Replication Properties of Clade A/C Chimeric Feline Immunodeficiency Viruses and Evaluation of Infection Kinetics in the Domestic Cat[▽]

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Feline immunodeficiency virus (FIV) causes progressive immunodeficiency in domestic cats, with clinical course dependent on virus strain. For example, clade A FIV-PPR is predominantly neurotropic and causes a mild disease in the periphery, whereas clade C FIV-C36 causes fulminant disease with CD4⁺ T-cell depletion and neutropenia but no significant pathology in the central nervous system. In order to map pathogenic determinants, chimeric viruses were prepared between FIV-C36 and FIV-PPR, with reciprocal exchanges involving (i) the 3' halves of the viruses, including the *Vif*, *OrfA*, and *Env* genes; (ii) the 5' end extending from the 5' long terminal repeat (LTR) to the beginning of the capsid (CA)-coding region; and (iii) the 3' LTR and *Rev2*-coding regions. Ex vivo replication rates and in vivo replication and pathologies were then assessed and compared to those of the parental viruses. The results show that FIV-C36 replicates ex vivo and in vivo to levels approximately 20-fold greater than those of FIV-PPR. None of the chimeric FIVs recapitulated the replication rate of FIV-C36, although most replicated to levels similar to those of FIV-PPR. The rates of chloramphenicol acetyltransferase gene transcription driven by the FIV-C36 and FIV-PPR LTRs were identical. Furthermore, the ratios of surface glycoprotein (SU) to capsid protein (CA) in the released particles were essentially the same in the wild-type and chimeric FIVs. Tests were performed in vivo on the wild-type FIVs and chimeras carrying the 3' half of FIV-C36 or the 3' LTR and *Rev2* regions of FIV-C36 on the PPR background. Both chimeras were infectious in vivo, although replication levels were lower than for the parental viruses. The chimera carrying the 3' half of FIV-C36 demonstrated an intermediate disease course with a delayed peak viral load but ultimately resulted in significant reductions in neutrophil and CD4⁺ T cells, suggesting potential adaptation in vivo. Taken together, the findings suggest that the rapid-growth phenotype and pathogenicity of FIV-C36 are the result of evolutionary fine tuning throughout the viral genome, rather than being properties of any one constituent.

Feline immunodeficiency virus (FIV) is a lentivirus that infects both free-range domestic and feral cats worldwide (40), leading to an AIDS-like acquired immunodeficiency disease (11, 15, 27). The FIV infection and disease pattern closely resembles that of human immunodeficiency virus type 1 (HIV-1) infection in humans (26, 27, 43), and subtype classifications similar to those for HIV have been established for FIV (34). Thus, in-depth study of the fundamental molecular mechanisms of virus replication and pathogenesis of FIV may aid in the development of intervention strategies relevant to treatment of AIDS in both cats and humans.

Similar to the primate lentiviruses, FIV exhibits structural and functional diversity that dictates ex vivo and in vivo growth rate, host cell range, and, ultimately, extent and nature of pathogenicity in vivo. Correlates between ex vivo and in vivo phenotypes have not been established and would greatly aid in the use of the model for assessment of treatment modalities. The majority of FIV subtypes identified to date fall into clades

A or B and exhibit various degrees of pathogenicity in animals (34). In contrast, at least one clade C isolate causes a high disease incidence and severity, with approximately 60% mortality within 18 weeks postinfection (6, 7, 9, 24). We previously isolated and characterized a highly pathogenic FIV clone (FIV-C36) of this clade C isolate, which generated severe acute immunodeficiency disease in young cats (6). Interestingly, dams nursing infected kittens also became infected, with resultant high viral load and disease, demonstrating for the first time lentivirus transfer from offspring to parent and underscoring the acute pathogenicity of FIV-C36.

As with the primate lentiviruses, the molecular basis for the varied pathogenic potentials of different isolates is not fully understood. Therefore, the present study was undertaken as an approach to elucidate the genetic elements that contribute to the pathogenic phenotype of FIV. We generated a panel of chimeric clones between the relatively neurotropic isolate FIV-PPR (28, 29) and the highly pathogenic FIV-C36 clone and report here a comparison of the ex vivo, as well as in vivo, properties of these clones.

MATERIALS AND METHODS

Cloning of constructs. The cytomegalovirus (CMV)-chloramphenicol acetyltransferase (CAT), PPR-long terminal repeat (LTR), and ΔPPR-CAT constructs were previously described (5). The FIV-C36-LTR construct, in which the FIV-C36 LTR was cloned upstream of the identical CAT gene, was also prepared.

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For preparation of chimeric constructs, a second NdeI site (silent mutation at nucleotide [nt] 5000) was introduced into the wild-type FIV-PPR clone (28) by site-directed mutagenesis. This allowed a swapping of the NdeI fragments containing the viral genes *vif*, *orfA*, and *env* between FIV-PPR and FIV-C36 (see Fig. 4). The 3' region of this clone was "recloned" back into the original FIV-C36 backbone and used to verify that the results of the in vitro infection studies were not due to cloning errors. A similar fragment exchange was performed using a common XhoI site at nt 1460 and an external restriction site located 5' of the 5' LTR. This constituted a partial cut for the FIV-PPR clone, which contains an additional XhoI site at nt 502. All chimeric clones were transformed in MAX Efficiency Sthl2 competent *Escherichia coli* (Invitrogen, Carlsbad, CA) and grown at 30°C in order to avoid instability and recombination of lentiviral sequences. All constructs were verified for correct sequences by restriction enzyme analysis and sequencing by the TSRI Center for Nucleic Acid Research.

Cell lines and T-cell separation. Crandell feline kidney (CrFK) cells overexpressing feline receptor CD134 (herein referred to as GFox cells) (3) and interleukin 2-dependent 104-C1 T cells were maintained as previously reported (19). The separation of the CD4⁺ and CD8⁺ T-cell populations was conducted using fresh specific-pathogen-free (SPF) feline blood as described previously (19).

Transfection and infection. CrFK cells (10⁵) were plated in six-well plates and allowed to attach overnight. Transfections were conducted using 1 µg of DNA and Eugene6 (Roche, Indianapolis, IN) according to the manufacturer's instructions. Cells were incubated for 48 h, trypsinized and transferred to T 75 flasks, and then fed 30 ml complete medium. Cultures were fed with fresh medium, but fresh cells were not added during this time period. Supernatant was analyzed for virion production every 4 days. After 10 to 14 days in culture, supernatant was harvested from cells and the final reverse transcriptase (RT) activity of the cell-free supernatant was measured as described previously (19). This stock was used for T-cell infections as described in the following paragraph and for in vivo challenge, described further on in Materials and Methods.

T cells (2 × 10⁴ 104-C1 cells, peripheral blood mononuclear cells [PBMCs], CD4⁺ cells, or CD8⁺ cells) were incubated in 96-well plates and infected with respective viral supernatants (10⁴ 50% tissue culture infectious doses [TCID₅₀]) in quadruplicate. Cells were incubated at 37°C in 5% CO₂. RT activity was measured, and cells were split 1:6 every ~5 days.

Western hybridization. For viral Western blot hybridization, viral supernatants normalized to 2 × 10⁵ cpm RT activity were centrifuged at 6 × 10⁴ rpm for 30 min at 4°C. The viral pellets were resuspended in 60 µl phosphate-buffered saline (PBS) before undergoing three freeze-thaw cycles. Volumes of 20 µl 8 M urea and 20 µl Laemmli buffer (18) were added to the viral mixture, and 30 µl was loaded on a 10 to 20% Tris-glycine gel and carried through Western blot procedures as described previously (4).

Preparation of cell extract for the CAT assay. LTR-CAT constructs (1 µg) were transfected into 10⁵ CrFK cells (see descriptions of cell lines and transfection) seeded the night before in six-well plates. After 48 h, cells were washed in PBS three times and incubated in 500 µl TEN buffer (40 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0], 150 mM NaCl) for 5 min at room temperature. Cells were scraped off the plate surface, transferred to a microcentrifuge tube, and spun for 1 min. The cell pellet was resuspended in 100 µl 0.25 M Tris-HCl [pH 8.0] and subjected to three freeze-thaw cycles, with vortexing after each thaw cycle. Cell lysates were then heated at 60°C for 10 min and centrifuged for 2 min. Supernatants were transferred to a fresh tube to be assayed for CAT activity.

CAT assay. The following reaction mixture was prepared for each sample: 50 µl cell extract, 1 µl [¹⁴C]chloramphenicol (at 0.05 mCi/ml), 1 µl *n*-butyryl coenzyme A, 0.25 M Tris-HCl [pH 8.0] for a final volume of 100 µl. Reaction mixtures were incubated at 37°C for 2 h. Three hundred microliters of mixed xylenes was then added to reaction tubes and vortexed for 30 s. Tubes were centrifuged for 3 min to achieve good separation of phases. Two hundred microliters of the upper phase was carefully removed into scintillation vials holding 10 ml liquid scintillation fluid. The counts per minute (cpm) of the butyrylated chloramphenicol products were measured.

Preparation of immunoadhesins. Construction of immunoadhesins containing the SU protein of FIVs expressed in-frame with human Fc have been previously described (4).

Stable cell lines expressing the FIV-PPR and FIV-C36 Fc-SU immunoadhesins were prepared by transfection of CHO-K1 cells, followed by growth in the presence of 25 µM methionine sulfoximine. Cells that survived this selection were then assayed for immunoadhesin expression by incubation of supernatants with immobilized *Staphylococcus* protein A, followed by washing and Western blot analyses to characterize Fc-containing proteins specifically bound to the beads. Large quantities of immunoadhesins were then obtained by scale-up shifting and purification using larger immobilized protein A columns.

Cytofluorimetric analyses using immunoadhesins. Specific binding of immunoadhesins to a variety of cells was carried out by fluorescence-activated cell sorting (FACS) analyses, with Fc-only constructs serving as negative controls. In general, 3 × 10⁵ cells were incubated for 1 h at 4°C with 1 µg immunoadhesin in PBS plus 0.1% bovine serum albumin. The cells were then washed twice with cold PBS and labeled with a 1:100 dilution of fluorescein-conjugated antibody to human immunoglobulin G1 Fc (Cappel/MP Biomedicals, Solon, OH) for 1 h at 4°C. The cells were washed in PBS and analyzed by flow cytometry on a FACScan, using CellQuest software (BDIS, San Jose, CA). Where indicated, the CXCR4 antagonist, AMD3100, was typically added to the cells prior to incubation with immunoadhesins, and the samples were then analyzed as described above.

Overlay assay for detection of immunoadhesin binding to receptors. Overlay assays that allow detection of receptor binding to proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes were performed as detailed elsewhere (4).

In vivo infections. Twenty-four 14- to 16-week-old SPF cats (Cedar River Laboratories, Ames, IA, and an SPF cat colony at Colorado State University [CSU], Fort Collins, CO) were housed in barrier rooms in CSU facilities accredited by AAALAC International. Procedures were approved by the CSU IACUC prior to initiation. Four groups of cats (*n* = 5) randomized by age and sex were inoculated both orally (0.5 ml) and intravenously (0.5 ml) with 10^{3.5} TCID₅₀ particles/ml of either FIV-PPR, PCenv, PC3' LTR, or FIV-C36, propagated, and titrated as described above. Four cats were administered media only as noninfected controls. Animals were examined daily for evidence of clinical disease, and physical exams were performed during blood collections. Blood samples were collected at days -7, 7, 10, 14, 21, 30, 35, 42, 62, 77, 102, 132, and 156 postinoculation (p.i.) on unanesthetized animals. Serum and plasma samples were collected by centrifugation and PBMCs purified from blood (EDTA-treated blood) on Ficoll gradients as previously described (5).

Hematologic analysis and immunophenotyping. Complete leukocyte and red blood cell counts were determined from EDTA blood by using a Z1 series coulter counter. Differential leukocyte counts were determined manually from stained smears, and absolute neutrophil and lymphocyte counts were calculated as described previously (38). CD4⁺ and CD8⁺ surface antigens were measured by flow cytometric analysis with a CyAn cell sorter (Dako Cytomation, Glostrup, Denmark) (38) and results analyzed using the Summit software package (Dako Cytomation). To determine absolute CD4⁺ and CD8⁺ cell counts, total lymphocyte counts were multiplied by percentage of fluorescein isothiocyanate (CD4) or phycoerythrin (CD8) fluorescing cells. Blood samples collected from all cats 7 days prior to infection were used to establish baseline values. Statistical analysis of cell subsets was performed using Prism 4 (GraphPad Software, Inc., San Diego, CA). Repeated-measure analysis of variance was used to determine if significant differences (*P* < 0.05) could be attributed to treatment or time. If a significant difference was detected, individual comparisons of groups or time points were conducted using two-tailed Student *t* test analysis.

FIV proviral DNA and plasma RNA quantitation. Viral RNA was extracted from plasma collected from EDTA-treated whole blood following centrifugation. Plasma was frozen at -70°C until processing. RNA was purified from 140 µl of plasma, using a QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Superscript II (Invitrogen) was implemented in reactions with random hexamers (Invitrogen) added and treated with RNase Out (Invitrogen) for preparation of cDNA from viral RNA. Genomic DNA was extracted from PBMCs purified on a Histopaque-1077 (Sigma, St. Louis, MO) gradient, using a DNeasy tissue kit (Qiagen). Real-time PCR was performed with an iCycler thermocycler (Bio-Rad, Hercules, CA) to detect both proviral DNA and plasma viremia, using AmpliTaq Gold DNA polymerase-containing TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA). Derivation of FIV-A and FIV-C primer/probe sets and sequences has previously been described (26). PCRs in a total volume of 25 µl consisted of 12.5 µl master mix, 0.5 µl each of 20 µM forward and reverse primers, 0.2 µl of 10 µM probe, and 5 µl of the template. After 2 min at 50°C, the AmpliTaq Gold DNA polymerase was activated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Threshold cycle values were defined as the point at which the fluorescence passed a threshold limit. Calculation of FIV proviral copy number was performed using a standard curve generated from dilutions of a subcloned *gag* PCR product. To calculate copy number of viral RNA in plasma, a standard curve was generated by diluting FIV-PPR virus stock in naïve cat plasma, preparing cDNA as described above, and comparing the threshold cycle values to those of the subcloned *gag* standard.

FIG. 1. Measure of relative levels of transcription driven by PPR and FIV-C36 LTRs. Feline fibroblasts were transfected in triplicate with 500 ng of CMV-LTR, nonfunctional PPR-LTR (Δ PPR), wild-type PPR-LTR, or wild-type FIV-C36-LTR constructs. Cell extracts were harvested at 48 h posttransfection, and relative levels of CAT activities were determined, as outlined in Materials and Methods. No significant differences were noted in the levels of CAT transcription driven by the two viral LTRs.

RESULTS

LTR-driven transcription by FIV-PPR and FIV-C36 LTRs. We assessed the relative rates of transcription driven by the FIV-PPR and FIV-C36 LTRs as a possible explanation for the differential replication rates of the two FIVs. The LTRs encode the structural elements to which cellular transcription factors bind and facilitate virus replication (22, 25, 28, 36, 39). The 5' LTR of each isolate was cloned upstream of the CAT gene and transfected into feline CrFK cells as described in Materials and Methods. A 47-nt U3 deletion mutant for the FIV-PPR LTR (Δ PPR) (5) was used as a negative control, and CMV promoter-driven CAT transcription served as a positive control. Cell extracts were harvested at 48 h posttransfection and tested in a CAT assay. The results showed virtually equivalent CAT transcription rates for FIV-PPR and FIV-C36 (Fig. 1, showing 500-ng LTR data), which does not support differential transcription rates as an explanation for the observed differences in virus replication.

Assessment of receptor binding by FIV-C. FIV-C may have distinct receptor binding properties relative to FIV-PPR that might explain its more rapid growth properties and pathogenicity. Previous studies have shown that FIV-PPR and other domestic cat FIVs enter cells via the chemokine receptor CXCR4 (31, 42) but that primary binding occurs via CD134 (3, 33), similar to what occurs with HIV-1 binding to CD4 (2, 16). In order to assess FIV-C envelope binding to CD134, an immunoadhesin was prepared by attaching a human Fc domain to the N terminus of FIV-C36 SU, similar to constructs prepared using FIV-PPR and FIV-34TF10 in past studies (4) (also see Materials and Methods). We then used FIV-C Fc-SU and FIV-PPR Fc-SU in both FACS (Fig. 2) and overlay binding assays (Fig. 3) to compare the binding specificities of the two Envs. The findings revealed that the levels of binding of the two Envs to CD134 were indistinguishable, as indicated by FACS positivity on CD134^{hi}/CXCR4^{lo} 104-C1 cells (Fig. 2) and reactivity with the 45-kDa CD134 molecule in 104-C1 cell lysates in an overlay assay (Fig. 3). Binding of both FIV-PPR and FIV-C SU to CXCR4 on 3201 cells (CXCR4^{hi}/CD134^{neg}) (Fig. 2) as well as infection by both viruses is inhibited by the CXCR4 antagonist AMD3100, consistent with entry being mediated by the CXCR4 chemokine receptor (4).

Generation of PPR/C36 chimeric clones. We prepared a series of chimeric constructs between FIV-PPR and FIV-C36 in an attempt to map molecular determinants of the distinct phenotypes exhibited by these two clones (6).

We introduced an NdeI site into the center of FIV-PPR (base 5000) at the equivalent site, where an NdeI site was already present in FIV-C36 (Fig. 4A). The introduction of this NdeI site did not change the amino acid sequence of FIV-PPR in this region, which resides within the integrase-coding region, and the mutation was verified by sequencing (data not shown). We then used the NdeI sites to swap out the fragments between the two FIV clones, generating “mirror image” chimeric clones (Fig. 4A) (PCenv and CPenv). The resulting clones had either a FIV-PPR backbone, including *gag*, *pol*, and both 5' and 3' FIV-PPR LTRs, with 3' accessory genes *vif* and *orfA* as

Cell	104-C1	104-C1	3201	3201
Protein	FC-SU-PPR	FC-SU-C	FC-SU-PPR	FC-SU-C

FIG. 2. Binding of FIV Fc-SU to 104-C1 and 3201 cells. Cells were incubated with the indicated Fc-SU adhesins, followed by detection of protein binding to the cell by flow cytometry using anti-human Fc antibodies (solid line). Binding to 104-C1 cells is indicative of SU binding to the primary binding receptor CD134. Binding to 3201 cells is via the entry receptor CXCR4. Specific binding was demonstrated by preincubation of 3201 cells with the CXCR4 antagonist AMD3100 before addition of SU (dotted line). Cells incubated without Fc-SU protein are shown in solid gray.

FIG. 3. Overlay assay demonstrating binding to CD134 by FIV-C SU. Proteins from total cell extracts of feline 104-C1 T cells and 3201 cells were transferred to nitrocellulose membranes and exposed to Fc-SU adhesins as previously described (3) (also see Materials and Methods), and binding was detected by chemiluminescence. Both FIV-PPR and FIV-C36 SU (gp95) bound specifically to the 45-kDa CD134 primary binding receptor, present on 104-C1 cells but absent on 3201 cells.

well as *env* and 128/147 nt of the *rev* response element from FIV-C36 (PCenv), or the FIV-C36 backbone with 3' genes derived from FIV-PPR (CPenv) (Fig. 4A). Note that the two exons of *rev* are also recombinant between FIV-PPR and FIV-C36 in these two chimeras, with FIV-PPR *rev1* and FIV-C36 *rev2* in CPenv and the reverse order in PCenv (Fig. 4A). We also generated chimeras in which the 5' 1,460 bp were switched between FIV-PPR and FIV-C36 (Fig. 4A) (PCgag and CPgag) by utilizing a shared *XhoI* site within the 5' end of the CA-coding region (Fig. 4A). The two "gag" chimera clones thus carry the alternate 5' LTR, intergenic, and MA-coding regions as well as the first 240 bp of the CA-coding region on each parental background. The *XhoI* junction results in an in-frame ligation, allowing for a functional CA protein that itself represents a chimeric entity between FIV-PPR and FIV-C36. However, the 5' 240 bp of CA encodes only four amino acids that are divergent between the FIV-PPR and FIV-C36 CA proteins. The same holds true in reverse for the FIV-C36 backbone clone (CPgag), which contains a 5' PPR LTR, a PPR MA gene, and a recombinant CA gene. Note that the LTR sequence after reverse transcription during first-round replication is that of the 3' LTR, in this case contributed by FIV-PPR. An additional mutant, in which only *rev2* and the 3' LTR of FIV-C36 were placed on the FIV-PPR backbone, was also constructed (Fig. 4B). As described above, first-round replication of this chimera generated a FIV-PPR backbone, with FIV-C36 LTRs and a chimeric FIV-PPR/C36 *rev* gene. All clones were verified by direct nucleotide sequencing (data not shown).

DNAs encoding each of the above-mentioned clones and the two wild-type constructs were individually transfected into

FIG. 4. Schematic representation of different recombinant clones of PPR and FIV-C36. (A) PPR sequences are shown in white, and FIV-C36 sequences are in black. PCenv and CPenv were generated by exchanging the ~5 kb between the *NdeI* restriction sites of either isolate with those of the opposite isolate. PCgag and CPgag contain the 5' LTR, the MA gene, and two-thirds of the CA gene of either isolate in the backbone of the other isolate. Note: the second exon of *rev* is of the opposing isolate, making *rev* chimeric as well. RRE, *rev* response element. (B) The FIV-C36 3' LTR including the second exon for *rev* was cloned into the PPR backbone.

FIG. 5. Relative infection of feline T-cell line 104-C1 and total PBMCs by wild-type and chimeric FIVs. (A) Relative infection of 104-C1 T cells. d, day. (B) Relative infection of total PBMCs. Cells were infected with 10^2 TCID₅₀ of each virus, and progression of infection was determined by monitoring RT activity in the culture supernatant. The genomic structures of the various chimeric and wild-type FIVs are shown in Fig. 4.

GFox cells. RT levels of resultant supernatants varied markedly, with the chimeric constructs producing substantially less released virus than the wild-type constructs (see below). The virus-containing pellets (centrifuged at $100,000 \times g$) from the supernatants were normalized to 2×10^5 cpm RT activity for Western blot comparisons of relative SU and CA expression levels. No significant differences were noted in SU/CA ratios with any of the constructs (not shown).

Relative levels of infection of different cell types by wild-type and chimeric FIVs. We next determined the relative infectivities of the chimeras to the parental isolates by using both cell lines and PBMCs. Virus supernatants were modified for TCID₅₀, and infections were then performed on four infection targets, including 104-C1, PBMCs, and homogeneous populations of CD4⁺ or CD8⁺ T cells sorted from the total PBMC pool as described above. Previous results indicated that FIV adapts over time to cell culture conditions (28); therefore, experiments were terminated at 14 days p.i. (dpi). The data obtained with the different recombinants varied with target cell. By 4 to 10 dpi, RT counts measured for FIV-C36 in

104-C1 (Fig. 5A), total primary PBMCs (Fig. 5B), and sorted CD4⁺ and CD8⁺ T cells (Fig. 6A and B, respectively) were significantly higher than those of either wild-type FIV-PPR or chimeric clones with peak RT values two to five times greater than those of either FIV-PPR or any of the chimeras. In contrast, infection by FIV-PPR was both lower and delayed within the same timeframe, consistent with a lower replication rate for the latter virus in vivo (13). Interestingly, chimeric FIV infections of the total PBMCs were less efficient than those of 104-C1 cells, consistent with heterogeneity in cell types and cell cycle in the PBMC pool (Fig. 5B). Growth restriction was less apparent in CD8⁺ lymphoblasts, with relatively high infectivity with FIV-C36 by 8 dpi (Fig. 6B). These differences may simply reflect the relative growth rates of the continuous cell line and the primary cell cultures.

None of the chimeric FIVs recapitulated the growth rate of FIV-C36 in any of the cells, although all replicated in the 104-C1 cell line. Consistent with the LTR-driven transcription experiments whose results are shown in Fig. 1, substitution of the FIV-C36 3' LTR into the FIV-PPR background (PC3'LTR) yielded a

FIG. 6. Relative infection of CD4⁺ and CD8⁺ T-cell subsets by wild-type and chimeric FIVs. (A) Relative infection of the feline CD4⁺ T-cell subset, sorted from total PBMCs. d, day. (B) Relative infection of the CD8⁺ T-cell subset, sorted from total PBMCs. Cells were infected with 10^2 TCID₅₀ for each virus, and progression of infection was determined by monitoring RT activity in the viral supernatant. The genomic structures of the various chimeric and wild-type FIVs are shown in Fig. 4.

chimera with growth rates that did not differ significantly from those of the parental FIV-PPR isolate (Fig. 5 and 6).

To exclude the possibility that these results were due to cloning errors, we recloned the 3' region from the PCenv chimera back into the original FIV-C36 backbone as described in Materials and Methods. The reconstructed wild-type FIV-C36 isolate replicated with kinetics identical to those of the original FIV-C36 clone (data not shown).

In vivo kinetics of chimeric PCenv and PC3'LTR clones. Based on ex vivo results and a focus on defining FIV-C36 pathogenicity, we compared two of the chimeras, PCenv and PC3'LTR, to the parental FIVs in vivo. Groups containing five cats each were infected with either wild-type or chimeric FIVs as detailed in Materials and Methods. Relative rates and levels of viremia were assessed over time by measuring both the number of viral RNA copies per milliliter plasma and the number of viral DNA copies detected per 10^6 PBMC DNA equivalents. Average numbers of viral RNA copies per milliliter peaked at day 14 following inoculation with FIV-PPR, FIV-C36, and FIVPC3'LTR, whereas PCenv showed delayed kinetics, with viral load reaching maximum levels at day 35 p.i. (Fig. 7A). FIV-C36 infections resulted in the greatest peak viremia (6.17×10^7 copies/ml).

PCenv circulating viral load approached that of FIV-PPR, with levels reaching 2.8×10^4 and 7.6×10^4 copies/ml, respectively. PC3'LTR plasma viremia peaked at 9.4×10^3 copies/ml, indicating significant attenuation relative to both parental con-

structs. At the time of necropsy, FIV-PPR plasma loads were lowest, averaging 25.4 copies/ml, followed by PC3'LTR, which averaged 74.5 copies/ml. PCenv and FIV-C36 retained higher levels, at 250 and 375 RNA copies/ml, respectively, by the end of the study (day 156 p.i.) (Fig. 7A).

Integrated proviral *gag* sequences were detected in PBMCs of all inoculated cats. Proviral load peaked by day 35 in cats infected with FIV-C36, FIV-PPR, and PC3'LTR. PCenv did not attain maximal viral load until day 77 p.i. and at that point approached proviral loads comparable to those of FIV-PPR (Fig. 7B). Mean peak numbers of proviral copies/ 10^6 PBMCs remained relatively constant for all groups until the end of the study as follows: PC3'LTR, 5.88×10^2 ; FIV-PPR, 3.47×10^3 ; PCenv, 6.53×10^3 ; and FIV-C36, 1.35×10^5 (Fig. 7B).

PCenv demonstrated interesting kinetics in that virus was nearly undetectable in both plasma and PBMCs at day 14, when other viral constructs were nearly maximal; however, by day 35 viral loads for this construct were comparable to or higher than those for parental strain FIV-PPR. This viral growth phenotype was consistent in all five animals and highly unusual relative to infection kinetics typically observed during FIV infection.

Hematologic effects of PCenv and PC3'LTR infections. The absolute CD4⁺ T-lymphocyte counts preinfection were normal ($>1,000/\mu\text{l}$) and similar to control levels in all groups (Fig. 8A). FIV-C36-infected cats had an early significant decline in CD4⁺ T cells that stabilized by day 77 p.i. CD4⁺

FIG. 7. Circulating and integrated virus during infections with wild-type and chimeric FIVs. Group averages for plasma viremia are shown as numbers of RNA copies/ml (A), and proviral loads are shown as numbers of copies/10⁶ PBMCs (B) over the course of the 156-day study. PCRs were performed in triplicate. Naïve cats had undetectable proviral levels (data not shown).

T-cell counts in PCenv-infected cats were significantly lower than those in controls at day 66 ($P = 0.073$), and in two of five cats, levels dropped below 1,000/ μ l by day 102 p.i., maintaining this depletion until the end of the study, at which time the group mean CD4⁺ T-cell level was significantly lower than that for the controls (Fig. 8A and 9A). In comparison, CD4⁺ T-cell depletions observed in FIV-PPR- and PC3'LTR-infected cats were delayed and milder. CD4/CD8 ratio declines reflected CD4⁺ T-cell depletion, i.e., FIV-C36-infected cats had the lowest ratios through the study starting at day 30 p.i., and after day 60, PCenv CD4/CD8 ratios were below control levels. FIV-PPR and PC3'LTR levels remained above or consistent with control means for the entire study (Fig. 8B and 9B). Toward the end of the study, the ratio for FIV-C36 infections dropped more sharply than that for other cats due to an increase in absolute CD8⁺ T-cell numbers (data not shown).

Neutropenia in cats is defined by circulating neutrophil values lower than 2,000/ μ l. While minor neutrophil decline occurred in some FIV-PPR (4/5) and PC3'LTR (three of five) infected cats and in two of five noninfected cats, it was mild and transient (Fig. 8C). All five FIV-C36-infected cats experienced marked neutropenia starting at day 30 p.i. that was statistically significant (Fig. 9C). Three of these animals recovered by the end of the study, while two remained neutropenic throughout the study. PCenv infections also resulted in prolonged neutropenia in three cats that was statistically significant at some time points but was delayed compared to that for FIV-C36, appearing between days 77 and 102 p.i. and persisting until the end of the study (day 156 p.i.) (Fig. 9C).

DISCUSSION

We performed a series of analyses comparing FIV-PPR and FIV-C36 in an attempt to localize the molecular determinants of their unique pathologies. We assessed (i) the relative rates of transcription driven by the LTRs of these two FIV strains, (ii) the ability of envelope SU to bind the primary receptor CD134 and the entry receptor CXCR4 for each strain, and (iii) the ratios of Env SU to CA viral proteins isolated from virions. Further, we generated a panel of chimeras by swapping genomic regions of each molecularly cloned parental isolate and tested (i) ex vivo replication capacity in primary cell populations and cell lines and (ii) the in vivo infectivity and pathogenicity of two of the chimeric constructs relative to those of the parental strains.

The results indicate that the differences in infection phenotypes, either ex vivo or in vivo, cannot be explained on the basis of differential rates of promoter/enhancer-driven transcription. The in vitro rates of CAT transcription driven by FIV-C and FIV-PPR were essentially identical (Fig. 1), and a chimera wherein the FIV-C36 LTR was substituted into FIV-PPR did not recapitulate either ex vivo or in vivo replication properties of FIV-C36.

Both FIV-PPR and FIV-C36 as well as other FIVs tested utilize CXCR4 as an entry receptor and CD134 as a primary binding receptor to facilitate high-affinity binding to CXCR4 (3, 4, 31, 33, 41, 42). Although there are likely some distinctions in relative affinity between CD134 interactions, neither the binding studies nor the ex vivo infection analyses revealed significant differences in receptor utilization that would explain the distinctions in infection level between the isolates. We also

FIG. 8. Time course of hematologic changes in domestic cats infected with parental or chimeric FIVs. Blood was sampled at various time points over the course of infection as described in Materials and Methods. Complete blood counts, differential leukocyte analysis, and CD4⁺ and CD8⁺ T-lymphocyte percentages in virus-inoculated cats ($n = 5/\text{group}$) or sham-inoculated control cats ($n = 4$) were calculated. CD4 counts (shown as numbers of cells/ μL) (A), CD4/CD8 ratios (B), and neutrophil counts (shown as numbers of cells/ μL for group means) (C) are demonstrated over the course of the study. For ease of viewing, error bars are shown for the control group only. P values were calculated as described in Materials and Methods. Statistically significant values relative to normal control values are indicated by asterisks (*, P between 0.05 and 0.07; **, P between 0.01 and 0.05; ***, $P < 0.01$).

considered that the relative resistance of SU to degradation could influence viral infectivity. For example, if FIV-C36 Env SU were more stable than FIV-PPR SU, it could have a potentially longer “half-life” in the host, remain in circulation for a longer period of time, and result in the higher plasma viremia levels observed in FIV-Cpg domestic cat infections. However, comparison of SU-to-CA ratios in FIV-C36 versus FIV-PPR did not reveal differences supportive of this mechanism.

The results of ex vivo infection of cell lines and primary cell populations revealed that FIV-C36 replicates to two- to five-fold-greater levels than FIV-PPR or any of the C36/PPR chimeras used in short-term culture. This difference was observed in the interleukin 2-dependent T-cell line 104-C1, total PBMCs, and both CD4⁺ and CD8⁺ T-cell populations sorted from PBMCs. Infection of these cell populations is consistent with previous reports of localization of FIV in both CD4⁺ and

CD8⁺ cell compartments ex vivo (1) as well as cells sorted from infected cats (8, 10). The increased level of infection with FIV-C compared to that with FIV-PPR mimics observations with in vivo infections, with either uncloned FIV-Cpg (7, 9) or cloned FIV-C36 (6). The increase in FIV-C infection rate translates to an approximately 2-log increase in peak viral load in vivo (Fig. 7) (6, 7, 9). Given the ex vivo findings, the results imply that the advantage that clade C exhibits in vivo is not simply a function of increased immunological privilege in the intact animal.

Every FIV-C36 genome chimera had attenuated ex vivo growth characteristics relative to FIV-C36. Further, FIV-PPR with FIV-C36 genomic substitutions did not increase in replicative capacity ex vivo, even when FIV-C36 *env* was substituted for this element in FIV-PPR. We conclude from this observation that the high-titer growth rate of FIV-C36 is dictated by

FIG. 9. Peripheral T-cell and neutrophil kinetics for individual cats infected with parental or chimeric FIVs. Blood was sampled at various time points over the course of infection, as described in Materials and Methods. While group means were not statistically different (Fig. 8), individual animal measurements at days 102 and 156 p.i. demonstrate trends for CD4 depletion (A) and CD4/CD8 ratio decrease (B) in FIV-C36 and PCenv versus levels in other groups. Further, neutrophil counts at days 30, 102, and 156 p.i. demonstrate neutropenia in FIV-C36 and PCenv versus levels in other groups (C). For ease of viewing, reference points are illustrated by horizontal bars placed at 1,000 CD4⁺ T cells/μL, a CD4/CD8 ratio of 1.5, and 2,000 neutrophils/μL. ni, no infection (control).

multiple genomic regions and gene products rather than one single gene. This observation underscores the dynamic nature of viral evolution whereby replication potential is contingent on many regions of the viral genome. A given nucleotide sequence may influence replication either via amino acid sequence and resultant protein structure/function or through secondary structural changes in viral transcripts that influence transcription rates and/or genomic stability. Manipulations, as reported here, can readily impair virus infectivity but can rarely, if ever, enhance the innate replicative ability that has been shaped by evolutionary tuning in the host. Although replication levels in different cell types varied among chimeric constructs, no distinctions in ex vivo host cell range were detected to suggest differential replication in specific cell subsets. However, the venues tested here are not exhaustive and further examination of host cell range in vivo is still warranted.

The findings with chimeric FIVs may have parallels with previous work involving HIV/simian immunodeficiency virus (SHIV) chimeras (14, 17, 20, 21, 30). In these studies, initial SHIVs replicated ex vivo and in macaques but were not highly pathogenic on primary passage (20, 21, 30). However, pathogenicity increased upon serial in vivo passage (30), as did

quasispecies diversity with respect to receptor usage and host cell range (17). Other studies have suggested a correlation with a particular threshold viral load for SHIV and the level of in vivo pathogenicity (37), which may prove to be the key as to why the high-level-replicating FIV-C36 isolate also exhibits increased pathogenicity in the periphery. It will be of interest to determine whether similar results will be noted with FIV chimeras after serial in vivo passage.

Given the findings outlined above, it seems plausible that FIV-C36 replication may be less affected by host restriction factors recognized to influence retroviral replication. For example, FIV-C36 may be less prone to restriction by the Trim 5α class of proteins that block uncoating of capsid and inhibit cDNA transcription of viral sequences in primate lentiviruses (12, 32, 35). Human and rhesus macaque monkey Trim 5α proteins have been shown to restrict FIV replication (32), implying that similar mechanisms are extant during FIV infection. Higher net replication rates may be attributed to successful bypassing or surviving of the Trim 5α restriction, allowing intact FIV-C36 to reach the cell nucleus with greater ease and speed, thus leading to better integration, transcription, and assembly of virions. Although inclusion of FIV-C Vif did not

impart clade C growth potential to the chimeric FIVs studied here, a more efficient interaction with the family of APOBEC molecules may also contribute to increased clade C replication by reducing cytidine deamination in newly synthesized viral DNA (23). Additional innate levels of control of viral infection that may also be factors in influencing relative FIV replication rates will likely be discovered.

The viral kinetics and virulence characteristics of FIV-C36 and FIV-PPR molecular clones in vivo were similar to those observed previously for uncloned field isolates. FIV-C36 displayed an accelerated disease phenotype compared to that of FIV-PPR during infections of domestic cats. Plasma viremia, along with PBMC proviral load, peaked at the same time p.i. for parental isolates but reached much higher levels in FIV-C36 infections. Additionally, depletion of peripheral CD4⁺ T lymphocytes and neutrophils was early and dramatic during FIV-C36 infections, while FIV-PPR-infected cats had relatively stable counts relative to uninfected controls. While the PC3'LTR chimera was less robust in vivo than either parental chimera, suggesting that this substitution decreased fitness relative to either parental strain, FIV-PCenv dynamics were unique in exhibiting delayed replication and plateau phase kinetics and hematologic effects that ultimately were intermediate between parental strains. This observation may indicate that this chimera gained an enhanced replicative capacity during the course of in vivo passage and suggests that the FIV-C36 3' genomic elements *vif*, *orfA*, and *env* and a portion of the *rev* response element may contribute to in vivo virulence phenotype. For example, the bone marrow tropism and neutropenia classically noted during FIV-CPG infection may be dictated by bone marrow cell susceptibility mediated through *env* or *vif*.

Certain aspects of the in vivo pathology noted for each strain may simply be explained on the basis of differential virus loads as noted above. However, the unique involvement of FIV-PPR and the apparent exclusion of FIV-C involvement in central nervous system pathology do not fit with this scenario. In addition to further probing of intracellular restriction factors that might inherently limit replication of certain viral strains, it will be of interest to determine whether the distinct pathologies for each virus can be defined in terms of specific viral genes, resulting in unique cell tropisms and kinetics in vivo.

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REFERENCES

- Brown, W. C., L. Bissey, K. S. Logan, N. C. Pedersen, J. H. Elder, and E. W. Collisson. 1991. Feline immunodeficiency virus infects both CD4⁺ and CD8⁺ T lymphocytes. *J. Virol.* **65**:3359–3364.
- Dagleish, A. G., P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss. 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* **312**:763–767.
- de Parseval, A., U. Chatterji, P. Sun, and J. H. Elder. 2004. Feline immunodeficiency virus targets activated CD4⁺ T cells by using CD134 as a binding receptor. *Proc. Natl. Acad. Sci. USA* **101**:13044–13049.
- de Parseval, A., and J. H. Elder. 2001. Binding of recombinant feline immunodeficiency virus surface glycoprotein to feline cells: role of CXCR4, cell-surface heparans, and an unidentified non-CXCR4 receptor. *J. Virol.* **75**:4528–4539.
- de Parseval, A., and J. H. Elder. 1999. Demonstration that orf2 encodes the feline immunodeficiency virus transactivating (Tat) protein and characterization of a unique gene product with partial Rev activity. *J. Virol.* **73**:608–617.
- de Rozières, S., C. K. Mathiason, U. Chatterji, U. Rolston, E. A. Hoover, and J. H. Elder. 2004. Characterization of a highly pathogenic molecular clone of feline immunodeficiency virus clade C. *J. Virol.* **78**:8971–8982.
- de Rozières, S., C. H. Swan, D. A. Sheeter, K. J. Clingerman, Y. C. Lin, S. Huitron-Resendiz, S. Henriksen, B. E. Torbett, and J. H. Elder. 2004. Assessment of FIV-C infection of cats as a function of treatment with the protease inhibitor, TL-3. *Retrovirology* **1**:38.
- Dean, G. A., S. Himathongkham, and E. E. Sparger. 1999. Differential cell tropism of feline immunodeficiency virus molecular clones in vivo. *J. Virol.* **73**:2596–2603.
- Diehl, L. J., C. K. Mathiason-Dubard, L. L. O'Neil, L. A. Obert, and E. A. Hoover. 1995. Induction of accelerated feline immunodeficiency virus disease by acute-phase virus passage. *J. Virol.* **69**:6149–6157.
- English, R. V., C. M. Johnson, D. H. Gebhard, and M. B. Tompkins. 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. *J. Virol.* **67**:5175–5186.
- English, R. V., P. Nelson, C. M. Johnson, M. Nasise, W. A. Tompkins, and M. B. Tompkins. 1994. Development of clinical disease in cats experimentally infected with feline immunodeficiency virus. *J. Infect. Dis.* **170**:543–552.
- Hatzioannou, T., D. Perez-Caballero, A. Yang, S. Cowan, and P. D. Bieniaz. 2004. Retrovirus resistance factors Ref1 and Lvl are species-specific variants of TRIM5alpha. *Proc. Natl. Acad. Sci. USA* **101**:10774–10779.
- Huitron-Resendiz, S., S. de Rozières, M. Sanchez-Alvarez, B. Bühler, Y. C. Lin, D. L. Lerner, N. W. Henriksen, M. Burudi, H. S. Fox, B. E. Torbett, S. Henriksen, and J. H. Elder. 2004. Resolution and prevention of feline immunodeficiency virus-induced neurological deficits by treatment with the protease inhibitor TL-3. *J. Virol.* **78**:4525–4532.
- Igarashi, T., O. K. Donau, H. Imamichi, Y. Nishimura, T. S. Theodore, R. Iyengar, C. Erb, A. Buckler-White, C. E. Buckler, and M. A. Martin. 2007. Although macrophage-tropic simian/human immunodeficiency viruses can exhibit a range of pathogenic phenotypes, a majority of isolates induce no clinical disease in immunocompetent macaques. *J. Virol.* **81**:10669–10679.
- Ishida, T., T. Washizu, K. Toriyabe, S. Motoyoshi, I. Tomoda, and N. C. Pedersen. 1989. Feline immunodeficiency virus infection in cats of Japan. *J. Am. Vet. Med. Assoc.* **194**:221–225.
- Klatzmann, D., E. Champagne, S. Chamaret, J. Gruet, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier. 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* **312**:767–768.
- Kozirev, I. L., K. Ibuki, T. Shimada, T. Kuwata, T. Takemura, M. Hayami, and T. Miura. 2001. Characterization of less pathogenic infectious molecular clones derived from acute-pathogenic SHIV-89.6p stock virus. *Virology* **282**:6–13.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Lerner, D. L., C. K. Grant, A. de Parseval, and J. H. Elder. 1998. FIV infection of IL-2-dependent and -independent feline lymphocyte lines: host cells range distinctions and specific cytokine upregulation. *Vet. Immunol. Immunopathol.* **65**:277–297.
- Li, J. T., M. Halloran, C. I. Lord, A. Watson, J. Ranchalis, M. Fung, N. L. Letvin, and J. G. Sodroski. 1995. Persistent infection of macaques with simian-human immunodeficiency viruses. *J. Virol.* **69**:7061–7067.
- Luciw, P. A., E. Pratt-Lowe, K. E. Shaw, J. A. Levy, and C. Cheng-Mayer. 1995. Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). *Proc. Natl. Acad. Sci. USA* **92**:7490–7494.
- Miyazawa, T., M. Fukasawa, A. Hasegawa, N. Maki, K. Ikuta, E. Takahashi, M. Hayami, and T. Mikami. 1991. Molecular cloning of a novel isolate of feline immunodeficiency virus biologically and genetically different from the original U.S. isolate. *J. Virol.* **65**:1572–1577.
- Münk, C., J. Zielonka, H. Constabel, B. P. Kloke, B. Rengstl, M. Battenberg, F. Bonci, M. Pistello, M. Löchelt, and K. Cichutek. 2007. Multiple restrictions of human immunodeficiency virus type 1 in feline cats. *J. Virol.* **81**:7048–7060.
- Obert, L. A., and E. A. Hoover. 2000. Feline immunodeficiency virus clade C mucosal transmission and disease courses. *AIDS Res. Hum. Retrovir.* **16**:677–688.
- Olmsted, R. A., A. K. Barnes, J. K. Yamamoto, V. M. Hirsch, R. H. Purcell, and P. R. Johnson. 1989. Molecular cloning of feline immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **86**:2448–2452.
- Pedersen, N. C., C. M. Leutenegger, J. Woo, and J. Higgins. 2001. Virulence differences between two field isolates of feline immunodeficiency virus (FIV-APetaluma and FIV-CPGammara) in young adult specific pathogen free cats. *Vet. Immunol. Immunopathol.* **79**:53–67.

27. Pedersen, N. C., J. K. Yamamoto, T. Ishida, and H. Hansen. 1989. Feline immunodeficiency virus infection. *Vet. Immunol. Immunopathol.* **21**:111–129.
28. Phillips, T. R., R. L. Talbott, C. Lamont, S. Muir, K. Lovelace, and J. H. Elder. 1990. Comparison of two host cell range variants of feline immunodeficiency virus. *J. Virol.* **64**:4605–4613.
29. Prospéro-García, O., N. Herold, T. R. Phillips, J. H. Elder, F. E. Bloom, and S. J. Henriksen. 1994. Sleep patterns are disturbed in cats infected with feline immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **91**:12947–12951.
30. Reimann, K. A., J. T. Li, R. Veazey, M. Halloran, I. W. Park, G. B. Karlsson, J. Sodroski, and N. L. Letvin. 1996. A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate *env* causes an AIDS-like disease after in vivo passage in rhesus monkeys. *J. Virol.* **70**:6922–6928.
31. Richardson, J., G. Pancino, R. Merat, T. Leste-Lasserre, A. Moraillon, J. Schneider-Mergener, M. Alizon, P. Sonigo, and N. Heveker. 1999. Shared usage of the chemokine receptor CXCR4 by primary and laboratory-adapted strains of feline immunodeficiency virus. *J. Virol.* **73**:3661–3671.
32. Saenz, D. T., W. Teo, J. C. Olsen, and E. M. Poeschla. 2005. Restriction of feline immunodeficiency virus by Ref1, Lv1, and primate TRIM5 α proteins. *J. Virol.* **79**:15175–15188.
33. Shimojima, M., T. Miyazawa, Y. Ikeda, E. L. McMonagle, H. Haining, H. Akashi, Y. Takeuchi, M. J. Hosie, and B. J. Willett. 2004. Use of CD134 as a primary receptor by the feline immunodeficiency virus. *Science* **303**:1192–1195.
34. Sodora, D. L., E. G. Shpaer, B. E. Kitchell, S. W. Dow, E. A. Hoover, and J. I. Mullins. 1994. Identification of three feline immunodeficiency virus (FIV) *env* gene subtypes and comparison of the FIV and human immunodeficiency virus type 1 evolutionary patterns. *J. Virol.* **68**:2230–2238.
35. Stremlau, M., C. M. Owens, M. J. Perron, M. Kiessling, P. Autissier, and J. Sodroski. 2004. The cytoplasmic body component TRIM5 α restricts HIV-1 infection in Old World monkeys. *Nature* **427**:848–853.
36. Talbott, R. L., E. E. Sparger, K. M. Lovelace, W. M. Fitch, N. C. Pedersen, P. A. Luciw, and J. H. Elder. 1989. Nucleotide sequence and genomic organization of feline immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **86**:5743–5747.
37. Ten Haaf, P., B. Verstrepen, K. Uberla, B. Rosenwirth, and J. Heeney. 1998. A pathogenic threshold of virus load defined in simian immunodeficiency virus- or simian-human immunodeficiency virus-infected macaques. *J. Virol.* **72**:10281–10285.
38. Terwee, J. A., J. K. Yactor, K. S. Sondgeroth, and S. Vandewoude. 2005. Puma lentivirus is controlled in domestic cats after mucosal exposure in the absence of conventional indicators of immunity. *J. Virol.* **79**:2797–2806.
39. Thompson, F. J., J. H. Elder, and J. C. Neil. 1994. Cis- and trans-regulation of feline immunodeficiency virus: identification of functional binding sites in the long terminal repeat. *J. Gen. Virol.* **75**:545–554.
40. Troyer, J. L., J. Pecon-Slatery, M. E. Roelke, W. Johnson, S. Vandewoude, N. Vazquez-Salat, M. Brown, L. Frank, R. Woodroffe, C. Winterbach, H. Winterbach, G. Hemson, M. Bush, K. A. Alexander, E. Revilla, and S. J. O'Brien. 2005. Seroprevalence and genomic divergence of circulating strains of feline immunodeficiency virus among *Felidae* and *Hyaenidae* species. *J. Virol.* **79**:8282–8294.
41. Willett, B. J., E. L. McMonagle, S. Ridha, and M. J. Hosie. 2006. Differential utilization of CD134 as a functional receptor by diverse strains of feline immunodeficiency virus. *J. Virol.* **80**:3386–3394.
42. Willett, B. J., L. Picard, M. J. Hosie, J. D. Turner, K. Adema, and P. R. Clapham. 1997. Shared usage of the chemokine receptor CXCR4 by the feline and human immunodeficiency viruses. *J. Virol.* **71**:6407–6415.
43. Yamamoto, J. K., H. Hansen, E. W. Ho, T. Y. Morishita, T. Okuda, T. R. Sawa, R. M. Nakamura, and N. C. Pedersen. 1989. Epidemiologic and clinical aspects of feline immunodeficiency virus infection in cats from the continental United States and Canada and possible mode of transmission. *J. Am. Vet. Med. Assoc.* **194**:213–220.