-free DMEM the day before the assay, and the culture supernatants were harvested. The harvested pseudotype viruses were incubated with fresh human or cat serum for 1 h at 37 °C. After incubation, the viruses were serially diluted and inoculated to target cells in the presence of 8 μg/ml of polybrene. Four hours after inoculation, the culture medium was replaced with fresh medium and the cells were incubated for an additional 2 days before X-gal staining. Since we found that HEK293 and AH927 cells were sensitive to lysis by fresh sera from cats and humans, respectively (data not shown), we used homologous target cells for the serum sensitivity test (i.e. HEK293 for the human serum and AH927 for the cat serum). FeLV-B(*lacZ*)/2SC was resistant to human serum but efficiently inactivated by cat serum (Table 2). In contrast, FeLV-B(*lacZ*)/AH was efficiently inactivated by human serum, however the viruses were relatively resistant to cat serum (Table 2). Heat-inactivated human and cat sera did not inactivate the pseudotype viruses at all (data not shown).

Finally, the xenoantigenicity of the cell lines was examined by flow cytometric analyses. Cells were harvested after 0.05% EDTA treatment for 10 min at 37 °C. After being washed with phosphate-buffered saline (PBS), the cells were reacted with 10% heat-inactivated cat or human serum in serum-free DMEM on ice for 40 min. The cells were washed with PBS, and then reacted with fluorescein isothiocyanate-labeled anti-human whole immunoglobulin (Ig) (Molecular Probe, Eugene, OR, USA) or anti-feline whole Ig (ICN Biomedical, Aurora, OH, USA) at a final concentration of 0.002 μg/ml on ice for 40 min. After being washed with PBS, the cells were resuspended in 500 μl of PBS, and analyzed using a FACSCalibur (Becton Dickinson, San Jose, CA, USA). The data were analyzed using CellQuest software (Becton Dickinson). Human HEK293 cells did not react with sera from humans or cats (Fig. 3A). Feline AH927 cells reacted strongly with human serum but weakly with cat serum (Fig. 3C). The reactivity to the sera was not affected by the expression of MLV Gag-Pol and FeLV-B Env proteins in the cells (Fig. 3B and D).

Table 2
Sensitivity of viruses for human and feline serum

Pseudotype virus	Target	Serum	Titer (ffu/ml)[a]	Percentage of reduction
FeLV-B(*lacZ*)/2SC	HEK 293	Control[b]	800	
		Human	800	0
	AH927	Control	1500	
		Cat	366	76
FeLV-B(*lacZ*)/AH	HEK293	Control	2500	
		Human	<10	100
	AH927	Control	7500	
		Cat	5030	33

[a] Averages of titers of *lacZ* pseudotype viruses in three independent experiments are shown.
[b] Pseudotype viruses were reacted with serum-free DMEM.

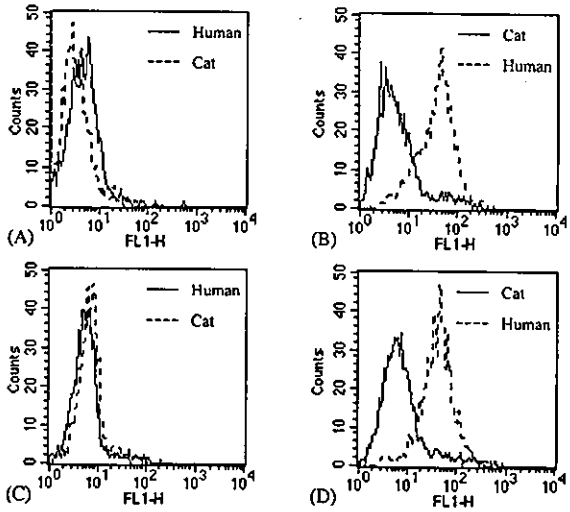

Fig. 3. Xenoantigenicity of HEK293, AH927 and their derivatives. HEK293 (A) and 2SC-1/*lacZ*/pFBGAHF cells (B) were reacted with heat-inactivated sera from humans (bold line) and cats (dotted line). AH927 (C) and AHCeB/lacZ/pFBGAHF cells (D) were reacted with heat-inactivated sera from cats (bold line) and humans (dotted line).

In this study, we newly established two retrovirus packaging cell lines, AHCeB7 and 2SC-1, from feline AH927 and human HEK293 cells, respectively. After the introduction of a lacZ vector and FeLV-B Env expression plasmid, these cells produced LacZ pseudotype viruses. Using these pseudotype viruses, we compared the sensitivities of the viruses to fresh sera from humans and cats, and found that usage of feline cells as packaging cells is suitable for in vivo gene therapy in cats.

The most important difference at the cell surface between humans and other mammals except apes and old world monkeys is the existence of Gal(α1-3)Gal terminal carbohydrates ((α1-3)Gal) (Galili et al., 1985, 1987). Since humans and old world monkeys lack a functional (α1-3)galactosyltransferase (Larsen et al., 1990; Galili and Swanson, 1991), they have no (α1-3)Gal terminal structure and develop abundant anti-(α1-3)Gal antibodies in their sera

(Galili et al., 1985). Virions released from nonprimate cells incorporate the (α1-3)Gal (Takeuchi et al., 1996, 1997). Retroviruses bearing the (α1-3)Gal epitope are immediately inactivated by human serum (Rother et al., 1995).

Here, we also confirmed that human serum efficiently inactivated the pseudotype viruses from feline AH927 cells but not those from human HEK293 cells. On the other hand, cat serum efficiently inactivated the pseudotype viruses from HEK293 cells. Since cat serum did not react with 2SC-1/*lacZ*/FBGAHF cells in the flow cytometric analyses (Fig. 3) and heat-inactivated cat sera did not inactivate the pseudotype viruses, we considered that most of the pseudotype viruses have been inactivated by complement in an Ab-independent manner, i.e. alternative or MBP complement pathway. To determine which pathway is involved in the neutralization, the effects of depletion of Ca^{2+} and Mg^{2+} were examined. Ca^{2+} is essential for the classical and MBL complement pathways, whereas the alternative pathway can be activated in the presence of Mg^{2+} instead of Ca^{2+}. Contrary to our expectation, no effects were observed when both Ca^{2+} and Mg^{2+} or Ca^{2+} alone were chelated by 10 mM EDTA or EGTA, respectively (data not shown). These results suggested that certain temperature-sensitive factor(s) other than complements might be involved in this neutralization although the nature of the factor(s) is still unknown at present.

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NOTE *Virology*

High Genetic Stability of TM1 and TM2 Strains of Subtype B Feline Immunodeficiency Virus in Long-Term Infection

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ABSTRACT. To know the genetic changes of feline immunodeficiency virus (FIV) in long-term infection in cats, we inoculated three specific pathogen-free cats with FIV isolates and determined a partial *env* sequence covering the V3-V5 region. In 2 cats infected with subtype B strains TM1 and TM2, only one amino acid change in region V3 was observed at 9 years post infection (y.p.i.), and no nucl eotide substitutions were observed between 9 and 10 y.p.i., indicating that these strains are genetically stable. On the other hand, in a cat infected with subtype A strain Petaluma at 8.7 y.p.i., 3 nucleotide insertions (one amino acid insertion) in region V5, and 1 sy nonymous nucleotide substitution and 2 non-synonymous nucleotide substitutions in region V5, were observed.

KEY WORDS: FIV, mutation, subtype.

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A hallmark of human immunodeficiency virus (HIV) infection is the rapid generation and turnover of viral variants, resulting in a high degree of sequence diversity within and between infected individuals [14]. Immune surveillance [15] and viral cell tropism [10] are examples of plausible selective forces that may be shaping HIV diversity *in vivo*.

Feline immunodeficiency virus (FIV) infection in cats is an important animal model for lentiviral vaccine development and antiviral therapy since FIV causes selective loss of the CD4+ T cell subset and acquired immunodeficiency syndrome (AIDS) in naturally infected host species [2]. Similar to HIV, FIV has considerable sequence variation in the *env* gene, and the third to fifth variable regions (V3 to V5) of *env* contain an immunodominant neutralization domain and a determinant of cell tropism [3, 12, 13]. Based on the sequence diversity in V3 to V5, FIV isolates have been classified into 5 subtypes, A to E [8, 11]. In the present study, we estimated the mutation rates of the V3-V5 region in long term infections of over 8 years duration. Our results showed remarkable genetic stability among subtype B FIV isolates in cats.

Three specific pathogen-free (SPF) cats aged 5.5 months were injected intraperitoneally with primary peripheral blood mononuclear cells (PBMCs) infected with the FIV subtype A strain Petaluma (Cat 105) which was isolated from a cat with an immunodeficiency-like disease [9], or 0.5 m*l* of the peripheral blood of cats naturally infected with subtype B strains TM1 and TM2 (Cats 103 and 104, respectively) [7]. These cats were kept separately in isolation units during the experiment. As we reported previously [6], Cat 105 died from immunodeficiency-like diseases with remarkable a decrease in the CD4/CD8 ratio at 8 years and 8 months after infection. FIV was isolated from Cats 103, 104 and 105 all through the experimental period.

PBMCs were isolated from Cats 103 and 104 at 3 weeks post infection (w.p.i.) and 9 and 10 years p.i. (y.p.i.). PBMCs of Cat 105 were isolated at 3 w.p.i. and 8.7 y.p.i., a week before the death. The isolated PBMCs were stored in liquid nitrogen until the genomic DNA was isolated. For sequencing analyses, total cellular DNA was extracted from the PBMCs with a QIAamp blood kit (QIAGEN, Hilden, Germany) and the part of the *env* gene encompassing the region from V3 to V5 was amplified using the primers HV3f and HV5r and subjected to direct sequencing analysis as described previously [8]. Three independent PCR amplifications were carried out for each of the DNA templates. Each PCR amplification yielded identical results.

The 627 bp nucleotide sequence covering the V3-V5 region from each of the cats was determined and the three were compared. In Cats 103 and 104, no synonymous but non-synonymous substitutions were observed (Fig. 1). The amino acid changes in both cats were located in region V3 at 9 y.p.i. The sequences from Cats 103 and 104 at 10 y.p.i. revealed no nucleotide substitutions between 9 and 10 y.p.i. On the other hand, 3 nucleotide insertions and 3 nucleotide substitutions were observed in Cat 105 at 8.7 y.p.i. One nucleotide change between V3 and V4 was a synonymous substitution, but the others resulted in two amino acid sub-

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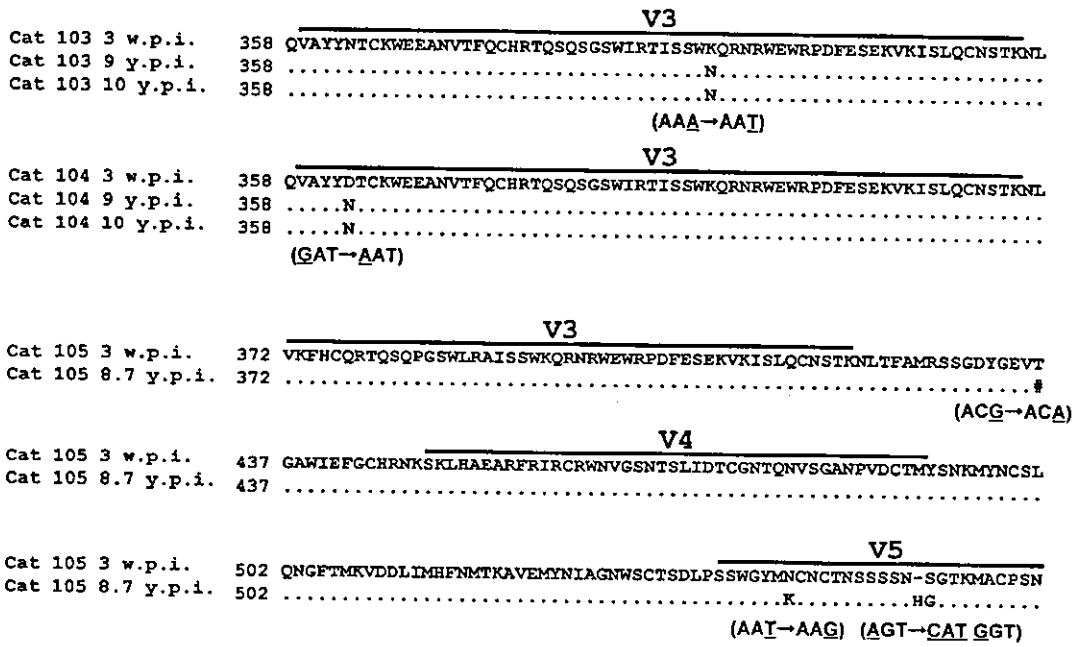

Fig. 1. Comparison of the amino acid sequence of the V3-V5 region of the FIV *env* gene for isolates within single hosts through long term infection. Identical amino acids are indicated by dots (•), and gaps are indicated by bars (–). The position of the synonymous substitution in Cat 105 is indicated by #. Nucleotide substitutions and insertions in the amino acid codons are indicated as und erlined in parentheses. Since there are few amino acid substitutions in the three cats, completely conserved sequences in the V3-V5 regon are omitted.

stitutions and one amino acid insertion in the V5 region.

As we reported previously [7], peripheral blood samples from domestic cats naturally infected with FIV strains TM1 and TM2 were used to directly inoculate SPF Cats 103 and 104, respectively. Although we had expected to find a high degree of mutation through long term infection, only one non-synonymous substitution was observed in the V3-V5 region in both Cats 103 and 104 at 10 y.p.i., suggesting remarkable genetic stability among the viruses *in vivo*.

Balfe *et al.* [1] examined a cohort of hemophiliacs who were infected with the same source of HIV type 1 (HIV-1) and estimated the mutation rate of the sequence to be 0.4% nucleotide substitutions per site per year in the V4-V5 region and 0.5% per year in the V3 region. Greene *et al.* [4] reported that the rate of mutation in the V1-V2 *env* region of a subtype A FIV isolate was 0.34% per year, which is comparable to that of HIV-1. However, in the present study, the mutation rates of both TM1 and TM2 (subtype B) were estimated to be only 0.015% per year. The nucleotide mutation rate of strain Petaluma (subtype A) (0.11% per year) was about six times that of TM strains. Cat 105 developed an AIDS-like disease 8 years after infection, whereas both Cats 103 and 104 remained asymptomatic for over 10 years, suggesting a correlation between disease progression and nucleotide substitution rates. The low genetic diversity rates may, in part, be ascribed to the long term non-progression without viremia in the infected cats even at 8 years post-infection [6]. Although it is still unknown whether this genetic stability of the strains is subtype-specific, it is of note that subtype B isolates are considered to be more host-adapted than subtype A isolates [11], leading to higher genetic stability in cats.

In conclusion, we found the mutational rates of the subtype B isolates, TM1 and TM2 strains, to be lower than expected. Because of their genetic stability and low virulence, modified TM2-type viruses [5] might be a good candidate for an attenuated live vaccine against FIV.

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Original article

T cell subpopulations mediating inhibition of feline immunodeficiency virus replication in mucosally infected cats

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Abstract

Feline immunodeficiency virus (FIV) infection induces an increase in two subpopulations ($CD8\alpha^+\beta^{low}$ and $CD8\alpha^+\beta^-$) within $CD8^+$ peripheral blood lymphocytes (PBLs) of cats. It is known that depletion of $CD8^+$ cells often results in augmentation of FIV proliferation in PBL culture, similarly to the case of human immunodeficiency virus. In this study, we attempted to define PBL subpopulations mediating antiviral activity in five cats intravaginally infected with a molecularly cloned FIV isolate. Several subpopulations ($CD8\alpha^+\beta^+$, $CD8\alpha^+\beta^-$, and $CD4^+$ cells) were shown to participate in inhibition of the FIV replication, at least in part, in a major histocompatibility complex-unrestricted manner. Moreover, the subpopulations showing anti-FIV activity were different among the individual cats.

Keywords: Cellular immune response; CD4; CD8; Feline immunodeficiency virus; Mucosal infection; Peripheral blood lymphocyte

1. Introduction

Feline immunodeficiency virus (FIV) [1], a member of the genus *Lentivirus*, infects domestic cats and causes an acquired immunodeficiency syndrome-like disease after a protracted asymptomatic phase of several years [2,3]. Both virus-specific cytotoxic T cell [4–9] and non-cytotoxic antiviral activities [6,10–18] are observed within $CD8^+$ peripheral blood lymphocytes (PBLs) of the infected cats in the early through the asymptomatic phase. These findings resemble human immunity in human immunodeficiency virus (HIV) infection [19]. Furthermore, FIV infection in cats can be achieved via genital and rectal mucosa [5,20–25]. Therefore, the FIV/cat system is a useful animal model to examine immunological responses in mucosal infection and to develop vaccines or antiviral therapies.

Previously, we reported an increase in two subpopulations, $CD8\alpha^+\beta^{low}$ and $CD8\alpha^+\beta^-$ cells, in $CD8^+$ PBLs of FIV-infected cats [26]. The $CD8\alpha^+\beta^{low}$ cells increase as early as 3–4 weeks post-infection [11,27] and are maintained through the asymptomatic phase. Bucci et al. [11] and Flynn et al. [6] reported the strong anti-FIV activity within $CD8\alpha^+\beta^{low}$ cells of both acute and chronic infections. A period of $CD8\alpha^+\beta^-$ cell expansion after infection has yet to be elucidated; however, the cells are often observed in relatively long-term infected cats [15,26]. Their role in FIV infection or correlation with disease progression has not been investigated, due to insufficient expansion of the cells in a short term after infection [15].

In this study, to better understand the cellular immunity in the mucosal infection, we investigated the antiviral activities of these $CD8^+$ subpopulations or other cells ($CD4^+$ lymphocytes) of cats mucosally infected with a molecularly cloned FIV isolate by two means, "depletion" and "reconstitution" assays.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FCM, flow cytometry; FIV, feline immunodeficiency virus; MHC, major histocompatibility complex; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor.

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2. Materials and methods

2.1. Experimental animals

The procedures used for inoculating specific-pathogen-free cats with FIV were reported previously [23,28]. Six female cats (cats 301–306), 5–7 months old, were used: cats 301 and 304, cats 302 and 305, and cats 303 and 306 were from the same litters, respectively. Briefly, these cats were inoculated via the vagina with MYA-1 cells (a feline T-lymphoblastoid cell line) [29] infected with FIV strain TM2. Consequently, five cats (301–305) became positive for proviral DNA in peripheral blood mononuclear cells (PBMCs) within 8 weeks post-inoculation. Four years after infection, anti-FIV antibodies were observed in these cats at a high level, as observed in the early phase of infection, and these cats were asymptomatic. Plasma samples of these cats were inoculated onto 2×10^5 MYA-1 cells at dilutions of 1:5 and 1:50, and the cells were cultured for 16 days; however, no evidence of viral infection was confirmed by indirect immunofluorescence assay of the cells [30] nor by enzyme-linked immunosorbent assay (ELISA) of the culture supernatants in any of the cases, indicating no or very low viral titers in plasma [31]. One cat (cat 306) did not become positive for provirus or antibody in spite of inoculation, indicating no establishment of infection in this animal. In this study, the five FIV intravaginally infected cats (cats 301–305) and one inoculated but uninfected control cat (cat 306) were used. Four years passed after FIV inoculation.

2.2. Depletion and culture of PBMCs (depletion assay)

PBMCs were isolated from heparin-treated peripheral blood with Ficoll-paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Aliquots were used to analyze the expression of two surface molecules, CD8α and CD8β, on PBLs by two color-flow cytometry (FCM), as described previously [26]. For depletion by panning, isolated PBMCs were divided into three and then incubated with no antibody (mock), anti-CD8αβ vpg9 (to deplete $CD8\beta^+$ but not $CD8\alpha^+\beta^-$ cells) or anti-CD8α 12A3 (to deplete all $CD8^+$ cells). After a wash, the cells were seeded on a Petri dish (Bio-Bik, Osaka, Japan), which had been pre-treated with goat anti-mouse IgG antibodies (Rockland, Gilbertsville, PA). Non-adherent cells were harvested by gentle washing of the dish, and aliquots were analyzed by FCM to estimate depletion efficiencies. The harvested PBMCs (1.5×10^5 cells) were mixed with MYA-1 cells (1×10^5 cells) as indicator cells, stimulated with concanavalin A for 3 days, and cultured for a further 9 days in the presence of interleukin-2. Culture supernatants were harvested at days 6, 9 and 12 for measurement of p24 FIV antigen by ELISA.

2.3. Reconstitution of PBMCs (reconstitution assay)

Isolated PBMCs were directly seeded on pre-treated Petri dishes to remove non-specifically adhered cells (mostly granulocytes and monocytes). Then non-adherent cells (lymphocytes) were harvested, labeled with adequate antibodies, and then panned as described above. In addition to non-adherent (target) cells, specific adherent (effector) cells were also harvested with cell scrapers and used in the cell culture. These effector and target cells were co-cultured at concentrations of 1×10^5 effector, 1.5×10^5 target, and 1×10^5 indicator cells per 1 ml, and then cultured as described for the depletion assay. When infected MYA-1 was used as the target, indicator cells were not added. The measurement of p24 was performed only at day 12, although for FIV-14-infected MYA-1, it was made at day 9.

2.4. Measurement of FIV p24 antigen

p24 antigen in culture supernatant was detected using a commercial kit (FIV Antigen Test Kit) (IDEXX, Westbrook, ME). In the depletion assay, an OD_{655} of more than 0.5 was regarded as positive for the proliferation of FIV. In the reconstitution assay, percent inhibition was calculated as follows: (p24 of target cells minus p24 of target cells co-cultured with effector cells)/(p24 of target cells minus p24 of effector cells without target cells) × 100 (%). In co-culture with infected MYA-1 as target cells, effector cells co-cultured with uninfected MYA-1 were used as the "target-absent effector". Antiviral activity was regarded as significantly positive when the percent inhibition was more than 50.

2.5. Antibodies

To deplete subpopulations of PBMCs by panning, anti-CD8αβ vpg9 (specific for αβ heterodimer) [27], anti-CD8α 12A3 [32], anti-CD3ε (unpublished), anti-CD4 44A8 [33] and anti-CD16 (unpublished) were used. For surface Ig^+ cell depletion, Petri dishes which were coated with a rabbit anti-cat IgG (Rockland) were used. For FCM analysis, fluorescein-isothiocyanate-labeled anti-CD8α antibody 10C7 and anti-CD4 4D9 [32,33], and phycoerythrin-labeled anti-CD8β antibody FT2 (Southern Biotechnology Associates, Birmingham, AL) were used.

2.6. Preparation of infected MYA-1 cells

Two infectious molecular clones, pTM219 (strain TM2) [34] and pFIV-14 (strain Petaluma) [35], were transfected into Crandell feline kidney cells by an electroporation method. Two days after transfection, each culture supernatant was inoculated onto MYA-1 cell culture. Fourteen days after infection, the supernatants were harvested and stocked in aliquots at –80 °C until use for infection of fresh MYA-1 cells at a multiplicity of infection of 0.01. The titers of virus stocks were determined as described previously [30]. In some experiments, these infected MYA-1 cells were used for co-culture with isolated PBMCs.

3. Results and discussion

3.1. Subpopulations of CD8$^+$ PBLs in intravaginally infected cats

In FCM, we observed reduced or diminished expression levels of CD8 β chain in CD8$^+$ PBLs in the infected cats, a unique characteristic of PBLs in FIV intraperitoneally infected cats [26]. Borderlines between high and low levels of CD8β were set arbitrarily to separate the two peaks, and then percentages were calculated. The percentages of CD8α$^+$β^{high}/β^{low}/β$^-$ within total PBLs were as follows: cat 301, 16%/15%/10%; cat 302, 25%/11%/16%; cat 303, 16%/19%/38%; cat 304, 10%/14%/4%; cat 305, 18%/12%/6%. In contrast, the control cat 306 exhibited a PBL composition of 21%/2%/2%, which was characteristic of uninfected cats [26]. No remarkable changes in these percentages were observed during this study (data not shown). This observation together with previous reports [6,11,15,26,27,36,37] revealed that FIV infection could be characterized by the reduction in the expression level of the CD8 β chain on peripheral blood CD8$^+$ lymphocytes irrespective of the infection route (intravaginal, intravenous or intraperitoneal) or viral strain used (Japanese, Swiss, British or American isolates). The reduction in β chain, but not α chain, has not been well documented in other viral infections in other animals; however, to our knowledge, similar changes in phenotype were reported in only two studies: Borna disease virus-infected cats [38] and HIV-infected patients [39]. The β chain reduction is not identical to a transient reduction in both α and β chains along with T cell receptor (TCR), which usually occurs after T cell interaction with a peptide-presenting major histocompatibility complex (MHC) class I molecule [40]. Recently, rat splenic γδ T cells were reported to reduce the expression of CD8β, but not CD8α, after in vitro co-stimulation through TCR and CD28 [41]. Unfortunately, no analysis of TCR expression on feline lymphocytes has been reported. The phenomenon of CD8 β chain reduction should also be well analyzed in various species.

3.2. CD8α$^+$β$^+$ and CD8α$^+$β$^-$ lymphocytes are involved in the anti-FIV activity

To examine the anti-FIV activity of the CD8$^+$ subpopulations, depletions of the subpopulations from PBLs were performed by panning. Representative FCM results of CD8β- or CD8α-depleted PBLs in the depletion assay are shown in Fig. 1. Mock treatment of PBMCs (Fig. 1a) had negligible effects on ratios of the CD8$^+$ subpopulations, when untreated PBMCs were stained in parallel and then subpopulation ratios were compared by FCM (data not shown). In each cat, cell populations positive for CD8β and CD8α after depletion of CD8β and CD8α were <1% (Fig. 1b) and <3% (Fig. 1c), respectively. While anti-CD8αβ vpg9 (used for depletion) slightly blocked the binding of anti-CD8β FT2 (used for FCM), vpg9 antibody was not detected

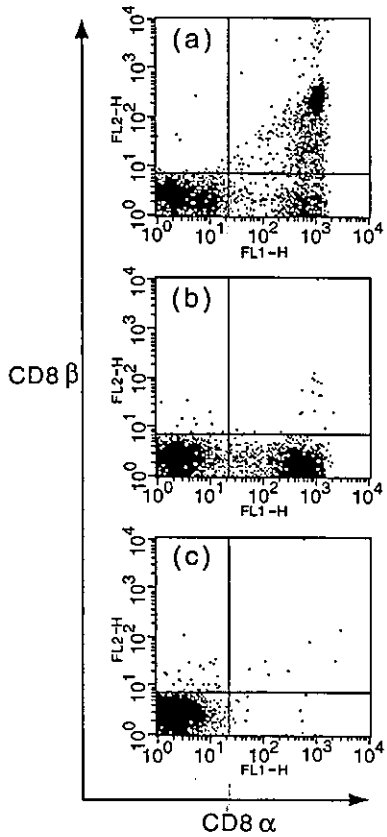

Fig. 1. Depletion of CD8β$^+$ or CD8α$^+$ cells from PBMCs of FIV-infected cats. Representative FCM results of cat 302 PBLs are shown. PBMCs were labeled with no mAb (mock), anti-CD8αβ or anti-CD8α, and depleted of the intended cell populations by the panning method. Then non-adherent cells were harvested as mock-treated (a), CD8β-depleted (b) and CD8α-depleted (c) PBLs and used for the depletion assay (Fig. 2).

in the depleted PBMCs; no binding of phycoerythrin-labeled anti-mouse antibody to the depleted cells was observed when analyzed in FCM (data not shown). Anti-CD8α 12A3 used for depletion did not block the binding of anti-CD8α 10C7 for FCM analysis (data not shown). From these facts, we concluded that the depletion of the cell populations observed in the FCM analysis was not due to epitope masking and that the depletion of the CD8β or CD8α population by the panning was properly performed. Further, our panning method used for the depletion also removed non-lymphoid cells such as monocytes and granulocytes that adhere to plastic dishes non-specifically; cells after the panning showed lymphocyte-specific light scatters in FCM (data not shown). Thus, the present study can be interpreted as an analysis of lymphocytes rather than mononuclear cells.

We co-cultured the depleted cells with indicator cells (MYA-1) and measured p24 antigen in the supernatants, as described in Section 2. As shown in Fig. 2, a striking increase in p24 antigen in supernatant was observed in several cases at day 12. In cat 301, neither mock nor CD8β depletion resulted in an increase in p24 antigen; however, CD8α depletion did.

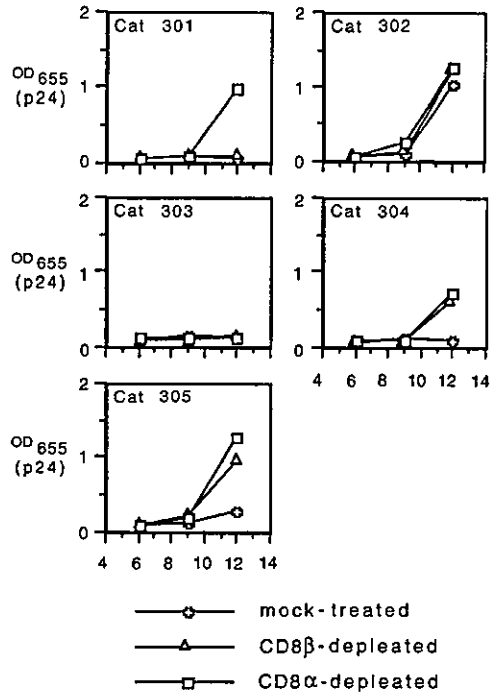

Fig. 2. Depletion assay: FIV replication in depleted PBLs. Mock-treated, CD8β-depleted and CD8α-depleted PBLs of FIV-infected cats (cats 301–305) were mixed with an FIV highly sensitive T-lymphoblastoid cell line (MYA-1 cells), stimulated by concanavalin A for 3 days and cultured for a total of 12 days. Culture supernatants were harvested on the days indicated and measured for the FIV p24 Gag antigens by ELISA. Experiments were performed in duplicate, and the averages of OD_{655} values are shown. A value greater than 0.5 was regarded as indicating positivity for FIV proliferation.

In cat 302, an increase in p24 was observed even in mock-treated PBLs. In cat 303, no increase in p24 was observed even in the CD8α-depleted cell culture. In cats 304 and 305, mock treatment did not result in increase in p24, while both CD8β and CD8α depletion did. These data indicate that anti-FIV activities were present within $CD8\alpha^+\beta^+$ in cats 304 and 305, and $CD8\alpha^+\beta^-$ lymphocytes in cat 301. These findings do not exclude the possibility that $CD8\alpha^+\beta^+$ lymphocytes in cats 301 and 303 have antiviral activity, because if other populations had enough antiviral activity, we should not have detected the effect caused by the depletion of $CD8\alpha^+\beta^+$ cells. While several groups reported suppressive activities within $CD8^+$ lymphocytes in FIV-infected cats, there are few studies mentioning the relationships between the different expression levels of the CD8 β chain and the suppressive activities. Two groups reported suppressive activities in purified $CD8\alpha^+\beta^{low}$ cells [6,11], and Gebhard et al. [15] obtained a similar result by the use of anti-CD8β and CD62L antibodies to sort the subpopulation. Concerning the $CD8\alpha^+\beta^{high}$ cells, the results by Flynn et al. [6] were inconsistent with those by others [11,15]: the former detected antiviral activities in the subpopulation, while the latter did not. We did not elucidate the distinct populations within $CD8\alpha^+\beta^+$ lymphocytes that had antiviral activity, because separation of $CD8\beta^{high}$ and $CD8\beta^{low}$ cells by sorting was difficult due to the unclear borderline between high and low levels of β-chain expression. As performed by Gebhard et al. [15], use of the CD62L (L-selectin) marker may be more suitable for the subdivision of responsible cells than use of the CD8β molecule.

The functional analyses of $CD8\alpha^+\beta^-$ cells have not been done, probably due to insufficient expansion of the subpopulation in a relatively short time (1–3 years) after FIV infection [15], while differential tissue dynamics of $CD8\alpha^+\beta^{high}$, β^{low} and β^- cells were also reported in neonatally infected cats [13]. However, at least regarding the suppression of the p24 increase in culture supernatants, we observed that $CD8\alpha^+\beta^-$ cells in one cat (cat 301) had suppressive activity, as did $CD8\alpha^+\beta^+$ cells in the other cats. More detailed studies will be required for these subpopulations in relation to a mechanism of the antiviral activity and maintenance of the asymptomatic phase or acquisition of immunodeficiency.

Thus, both $CD8\alpha^+\beta^+$ and $CD8\alpha^+\beta^-$ PBLs were shown to have antiviral activity. However, in the case of cat 302, which had a typical characteristic of $CD8^+$ PBLs of FIV-infected cats (Fig. 1a), there was no antiviral activity observed (Fig. 2). This means that $CD8\alpha^+\beta^{low}$ and $CD8\alpha^+\beta^-$ PBLs observed in the asymptomatic phase of FIV infection does not always show such activity in vitro. Considering that FIV could not be isolated from plasma of the animal (described in Section 2) and that the animal was asymptomatic like the others, neutralizing antibodies or other anti-virus mechanisms may be responsible for the regulation of FIV proliferation in cat 302 in vivo. Jeng et al. [18] and Hohdatsu et al. [16] also reported viral replication in undepleted PBMCs of infected cats, but without detailed analysis for CD8 phenotypes or viremia.

3.3. $CD4^+$ lymphocytes also can be responsible for anti-FIV activity

In the case of cat 303, the CD8 depletion from PBLs did not result in FIV replication (Fig. 2). There are two possibilities to explain the phenomenon: the cat did not have infected cells in the isolated PBMCs, or FIV could not replicate in the depleted PBLs for some reason. To determine whether the latter possibility is correct, we first co-cultured FIV (strain TM219 or FIV-14)-infected MYA-1 cells with CD8-depleted PBLs of cat 303. No increase in p24 was observed in the co-culture, although the peak of p24 production was observed at day 12 for TM2- and at day 9 for FIV-14-infected MYA-1 cells (data not shown). These results indicated that anti-viral activity was present in CD8-negative cells in cat 303. Next, we depleted other subpopulations ($CD3\varepsilon^+$, surface Ig^+, or $CD16^+$ cells) from the PBLs of cat 303 and then conducted co-cultures with FIV-infected MYA-1 cells. As shown in Fig. 3a, CD3ε depletion induced an increase in p24 in the FIV-14-infected MYA-1 cell culture. Because the $CD3\varepsilon^+$ population is mostly composed of $CD4^+$ and $CD8^+$ cells [42], depletion of $CD4^+$ or $CD8^+$ cells was also carried

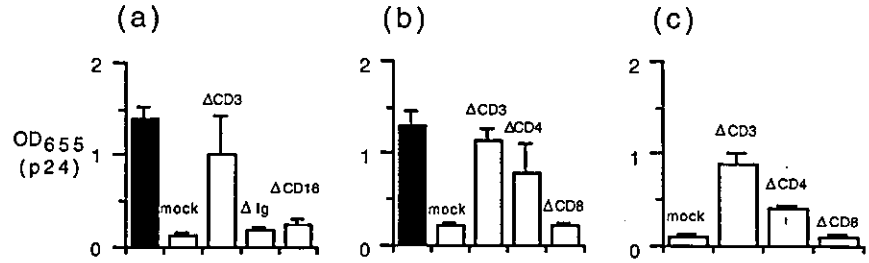

Fig. 3. Effects of depletion of other subpopulations in cat 303 PBLs on FIV replication. By the panning method, the PBMC subpopulations indicated were depleted from PBLs of cat 303 (for example, "ΔCD3" means CD3ε-depleted PBLs, and "mock" means PBLs labeled with no mAb for panning). These depleted cells were mixed with FIV-14-infected (a,b) or uninfected (c) MYA-1 cells and cultured. p24 antigens in culture supernatants were measured by ELISA at day 9 (a,b) or at day 12 (c). Black bars are results in infected MYA-1 cell culture without mixing of the depleted cells. Results are shown as the mean ± S.D. of quadruplicate data. The background (uninfected MYA-1 cell culture alone) is approximately 0.1 OD_{655}.

out. As shown in Fig. 3b, a p24 increase was observed in both CD3ε- and CD4-depleted, but not CD8α-depleted PBLs co-cultured with FIV-14-infected MYA-1. In the last experiment, uninfected MYA-1 was also used for the co-culture, and a significant increase in endogenous p24 was observed in the CD3ε-depleted cell culture (Fig. 3c). In addition, although not significant ($OD_{655} < 0.5$), an increase in p24 was observed in the CD4-depleted cell culture compared with the mock-treated cell culture. Hence, cat 303 certainly had infected cells in the periphery, and $CD4^+$ lymphocytes might be the principal effector hindering FIV replication in PBLs. The non-proliferation of endogenous or exogenous virus in cultures of CD8-depleted PBLs was due to this potent antiviral activity of $CD4^+$ lymphocytes. However, $CD8^+$ lymphocytes also might have weak antiviral activities in this cat, because CD3ε depletion (CD4 and CD8 double depletion [42]) induced more viral replication from an FIV-14-infected cell line than CD4 depletion (Fig. 3b, endogenous FIV replication was negligible, data not shown). These results suggest that more than two distinct populations in one individual can be involved in the suppression of FIV replication.

No replication of virus in CD8-depleted PBMCs of infected individuals, as in the case of cat 303, was occasionally reported in FIV [10,18] and HIV [43] studies. Several reasons for the phenomenon could be proposed; however, detailed immunological analyses for these individuals have not been done. We consider that $CD4^+$ PBLs, although less frequent than $CD8^+$ PBLs, can control lentivirus replication and that the infected individuals described above might control FIV or HIV replication by $CD4^+$ lymphocyte-mediated cytotoxicity [44–47], secretion of interferon-γ and β chemokines [48,49] or other unidentified mechanisms. The lack of a correlation between $CD8^+$ cell numbers and non-cytolytic activities [6,10,18] may be explained by the activity of these CD8-negative lymphocytes. Studies with larger numbers of cats will be needed to test this hypothesis.

3.4. CD4:CD8 ratios

FIV infection induces the decrease of CD4:CD8 ratios of PBLs of the infected cats, and the decreases are often used to estimate immunological disorders [2,4,27,31]. The CD4:CD8α ratios of the cats used in this study were as follows: cat 301, 0.77; cat 302, 0.59; cat 303, 0.33; cat 304, 0.95; cat 305, 0.59; cat 306, 0.77. Three out of five FIV-infected cats showed lower ratios than that of the uninfected one (cat 306). There seemed to be no apparent correlation between the CD4:CD8α ratios and the increases in CD8β-decreased subpopulations within $CD8^+$ PBLs or lymphocyte phenotypes responsible for inhibition of FIV replication. Surprisingly, cat 303, whose $CD4^+$ PBLs showed the potent antiviral activity (Fig. 3), had the lowest CD4:CD8α ratio among the cats.

3.5. Reconstitution assay

To further analyze the antiviral activities, a reconstitution assay was performed based on the results obtained from the depletion assay. $CD8\alpha^+$ cells from cats 301, 304 and 305 and $CD4^+$ cells from cat 303 were isolated by panning from each animal as effector cells that were regarded to suppress FIV replication. The purity of the effector cells for CD8α or CD4 was over 80% (data not shown). PBMCs depleted of the effector cells served as target cells where FIV could proliferate. Mock-treated PBMCs of cat 302 and FIV TM2- or FIV-14-infected MYA-1 cells were also used as target cells. These effector and target cells were mixed in all combinations, cultured, and then measured for p24 antigens in culture at day 12 or 9 (Table 1). Effector cells from cats 301, 303 and 305 inhibited p24 production from self PBMCs significantly (>50% inhibition), confirming the results in the depletion assay, while those from cat 304 did not. The failure of inhibition in cat 304 might be due to a technical error(s) which occurred during preparation of effector cells. Effectors from cats 301, 303 and 305 also inhibited FIV proliferation in non-self targets and in homologous (TM2) or heterologous (FIV-14) FIV strain-infected MYA-1 cells, although not in all combinations. These results suggest that the antiviral activity of $CD4^+$ as well as $CD8^+$ lymphocytes was mediated in a non-restricted manner by MHC, and possibly in an antigen-non-specific manner. No restriction of the activity to homologous isolates was reported [14]. The reason for the absence of suppressive activity of all effectors tested against viral replication in cat 302 PBLs is unclear.

Table 1
Reconstitution assay: co-culture of effector cells with self- or non-self-target cells from FIV-infected cats or with an in vitro-infected cell line (MYA-1)

		Inhibition (%) [a]						
		Target cell						
	Effector	Cat301 ΔCD8α [b]	Cat302 Whole	Cat303 ΔCD4	Cat304 ΔCD8α	Cat305 ΔCD8α	TM2-infected MYA-1 [c]	FIV-14-infected MYA-1
Cat301	CD8α [d]	74	15	99	21	95	61	16
Cat303	CD4	97	8	98	100	95	54	84
Cat304	CD8α	33	23	−19	46	43	29	19
Cat305	CD8α	94	−7	32	84	71	48	46

[a] Inhibition percentages were calculated as described in Section 2. More than 50% is regarded as significantly positive.
[b] CD8α-depleted PBLs of cat 301.
[c] MYA-1 cells infected with FIV strain TM2 at an MOI of 0.01.
[d] $CD8\alpha^+$ cells isolated from cat 301 PBLs.

In conclusion, we have demonstrated that various subpopulations of PBLs in FIV intravaginally infected cats ($CD8\alpha^+\beta^+$, $CD8\alpha^+\beta^-$, and $CD4^+$ phenotypes) are involved in the suppression of FIV replication, at least in part, in an MHC-non-restricted manner. A detailed understanding of immune responses in mucosal infection may help with vaccine or antiviral drug development against both FIV and HIV; therefore, further analysis of these cells carrying antiviral activities is important.

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1), rendering this loop more mobile; glutamate brings a further negative charge into the channel on the periplasmic side of the eyelet and may therefore change the permeation properties slightly. The exchange D91G of MspD removes the side chain defining the pore eyelet diameter (Fig. 4B) and should therefore increase the permeability decisively. N102D of MspD introduces a negative charge on the periplasmic side of the eyelet similar to an exchange in MspC. S103T and V104I should improve the stability of the chain at the goblet base, and the glutamine of A96Q projects into the periplasm. I68V is in the nonpolar core of the rim domain, whereas L8V and G141A strengthen the interface between these domains. Exchanges G1A, L2V, E5Q, W21A, D22E, K47T, and I49H are on the outer-domain surface about 20 Å below the goblet rim, varying the charge distribution and thus the expected interaction with components of the outer leaflet of the outer membrane. In particular, the removal of the protruding tryptophan should reduce the sugar-binding capacity (*29*). S136K and E139K on the rim change the antigenic properties of MspD compared with the other isomers. The interactions of MspA with the outer membrane may be analyzed in the crystal because the outer surface of four of the eight rim domains is freely accessible so that soaking experiments with membrane constituents are feasible.

Among the mycobacteria, *Mycobacterium tuberculosis* is carried by about two billion people and causes a higher death rate than any other infectious agent (*30*). *M. tuberculosis* and its close relative *Mycobacterium bovis* have porins with conductances of about 0.8 and 4 nS in 1 M KCl solutions that were detected in detergent extracts but could not be further characterized (*31*, *32*). These porins are thought to be crucial for drug efficacy because the three important drugs—isoniazide, ethambutol, and pyrazinamide—are small polar molecules (*6*) that, for instance, can easily permeate the channel constriction of MspA (Fig. 4B). Because porin sequences are highly variable (*33*), the lack of sequence similarity does not exclude a structural relationship to MspA. Therefore, it seems worthwhile to perform an MspA-based structure knowledge-directed search to identify the large porin in the *M. tuberculosis* genome. Any detected gene may then be expressed, purified, and crystallized like MspA.

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23. Experimental data are available as supporting material on *Science* Online.
24. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
25. The shear number S of a β barrel is derived by running from a given residue of a given β strand along the hydrogen bonds once around the barrel and counting the offset number of residues on return. For a regular 16-stranded β barrel, the square of the circumference is $(4900 + 115^2)$ Å^2, showing the dependence of the diameter on the shear number (*21*).
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SOM Text
Fig. S1
Tables S1 and S2
References

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Use of CD134 As a Primary Receptor by the Feline Immunodeficiency Virus

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Feline immunodeficiency virus (FIV) induces a disease similar to acquired immunodeficiency syndrome (AIDS) in cats, yet in contrast to human immunodeficiency virus (HIV), CD4 is not the viral receptor. We identified a primary receptor for FIV as CD134 (OX40), a T cell activation antigen and costimulatory molecule. CD134 expression promotes viral binding and renders cells permissive for viral entry, productive infection, and syncytium formation. Infection is CXCR4-dependent, analogous to infection with X4 strains of HIV. Thus, despite the evolutionary divergence of the feline and human lentiviruses, both viruses use receptors that target the virus to a subset of cells that are pivotal to the acquired immune response.

The primary 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 viral cell tropism and pathogenicity. For the primate lentiviruses, the viral receptor is CD4, targeting the virus to helper T cells, resulting in their depletion and the eventual development of acquired immunodeficiency syndrome (AIDS) (*1*). However, CD4 expression alone is insufficient to confer susceptibility to infection with human immunodeficiency virus (HIV), which also depends on the expression of coreceptors, principally the chemokine receptors CXCR4 and CCR5 (*2*). 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 (*3*). 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 (*4*).

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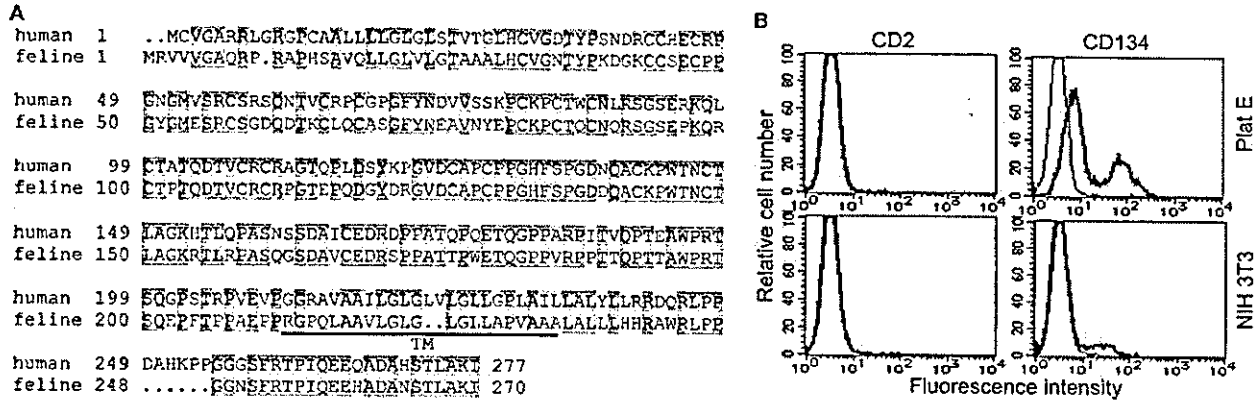

Fig. 1. Molecular cloning and characterization of feline CD134. (A) Alignment of feline and human CD134. Homologous amino acids are shaded light gray; identical residues are shaded dark gray. TM, transmembrane region. (B) Binding of FIV to CD134-expressing cells. Cell cultures transduced with CD134 or CD2 were incubated with culture supernatant from FIV-infected (thick line) or uninfected (thin line) cells, followed by FIV Env detection by flow cytometry.

The feline immunodeficiency virus (FIV) is unique among the nonprimate lentiviruses in that in its natural host species, the domestic cat, it induces a disease similar to AIDS, characterized by a progressive depletion of $CD4^+$ T lymphocytes (*5*). Consequently, an immunodeficiency syndrome develops that is characterized by wasting, neurological manifestations, chronic stomatitis and gingivitis, and an increased incidence of lymphoma (*6*). However, CD4 is not the primary receptor for FIV, suggesting that the virus may bind to an alternative molecule in order to target $CD4^+$ T cells (*7*). Partial elucidation of the mechanism of infection with FIV was provided by the discovery that primary isolates and laboratory strains of virus use CXCR4 as a cofactor for infection (*8*, *9*). However CXCR4 expression alone was insufficient to confer susceptibility to infection with primary isolates of FIV, indicating the existence of an as yet unidentified primary receptor for the virus.

To identify the primary receptor for FIV, we generated a cDNA library in the pMX retroviral vector from an interleukin-2 (IL-2)–dependent $CD4^+$ feline T cell line (MYA-1), which is highly susceptible to infection with FIV (*10*). After transduction of P3U1 murine myeloma cells with the library, cells capable of binding FIV were identified by "panning" with FIV-coated plates (*11*). Positive colonies were expanded, genomic DNA was prepared, and the cDNA insert was amplified from the retroviral vector by polymerase chain reaction. Of three cDNAs cloned from the library, only one showed reproducible binding to FIV when reexpressed in P3U1 cells and was characterized as the feline homolog of CD134 (DNA Data Bank of Japan accession no. AB128982). CD134 was first described as MRC OX-40, an antigen expressed on activated rat $CD4^+$ T lymphocytes and a member of the tumor necrosis factor–nerve growth factor receptor family (*12*, *13*). The feline CD134 cDNA identified here predicts a 270–amino acid protein with strong identity to human CD134 (Fig. 1A). To confirm that the feline CD134 protein binds FIV, we expressed the cDNA in the human cell line Plat-E and the murine cell line NIH-3T3. Specific FIV binding was detected on Plat-E and NIH 3T3 cells transduced with CD134 but not with cells expressing CD2 from the same vector (Fig. 1B).

To assess whether CD134 acts as a functional viral receptor, the feline large granular lymphoma cell line MCC (these cells are refractory to infection with primary strains of FIV) was stably transduced with a retroviral vector bearing the CD134 cDNA. Surface CD134 expression was detected by flow cytometry (Fig. 2A), whereas a ~45-kD species was detected by immunoblotting (Fig. 2B). CD134 expression rendered MCC cells permissive for infection with the five primary strains of FIV tested (Fig. 2C), whereas cells transduced with vector only remained nonpermissive. To quantify viral entry into the CD134-expressing cells, we prepared HIV pseudotypes bearing FIV Env proteins and carrying a luciferase gene. Each successful entry event would trigger luciferase production in the target cells. MCC-CD134 cells were permissive for infection with pseudotypes bearing the Env proteins of either the GL8 or TM2 strains of FIV (Fig. 2D),

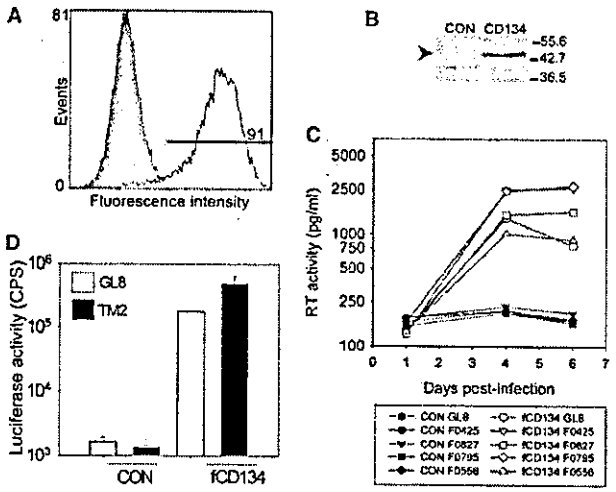

Fig. 2. CD134 expression renders feline cells permissive to infection with FIV. (A) Flow cytometric analysis of CD134 expression on MCC cells. Control antibody (solid histogram) and anti-CD134 (Ber-ACT35, open histogram) are shown. The percentage positive is shown. (B) Immunoblot analysis with rabbit antibody to rat OX40. MCC cells transduced with vector only (CON) were compared with cells transduced with the CD134 expression vector. A ~45-kD species was detected in CD134-transduced cells (arrowhead). (C) Feline (f) CD134 expression renders MCC cells permissive of productive infection with primary strains of FIV. MCC-CON or MCC-CD134 cells were infected with the GL8, F0425, F0827, F0795, and F0556 primary isolates of FIV. Reverse transcriptase activity in the culture supernatants was measured at 1, 4, and 6 days after infection. (D) Luciferase activity in MCC-CON or MCC-CD134 cells infected with HIV (FIV) luciferase pseudotypes bearing the GL8 or TM2 Envs (mean ± SE, $n = 4$ replicates).

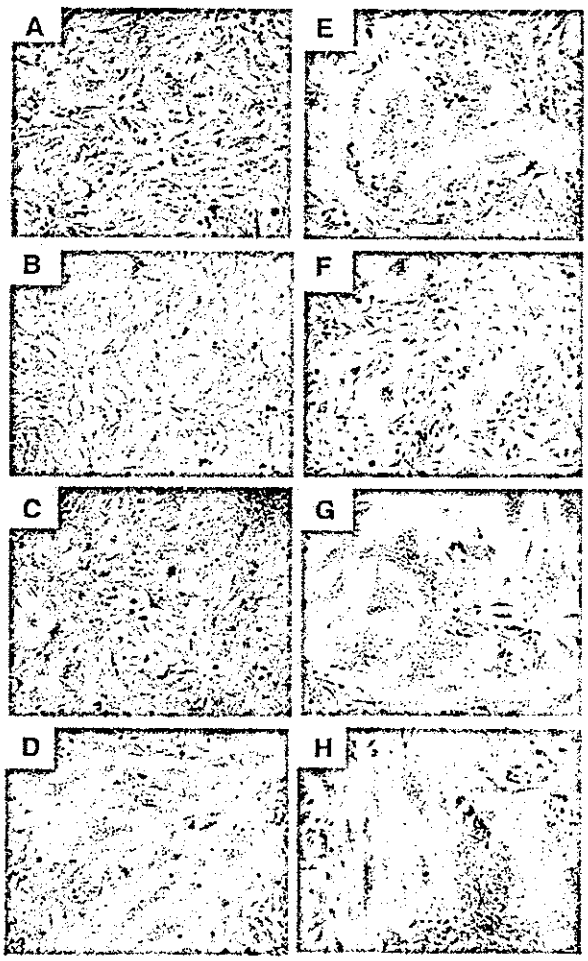

Fig. 3. CD134 expression renders AH927 cells permissive for syncytia formation mediated by FIV Env. FeLV-CR–expressing cells (A to D) or CD134-expressing cells (E to H) were transfected with pVR1012 expressing the GL8 [(A) and (E)], F0425 [(B) and (F)], F0827 [(C) and (G)], or PET [(D) and (H)] *env* genes.

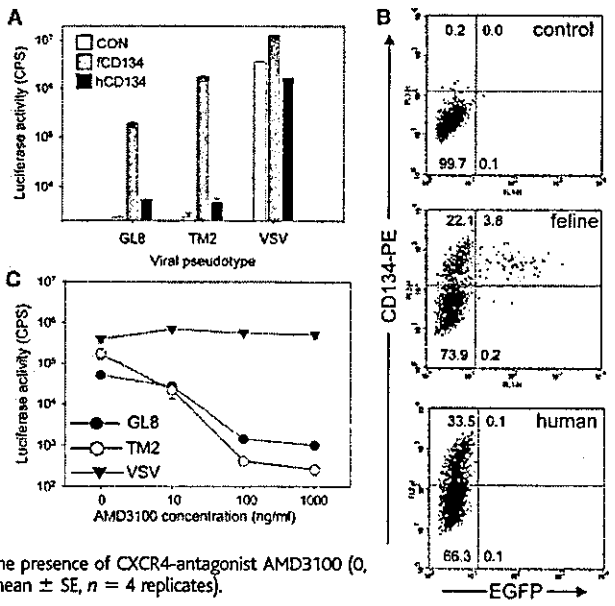

Fig. 4. CD134-mediated infection is species specific and CXCR4-dependent. (A) HeLa cells transduced with retroviral vectors bearing feline or human (h) CD134 cDNAs or a vector-only control were infected with HIV (FIV) (GL8 or TM2) or HIV (VSV) luciferase pseudotypes (mean ± SE, $n = 4$ replicates). (B) CD134-dependent infection of HeLa cells expressing feline CD134 by HIV (FIV) pseudotypes bearing a GFP marker gene. CD134 and GFP expression were analyzed by flow cytometry. (C) HeLa cells expressing feline CD134 were infected with HIV (FIV) (GL8 or TM2) or HIV (VSV) luciferase pseudotypes in the presence of CXCR4-antagonist AMD3100 (0, 10, 100, or 1000 ng/ml) (mean ± SE, $n = 4$ replicates).

confirming that CD134 expression overcame the block to viral entry into these cells. CD134 expression also relieved the block to infection in the feline fibroblast line AH927, whereas a control vector encoding the feline leukemia virus (FeLV)–C receptor (FeLV-CR) had no effect (fig. S1).

Syncytium formation occurs when Env expressed on the surface of a cell comes into contact with an adjacent cell expressing the viral receptor and coreceptor (*4*). Thus, transfection of cells expressing the viral receptor and coreceptor with *env* expression vectors should trigger syncytium formation. Accordingly, transfection of AH927-CD134 cells with constructs bearing the *env* genes from primary isolates (Fig. 3, E to G) led to the development of syncytia, whereas no syncytia were observed in the control cultures (Fig. 3, A to C) expressing the FeLV-CR. In contrast, FIV-PET *env* [derived from a FIV strain analogous to CD4-independent strains of HIV (*14*)] induced syncytium formation in both the CD134 (Fig. 3H) and FeLV-CR–expressing (Fig. 3D) cells, consistent with this Env interacting with CXCR4 in the absence of a primary receptor (*15*).

Human cells are refractory to infection with primary strains of FIV (*16*). We therefore examined the species specificity of the interaction between FIV and CD134. The human cell line HeLa was stably transduced with either feline or human CD134, and surface expression of CD134 was confirmed by flow cytometry (Fig. 4B). Although ectopic expression of feline CD134 rendered HeLa cells permissive for infection with primary FIV strain GL8 or TM2 pseudotypes (Fig. 4A), human CD134 expression had little effect. In contrast, the susceptibility of the feline and human CD134-expressing cells to HIV pseudotypes with a vesicular stomatitis virus glycoprotein (VSVG) envelope was broadly similar to that of the control HeLa cells. Similarly, feline, but not human, CD134 expression permitted infection with HIV pseudotypes bearing FIV envelopes and encoding green fluorescent protein (GFP) (Fig. 4B). These results suggest that the specificity of the interaction between the virus and its primary receptor may be a major determinant of the species specificity of FIV.

Because infection with both primary and cell culture–adapted strains of FIV is CXCR4-dependent (*8*, *9*), we determined whether CD134-dependent infection required coexpression of CXCR4. HeLa cells express high levels of CXCR4, and human CXCR4 is an efficient coreceptor for FIV (*17*). Infection of HeLa-CD134 cells with GL8 and TM2 pseudotypes was inhibited efficiently by the selective CXCR4 antagonist AMD3100, whereas infection with control HIV (VSV) pseudotypes was not affected (Fig. 4C), confirming an absolute requirement for both receptor and coreceptor.