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Adoptive Immunotherapy of Feline Immunodeficiency Virus with Autologous Ex Vivo-Stimulated Lymphoid Cells Modulates Virus and T-Cell Subsets in Blood

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The potential of immunotherapy with autologous virus-specific T cells to affect the course of feline immunodeficiency virus (FIV) infection was explored in a group of specific-pathogen-free cats infected with FIV a minimum of 10 months earlier. Popliteal lymph node cells were stimulated by cocultivation with UV-inactivated autologous fibroblasts infected with recombinant vaccinia viruses expressing either FIV *gag* or *env* gene products, followed by expansion in interleukin-2. One or two infusions of both Gag- and Env-stimulated cells resulted in a slow increase in FIV-specific gamma interferon-secreting T cells in the circulation of cats. In the same animals, viral set points fluctuated widely during the first 2 to 3 weeks after adoptive transfer and then returned to pretreatment levels. The preexisting viral quasispecies was also found to be modulated, whereas no novel viral variants were detected. Circulating CD4$^+$ counts underwent a dramatic decline early after treatment. CD4/CD8 ratios remained instead essentially unchanged and eventually improved in some animals. In contrast, a single infusion of Gag-stimulated cells alone produced no apparent modulations of infection.

In humans and in animal models, the induction and maintenance of virus-specific CD8$^+$-T-cell responses are crucial for the elimination of acute viral infections, such as influenza (26), and in controlling viral replication in persistent infections such as Epstein-Barr virus (EBV) and cytomegalovirus (CMV) infections (2, 27). Likewise, in persistent retroviral infections of humans and animals there is a close relationship between the presence of virus-specific cytotoxic T lymphocytes (CTL) and the control of retroviral replication (5, 7, 8, 18, 22, 24, 33, 35, 45). That virus-specific CTL are important in the control of human immunodeficiency virus (HIV) replication in people is supported by in vitro experiments (45) and by the frequent selection of viral mutants in vivo that are no longer recognized by CTL (11, 31) and consequently escape immune control. The use of histocompatibility complex tetramers presenting an immunodominant HIV peptide epitope has also shown that the numbers of HIV-specific T cells peak just after the level of viremia starts to decline after infection (44), implying that the CTL are responsible.

Recent improvements in antiretroviral chemotherapy have dramatically extended the life expectancy of HIV-infected individuals in the developed world. However, latent infection of memory CD4$^+$ T cells and possibly other cells provides a mechanism for lifelong persistence of the virus in treated patients so that virus load rapidly increases again after the withdrawal of chemotherapy (28, 34).

Adoptive immunotherapy with virus-specific T-cell clones, in an attempt to achieve a meaningful augmentation of adaptive immune responses, has proven to be a useful adjunct to chemotherapy for certain persistent viral infections in humans, including EBV and CMV (16, 30, 42). However, the same approach has met with mixed success in HIV-infected patients: some studies have shown modulations in viral burdens (3), and others have shown rapid progression to clinical disease associated with the selection of CTL escape mutant viruses (23).

Feline immunodeficiency virus (FIV) represents the only naturally occurring disease model for HIV, with an abundance and diversity of strains isolated from cats worldwide. Further, FIV is an important pathogen of its natural host species, resembles HIV in pathogenesis and immunobiology, and is recognized as an important model for evaluating novel approaches to lentiviral therapy and prophylaxis (4, 10, 41). In the present study, we evaluated the safety and the clinical, virologic, and immunological efficacy of adoptive transfer of autologous lymphoid cells restimulated in vitro with viral antigens in chronically FIV-infected domestic cats. Our aim was to evaluate the potential therapeutic value of this approach and, possibly, obtain hints about the importance of FIV-specific T cells in containing FIV. Treatment-naive cats infected with either of two independent isolates of FIV received one or two infusions of mixed Gag-stimulated lymphoid cells (SLC) and Env-SLC or a single infusion of Gag-SLC alone. All treatments were well tolerated, but only the mixed infusions produced detectable effects on infection course. The changes were most evident for 2 to 3 weeks posttreatment and consisted of rapid wide fluctuations in viral set points and in a drastic decrease in circulating CD4$^+$ counts. Subsequently, the virus stabilized again at prechallenge levels but CD4$^+$ counts remained low. Initially, CD4/CD8 ratios were little modified but improved eventually in some cats.

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TABLE 1. Experimental design

Cat ID	Infection status (virus strain; mo postinfection)	No. of SLC per infusion[a]		
		Gag stimulated	Env stimulated	Total
Single infusion of Gag- and Env-SLC				
BD	FIVGL-8; 30	2.0×10^7	2.0×10^7	4.0×10^7
BH	FIVPet; 10	2.0×10^7	2.0×10^7	4.0×10^7
BP	FIVPet; 10	1.1×10^7	1.2×10^7	2.3×10^7
Two infusions of Gag- and Env-SLC				
BB	FIVGL-8; 30	1.5×10^7	1.5×10^7	3.0×10^7
BT	FIVPet; 10	1.5×10^7	1.5×10^7	3.0×10^7
BV	FIVPet; 10	1.5×10^7	1.5×10^7	3.0×10^7
Single infusion of Gag-SLC alone				
AX	FIVGL-8; 55	4.0×10^7		4.0×10^7
BE	FIVGL-8; 30	4.0×10^7		4.0×10^7

[a] Intravenous infusion of SLC carried out on either day 0 or on days 0 and 7.

MATERIALS AND METHODS

Animals and infection. Due to the exploratory intent of the study, the number of experimental animals was kept to a minimum. These were eight treatment-naive female outbred specific-pathogen-free (SPF) domestic cats who had been experimentally infected with either the prototype Petaluma (FIV_{PET}) or the $Glasgow_8$ isolate of FIV (FIV_{GL-8}) 10 to 55 months prior to initiation of the experiment (Table 1). They were housed individually in a climatized animal facility and had ad libitum access to fresh water and a proprietary brand of cat food in accordance with European Community guidelines.

Preparation of the SLC. A skin biopsy sample and a popliteal lymph node were aseptically removed from each individual cat under general anesthesia and used to produce the stimulator fibroblasts and the SLC, respectively (13). For stimulator cell production, 24-well flat-bottom tissue culture plates were seeded with 10^5 fibroblasts per well in 1 ml of MEM ALPHA medium containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, Milan, Italy), 2 mmol of L-glutamine/liter, 100 IU of penicillin, and 100 μg of streptomycin per ml and incubated at 37°C in 5% CO_2. When fibroblasts were confluent, the medium was aspirated and the cells were inoculated with 5 PFU per cell of recombinant vaccinia virus (rVV) expressing the FIV Gag (12) or Env genes (37) or wild-type (wt) vaccinia virus in a total volume of 200 μl of complete MEM ALPHA medium. Infection was allowed to proceed for 3 h at 37°C to allow for optimal expression of FIV antigens (1). The fibroblasts were then irradiated for 300 s in a UV-cross-linker to inactivate the viral particles. For the production of Gag-SLC and Env-SLC, single cell suspensions prepared from the popliteal lymph nodes by gentle manual homogenization were added to the respective autologous stimulator cells at a ratio of ca. 10:1 in 2 ml of RPMI 1640 medium (Sigma-Aldrich) containing 10% heat-inactivated fetal bovine serum, 2 mmol of L-glutamine/liter, 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml (complete RPMI 1640 medium). The cultures were then incubated for 7 to 10 days at 37°C in 5% CO_2. Prior to reinfusion into the donor cats, in an effort to expand the cells which had responded to antigen stimulation and upregulated the interleukin-2 (IL-2) receptor, the SLC were harvested and cultured for a further 7 to 10 days in complete RPMI 1640 medium supplemented with human recombinant IL-2 (kindly provided by T. Miyazawa, University of Tokyo, and M. Hattori, University of Kyoto). However, to prevent the possible activation of LAK cells, the IL-2 content in the cultures was kept relatively low (100 IU/ml).

Quantification of functional Gag- and Env-specific T cells. Quantification of Gag- and Env-specific T cells in the SLC and in the peripheral blood mononuclear cells (PBMC) was achieved by enumerating the cells that reacted to the respective antigens by secreting gamma interferon (IFN-γ) in an enzyme-linked immunospot (ELISpot) assay. The wells of a 96-well filtration plate (Millipore MAHAS410) were coated overnight with 2 μg of murine anti-feline IFN-γ monoclonal antibody D9 (17) in 100 μl of phosphate-buffered saline (PBS) in a humidified atmosphere at 4°C. The wells were then washed four times with sterile PBS and incubated with 100 μl of complete RPMI 1640 medium for at least 1 h at 37°C to block any unreacted sites. The medium was then replaced with 100 μl of complete RPMI medium containing known numbers of the cells under scrutiny. Usually, a titration of cells was performed to give a range from 2×10^5 to 5×10^4 cells per well. To these wells, the rVV described above, expressing either the FIV *gag* or *env* gene products, were added with a multiplicity of infection of 2. The test was performed in duplicate, and the plates were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The wells were then washed four times with PBS containing 0.05% Tween 20 and further incubated with an affinity-purified biotinylated polyclonal sheep anti-feline IFN-γ antibody (17) at room temperature overnight. Unbound antibody was removed by four washes with PBS-Tween 20, and bound antibody was visualized by the addition of 0.4 μg of streptavidin-HRP (Vector Laboratories/DBA-Italia, Milan, Italy)/ml in PBS-Tween 20. After a final washing, the assay was developed by the addition of 100 μl of AEC chromogen (Vector Laboratories)/well according to the manufacturer's instructions, and the reaction was allowed to proceed until spots were visible to the naked eye. The plates were then washed in distilled water and air dried, and the frequencies of antigen-specific IFN-γ-secreting cells were determined by using a binocular dissecting microscope.

SLC infusions. As specified in Table 1, the cats were intravenously infused on either one or two occasions 7 days apart, and the infusions contained either Gag-SLC alone or Gag- and Env-SLC mixed in equal proportions immediately before administration. The cells transfused varied between 2×10^7 and 4×10^7 were >75% viable by trypan blue staining and were given in 10 to 20 ml of physiological saline.

Estimation of FIV DNA in cells. Genomic DNA was extracted from lymphocytes by using a commercial kit (QIAamp DNA Blood Minikit; Qiagen, Milan, Italy) and according to the manufacturer's instructions. Proviral DNA was quantified by real-time TaqMan PCR (TM-PCR) from 0.40 μg of genomic DNA (32). The p24 *gag* primers used have no mismatches with either FIV_{PET} or FIV_{GL-8} and have the following sequences: PU8-S, CACCAGCTAGGATGCAGTG TAGA (sense orientation, nucleotide positions 1375 to 1397, referred to as FIV_{PET}, GenBank accession no. NC_001482); PU8-P, TTGGCTGCCATAAA AGCTAAGTCTCCTCG (probe, nucleotide positions 1425 to 1453); and PU8-AS TGAATAATCTTCCTTAGCTCCTTGTCT (antisense, nucleotide positions 1467 to 1493). Serial 10-fold dilutions (10^1 to 10^7) of FIV_{PET} *gag* plasmid in a background of 1 μg of genomic DNA were used to produce a standard curve and determine the lowest limit of detection (10 copies).

Measurement of plasma viremia. Viral RNA was prepared from plasma samples by using the QIAamp Viral RNA kit (Qiagen). A total of 10 μl of RNA extracted from plasma was reverse transcribed and TM-PCR amplified (32). Serial 10-fold dilutions (10^2 to 10^7) of FIV_{PET} Gag RNA transcripts produced by runoff transcription were used to produce a standard curve. The sensitivity of the assay was 200 copies/ml of plasma, as evaluated by extracting and amplifying FIV-negative plasma spiked with serial 10-fold dilutions of FIV_{PET} Gag RNA transcripts.

Viral surface glycoprotein sequence analysis. For sequencing the surface glycoprotein, 10 μl of viral RNA extracted as described above from the plasma of infected cats was reverse transcribed and amplified with primers derived from FIV_{PET} (GenBank accession no. NC_001482). Synthesis of cDNA was carried out with avian myeloblastosis virus reverse transcriptase (Finnzymes; Celbio, Milan, Italy) and antisense primers SUSC-I (CCCTACATTCCATCTACATC; nucleotides 7647 to 7666) and SUT-I (CTAGTACTTGATGCTCTAATGT AAC; nucleotides 8267 to 8291), and the resulting product was amplified by

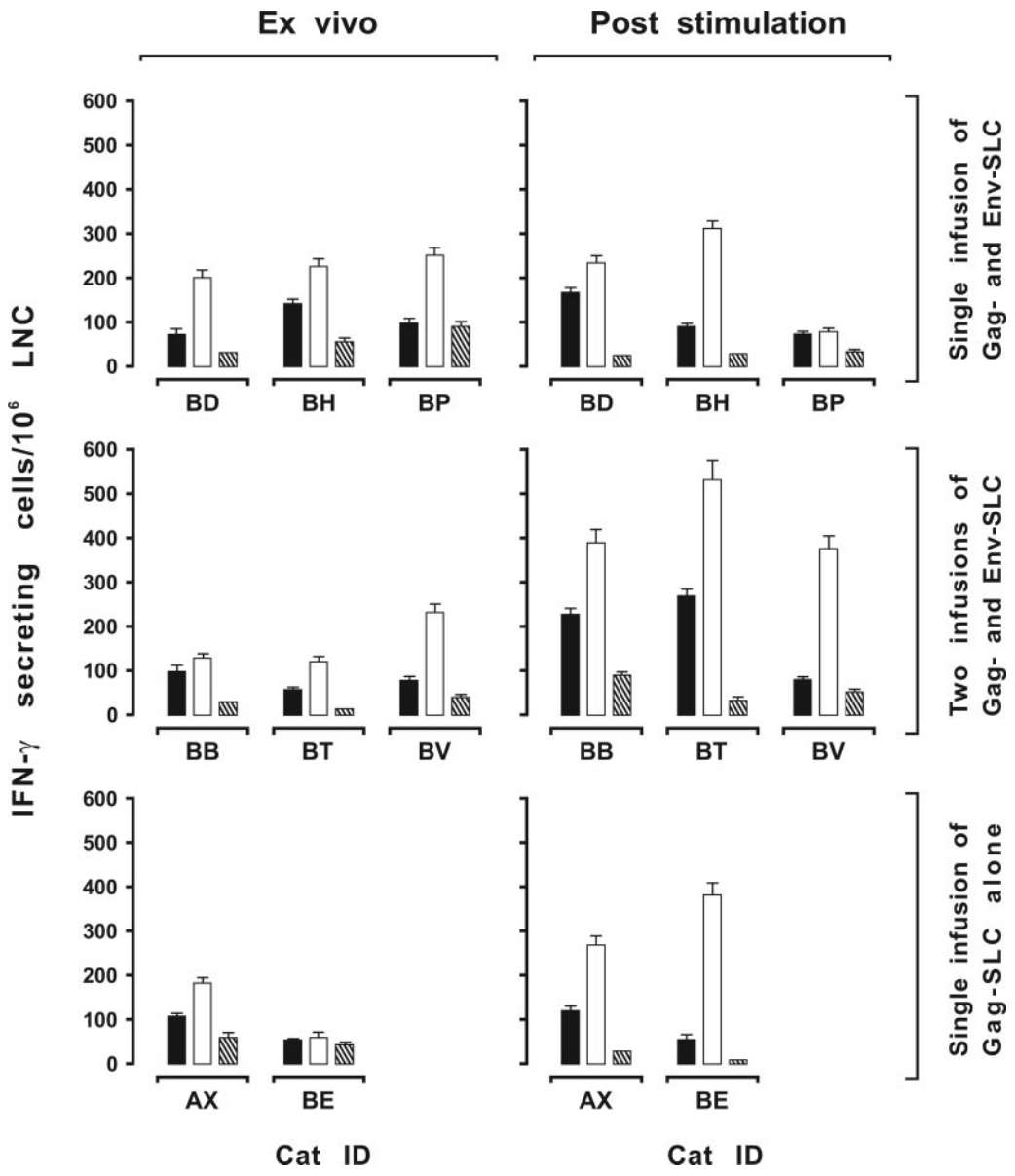

FIG. 1. Expansion of FIV-specific IFN-γ-secreting cells in culture. The frequencies of FIV Gag- (■), Env-specific (□), and control (▨) IFN-γ-secreting T cells were determined by ELISpot assays in popliteal lymph nodes (LNC) directly ex vivo and after in vitro restimulation. Restimulation was achieved by cocultivation with UV-inactivated autologous skin fibroblasts infected with rVVs expressing either FIV Gag or FIV Env gene products. The results shown represent the mean of duplicate cultures ± 1 standard error of the mean. As in the subsequent figures, the animals are grouped according to the therapeutic protocol subsequently received.

nested PCR. The cDNA obtained with SUSC-I primer was amplified with sense primers V12 (GCAGGTAAGTTTAGAAGAGCAAG; nucleotides 6503 to 6525) and SUSC-I (first-round PCR) and SUSC-II-S (CGCTCAGGTAGTAT GGAGACTTCC; nucleotides 6784 to 6807) and SUSC-II-AS (GCTCCCGTT ACTTCTCCATAATC; nucleotides 7559 to 7581) (second-round PCR). The cDNA obtained with SUT-I primer was amplified with sense primers V51 (AA ATGTGGATGGTGGAATCAA; nucleotides 7322 to 7342) and SUT-I (first-round PCR) and SUT-II-S (GAGCAATCTCGTCATGGAAACAAAG; nucleotides 7431 to 7455) and SUT-II-AS (CCTATAGCAGTAGCCCCCGTCCC TGC; nucleotides 8150 to 8175) (second-round PCR). Amplicons were either directly sequenced for determining the predominant variant or cloned into pCRII Topo TA plasmid vector (Invitrogen Italia, San Giuliano Milanese, Italy), and individual clones were sequenced for determining virus quasispecies. Sequencing was performed by using the automated ALF ExpressII DNA sequencer (Amersham Pharmacia Biotech, Cologno Monzese, Italy) and the Cy-5-labeled primers A-S (ACAGACCCATTACAAATCCCAC; nucleotides 7226 to 7247), A-AS (GTGGGATTTGTAATGGGTCTGT; nucleotides 7226 to 7247), SEQ-II-AS (CTTTTGTTTCCATGACGAGATTGCTC); nucleotides 7431 to 7455, V4-S (AACCTTTGCAATGAGAAGTT; nucleotides 7534 to 7553), and V4-AS (TACAAGACCAATTTCCAGCA; nucleotides 7849 to 7868). Sequence data were edited and aligned by BioEdit (version 6.0.5, http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Flow cytometry. Differential lymphocyte counts in the blood and in the SLC were performed by labeling with mouse monoclonal antibodies to feline CD4 (FE1.7B12), CD8α (FE1.10E9), and CD21 (CA2.1D6), all obtained from P. F. Moore, University of California at Davis. Bound primary antibodies were detected by using fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G1 (Space-Serotec, Milan, Italy), and the samples were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

TABLE 2. Phenotypic characterization of adoptively transferred SLC

Cat ID	% SLC with surface marker			FIV DNA copies/μg of SLC DNA	
	CD4	CD8	CD21	Gag stimulated	Env stimulated
Single infusion of Gag- and Env-SLC					
BD	30	25	26	26,777	84
BH	21	25	33	17,335	4,571
BP	30	27	13	89,260	18,166
Two infusions of Gag- and Env-SLC					
BB	22	33	17	15,660	75
BT	33	20	13	54,135	5,359
BV	32	28	15	596,947	499,638
Single infusion of Gag-SLC alone					
AX	10	15	7	150,216	ND[a]
BE	26	47	16	108,440	ND

[a] ND, not done.

Statistical analysis. Data were analyzed by using SigmaStat software (version 2.03; SPSS Software, Chicago, IL). Differences were considered significant if the P value was <0.05.

RESULTS

Characterization of the SLC to be reinfused. To generate SLC for adoptive immunotherapy, individual lymph node cells were cultured separately in the presence of UV-inactivated autologous fibroblasts infected with FIV Gag- or Env-expressing rVV for 7 to 10 days. The SLC were then expanded with IL-2 for a similar time period. To evaluate whether this protocol had been effective in stimulating FIV-specific T cells, the lymphoid cells from each individual cat were screened for the content in FIV Gag- and Env-specific IFN-γ-secreting cells by ELISpot analysis at the time the cultures were started and then again when they were harvested to be reinfused into the donor cats. The results (Fig. 1) showed that (i) when FIV antigen was present in the assay, the numbers of spot-forming cells were invariably higher than background, i.e., of the spot-forming cells produced when the corresponding cells were assayed in the presence of wt vaccinia virus; (ii) at initiation of the cultures, FIV-specific IFN-γ-secreting cells were detected in all cats at numbers that ranged from 5 to 75 per 10^6 cells for Gag and from 17 to 175 per 10^6 cells for Env; (iii) when the cultures were harvested, they demonstrated increased numbers of γ-IFN-producing cells specific for either Env (two of eight cats) or Gag (one of eight) or both (five of eight); and (iv) the response to Gag was generally much weaker than that to Env. Based on these findings, it was concluded that the procedure used to generate SLC from the FIV-infected cats had been effective at stimulating Env-specific T cells but less so at stimulating Gag-specific T cells. As a result, the Env-SLC that were eventually reinfused contained between 200 and 500 Env-specific IFN-γ-producing cells per 10^6, whereas the corresponding Gag-SLC contained 200 Gag-specific IFN-γ-producing cells per 10^6 at most.

At the time of adoptive transfer, the cell inocula were also phenotypically characterized for the proportions of $CD4^+$ T cells, $CD8^+$ T cells, and B cells by flow cytometry and for the content of FIV DNA by real-time TM-PCR. As shown in Table 2, all three lymphocyte subpopulations were invariably present, although their relative proportions varied somewhat in individual suspensions. The lowest proportion of $CD4^+$ T cells was observed in cat AX, who had been infected with FIV_{GL-8} for 55 months: in fact, for reasons that were not investigated but were probably related to the long-term infection with a particularly virulent virus as FIV_{GL8} (19), the majority of cultured cells from this animal failed to stain with any of the three markers investigated. As also shown in Table 2, proviral DNA was detected at variable numbers of copies in all of the SLC, regardless of the FIV antigen used for in vitro stimulation. It is, however, noteworthy that proviral copy numbers were markedly lower, up to 320-fold, in the Env-SLC than in the Gag-SLC. This indicated that Env stimulation had not only been more effective at generating Env-specific γ-IFN-secreting cells but had probably also achieved a more effective containment in vitro of the harbored FIV.

Gag- and Env-specific T-cell frequencies in the PBMC before and after adoptive immunotherapy. Five study cats received one infusion of SLC, and three were given two infusions 7 days apart, each containing between 2.3×10^7 and 4×10^7 cells. Furthermore, of the cats given a single infusion, two received Gag-SLC alone and three received equal numbers of Env-SLC and Gag-SLC (Table 1). As judged by the consumption of food and by daily physicals, the infusions were well tolerated by all animals. In an attempt to explore whether the adoptive immunotherapy modulated the host cellular immune function quantitatively or qualitatively, the frequencies of Gag- and Env-specific IFN-γ-secreting T cells in the blood of the study cats were monitored directly by ELISpot assays immediately before infusion and at selected times for 35 days thereafter. The results are shown in Fig. 2. Prior to adoptive transfer, IFN-γ-secreting cells were detected in most study cats, but their numbers were uniformly low, frequently in the range of background spot forming cells (0 to 80 per 10^6 PBMC), regardless of the FIV antigen used in the assay. In the very early days after adoptive immunotherapy, the numbers of FIV-specific IFN-γ-secreting T cells in the circulation of cats did not change appreciably or increased only slightly. More definite increases in circulating FIV-specific T cells were observed in some cats 7 to 10 days posttreatment, but this was transient and generally very moderate. However, by days 28 and 35 the

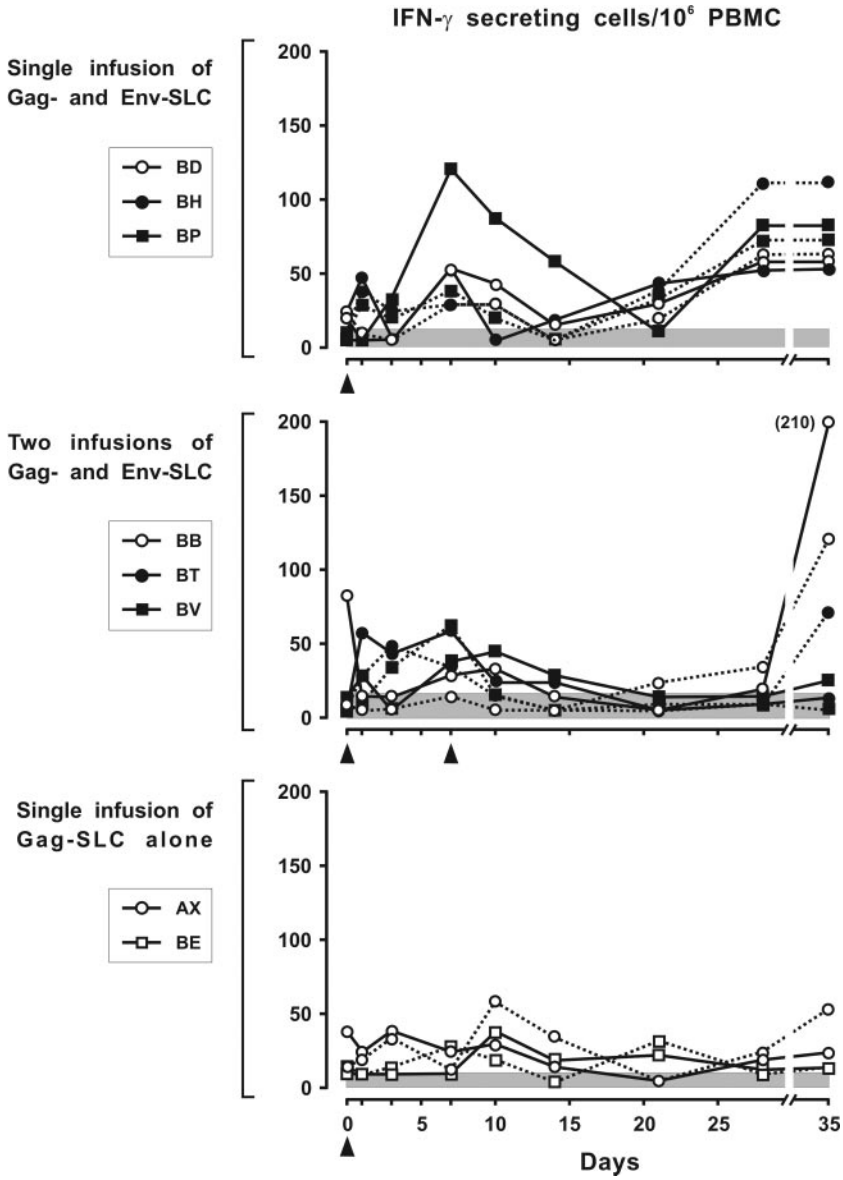

FIG. 2. Frequencies of FIV-specific IFN-γ-secreting cells in the blood of cats before and after adoptive immunotherapy. FIV Gag- and Env-specific IFN-γ-secreting T cells were enumerated directly in the PBMC by ELISpot assays prior to cell infusion and at intervals thereafter. Shaded areas are means ± the standard deviation of background spot-forming cells detected in the absence of FIV antigen in the assays. Arrowheads indicate the infusions.

numbers of T cells specific for Gag antigen, Env antigen, or both were substantially augmented. These increases did not appear to be dependent on the cats receiving a second infusion of SLC since they were also observed in cats who received only one infusion. In contrast, the increases appeared to be affected by the antigen used to stimulate the cells transfused since they were noted in the animals who had received both Gag-SLC and Env-SLC but not in the ones given Gag-SLC alone. In the former animals the effect was statistically highly significant ($P = 0.002$, by Friedman analysis).

Effect of adoptive immunotherapy on FIV loads. At selected intervals up to 80 days after the infusions, the study cats were assessed for plasma viral RNA and PBMC viral DNA copy numbers, as measures of the activity of FIV infection (Fig. 3). Consistent with the fact that the animals were in the steady-state phase of the infection, both parameters had stabilized at relatively low levels when the experiment was started. After adoptive transfer, the two cats who had received Gag-SLC alone underwent very little change in viral set points relative to pretreatment. In contrast, the posttreatment phase of all cats given one or two infusions of both Gag-SLC and Env-SLC was characterized by wide fluctuations of the viral parameters. The fluctuations were only partially in phase in individual cats; however, most lower values were concentrated in the very first days posttreatment and most peak values were concentrated at later times. This phase of perturbed infection balance lasted 2 to 3 weeks, after which plasma viremia levels and proviral copy numbers stabilized again around levels similar to the ones prior to treatment.

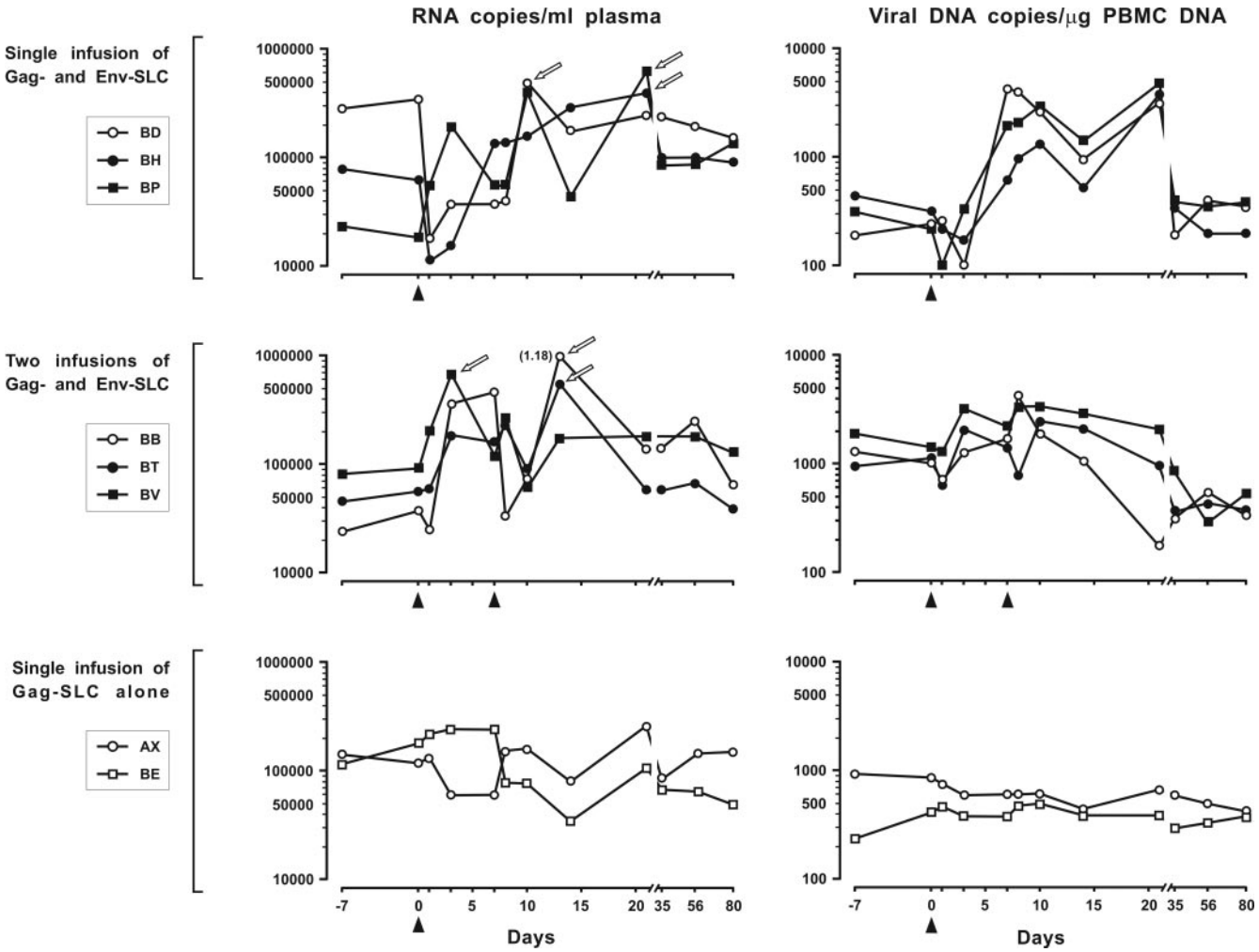

FIG. 3. FIV RNA and DNA loads produced by adoptive immunotherapy. Viral RNA in plasma and DNA in PBMC were measured by real-time TM-PCR. Arrows indicate the times when Env was sequenced; arrowheads indicate the infusions.

Effect of adoptive immunotherapy on viral surface glycoprotein sequence. Since it was plausible that the viral load fluctuations seen after infusion of mixed Gag- and Env-SLC were associated with surface modifications in the infecting virus, we directly sequenced the entire surface glycoprotein of paired viral samples obtained from all of the recipients at day 0 and at the time postinfusion of maximum plasma viremia (Fig. 3, arrows). Direct sequencing showed identical pre- and posttreatment sequences in three cats, and 2, 7, and 10 irregularly dispersed amino acid differences between the paired samples of the other three (BH, BV, and BT, respectively, all infected with FIV_{PET}). Because the observed interpair differences could reflect the emergence of novel viral variants or quantitative changes in the composition of the existent quasispecies, the paired samples of cats BT and BV were cloned, and 10 to 15 clones for each sample were sequenced. The results showed that, apart from one found in BV, all of the substitutions detected after treatment were already also present in the pretreatment samples (data not shown). However, the relative proportions of the sequences carrying one or the other residue were significantly different in the pre- and posttreatment samples, indicating that also in these animals adoptive immunotherapy had not led to the appearance of new variants but had simply modulated the preexisting viral quasispecies. It is, however, remarkable that of the amino acid positions involved five were shared between cats BT and BV (Fig. 4), suggesting the existence of at least some degree of consistency in such modulation.

Effect of adoptive immunotherapy on blood T-cell counts. At the start of the experiment, $CD4^+$-cell counts were below the range of normality only in three of the eight cats, and these were all infected by long time with FIV_{GL8}, known to be more virulent than FIV_{PET} (19). After the infusions, $CD4^+$-cell counts declined in all of the cats except the two given a single infusion of Gag-SLC, who already had very low pretreatment counts. The drop lasted for a couple of weeks, after which a certain recovery occurred in some cats. Similar fluctuations were also observed with $CD8^+$-cell counts. As a result, CD4/CD8 ratios, which were generally low before treatment, did not change until, in four cats given Env-SLC, an improvement became evident starting from the second week posttreatment

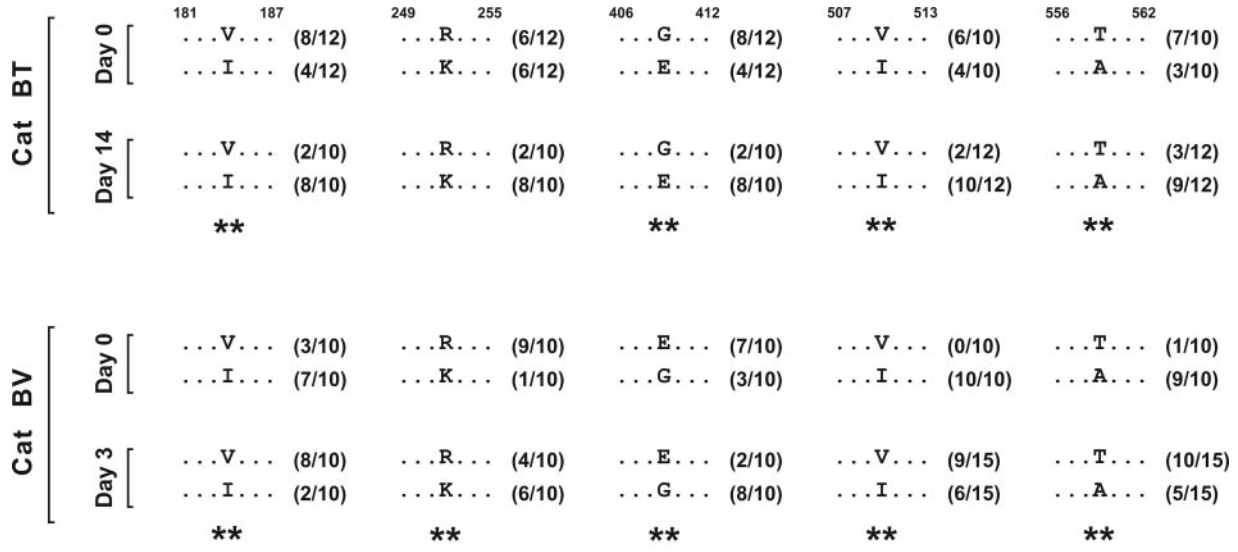

FIG. 4. Changes in the surface glycoprotein of FIV produced by adoptive immunotherapy in two cats. Paired virus samples obtained before and 14 days (cat BT) or 3 days after the first infusion (cat BV) were cloned and sequenced. Only the five amino acid positions found to be modulated in both cats are shown. In parentheses are the numbers of the indicated genotypes divided by the total numbers of clones sequenced. Asterisks indicate that the frequencies of the residues were significantly different ($P < 0.05$ [Fisher exact test]) in the pre- and postinfusion viral samples.

(Fig. 5). Cats who received a second infusion did not differ from cats receiving a single infusion.

DISCUSSION

This is the first report with the FIV model to explore the feasibility of immunotherapy of lentiviral infections with autologous lymphoid cells restimulated and expanded ex vivo. This kind of intervention has been considered as a possible therapeutic adjunct in the treatment of HIV infection (24) but has received little practical testing—and often under far-from-ideal conditions—in natural host systems. FIV is a well-characterized natural pathogen of domestic cats and can provide valuable insights about the usefulness of manipulating lentivirus infected hosts immunologically (4, 10, 41). Adoptive immunotherapy has been exploited with some success to treat chronic viral infections in humans refractory to antiviral chemotherapy (38) or to prevent the development of virus-induced disease in immunosuppressed transplant recipients (30). Also, studies on feline leukemia virus (FeLV), a commonly occurring gamma retrovirus of domestic cats, have revealed that adoptive immunotherapy was associated with very significant declines in FeLV proviral loads (14). Here, adoptive immunotherapy of FIV infection was evaluated with regard to the number of SLC infusions and the viral antigen used to restimulate the infused SLC (*gag* versus *env* products) in terms of safety and modulation of viral burden, viral quasispecies, and lymphocyte subsets in the peripheral circulation.

Study animals were SPF cats who had been FIV infected for a minimum of 10 months and were therefore in the steady-state infection (21). The SLC to be infused were generated by expanding autologous lymph node cells ex vivo by using UV-inactivated autologous fibroblasts infected with rVV expressing FIV *gag* (Gag-SLC) or *env* gene products (Env-SLC) as pulsing stimulus. Cell permanence in vitro was kept relatively short (14 to 20 days) to avoid the selection of T-cell subpopulations conditioned for in vitro culture and to ensure that viral epitope specificity of the transfused cells was representative of that found in vivo. Furthermore, infused cells were not depleted of $CD4^+$ T cells because a deficiency of the help provided by these cells may have been responsible for the failed long-term survival of infused CMV-specific $CD8^+$ T cells (43), and its presence was associated with improved clinical benefit in EBV immunotherapy (38). As determined by ELISpot analysis of IFN-γ-secreting cells, in vitro restimulation expanded the FIV-specific functionally responsive T cells in all cats. This effect was more marked for Env-specific T cells compared to the Gag-specific counterpart, possibly due to the higher numbers of the former present in the starting lymph nodes. It should, however, be noted that analyses of cat T-cell responses to FIV with the use of a single-cell assays have thus far been very few (6). The findings thus far have differed somewhat from reports for HIV and SIV (9, 25, 29) with regard to both the frequencies (higher in primate lentiviral infections) and the preferential targeting of reactive T cells (in contrast to what was observed here, Gag appears to be targeted more frequently than Env in primate lentiviral infections). These discrepancies might reflect inherent differences in the antiviral cell-mediated immune responses mounted by different lentivirus-infected hosts. Traditional bulk tests of cell-mediated immunity, such as antigen-specific lymphoproliferation and cytotoxic assays, have also generally given weak readings in the course of FIV infection (14). The sensitivity of our ELISpot assay also needs to be better evaluated, for example, versus similar assays with peptide-loaded (6) or virus-pulsed dendritic cells that in HIV have been reported to perform better than the ones with rVV (39, 40).

The infusions contained Gag-SLC alone or equal numbers of Gag-SLC and Env-SLC, and the mixed ones were given either once or twice. All regimens were apparently well toler-

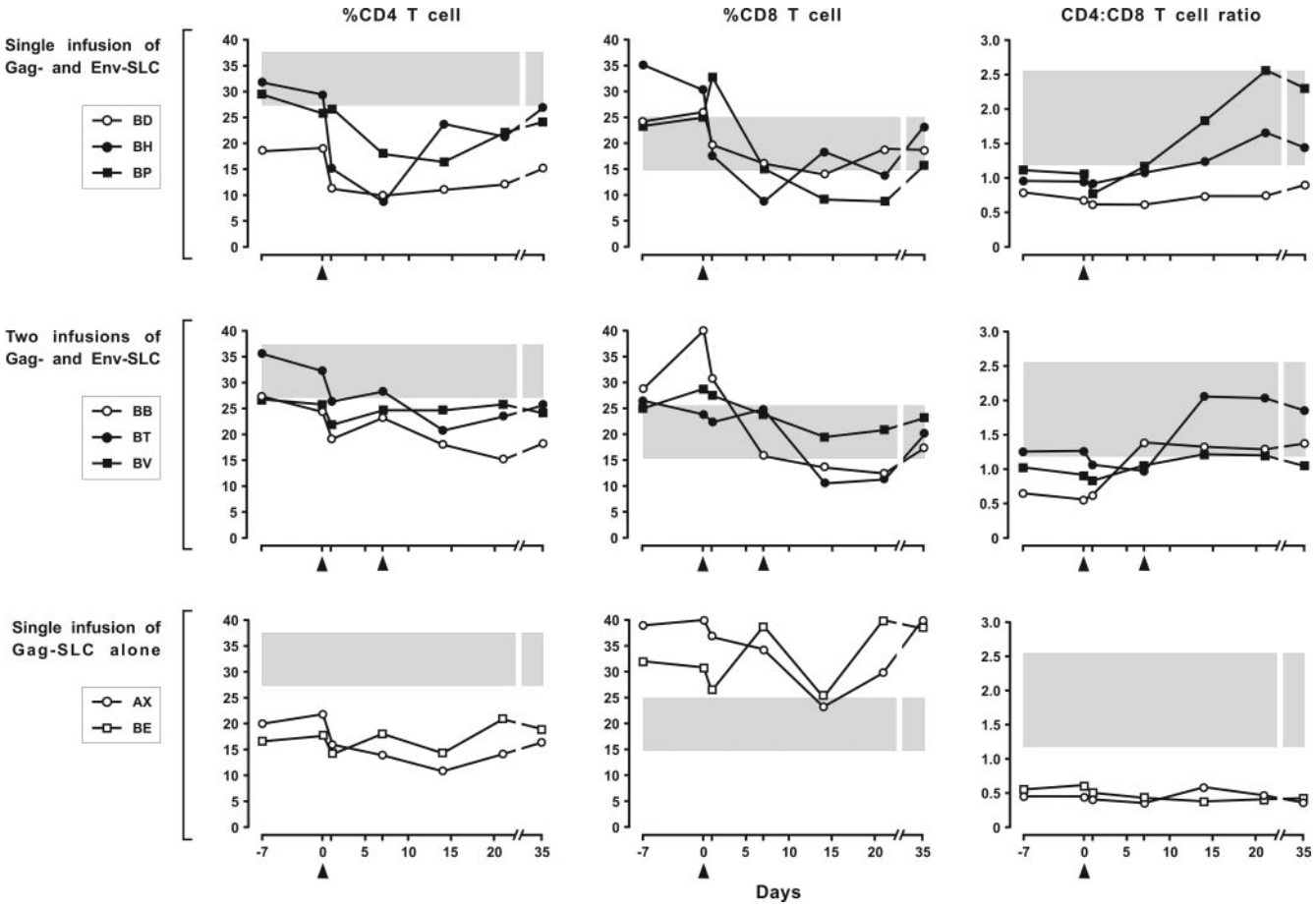

FIG. 5. Changes in circulating T-cell subsets produced by adoptive immunotherapy. $CD4^+$ and $CD8^+$ T cells were enumerated in the blood by flow cytometry with monoclonal antibodies. Shaded areas are the range of normality as determined in a large number of historical uninfected age-matched control cats. Arrowheads indicate the infusions.

ated, since no evidence of pyrexia, anorexia, dullness, or lethargy was observed in the treated cats. Prior to treatment, the recipients had low numbers of FIV-specific effector cells as determined by ELISpot analysis of PBMC with Gag and Env antigens, and these numbers increased only marginally soon after infusion, possibly indicating that most infused cells were at least initially trapped in solid tissues (3). From day 21 onward a steady increase in the numbers of FIV-specific IFN-γ-secreting T cells was, however, noted in the PBMC of the cats who had received both Gag- and Env-SLC but not in those given Gag-SLC alone. This indicated that the former infusions had indeed ameliorated antiviral cell-mediated responses in the infected cats, although rather slowly.

The effects of treatment on the viral set points followed an interesting pattern. The first 2 to 3 weeks were characterized by rapid fluctuations of both PBMC viral DNA and plasma RNA copy numbers that were considerably greater than generally observed in steady-state infected cats than in the ones under study. The reasons for these fluctuations remained unresolved, but a clear possibility is that they were triggered by the coexistence in the adoptively transferred cats of opposing forces that reduced or stimulated virus replication. Adoptive transfer certainly introduced new and possibly very active FIV-specific T cells, but most likely also increased the availability of FIV-permissive cells in the hosts due to the actual proliferating cells inoculated or through the release by these cells of cytokines that enhanced lymphoproliferation in general. The latter effect may have greatly expanded the pool of productively infected cells, especially since the infused cats received no antiviral drugs that would have contained FIV replication. It may not be by chance that most dips in viral set points occurred soon after the first infusions, when the beneficial effects were likely to be predominant, and that most peaks occurred instead later on when the virus-favoring mechanisms had had the time to become operative.

CTL epitope mutation in the escape of retroviruses from immune surveillance is well recognized (31). Indeed, expansion of an HIV Nef CTL escape mutant virus has been associated with the failure of at least one HIV adoptive immunotherapy trial (23). Furthermore, correlative data from vaccine studies have previously suggested that both FIV Gag- and Env-specific immune responses are important for protection (20) and that FIV Env-specific CTL are important in the maintenance of long-term anti-FIV immunity (15). The possibility was therefore also considered that the observed fluctuations, which appeared to be mediated mainly if not solely by Env-SLC, might

reflect the selective expansion of CTL escape mutant viruses. Sequencing studies performed in two cats at the time their plasma viral loads peaked posttransfer demonstrated no new amino acid residues throughout the entire viral surface glycoprotein relative to respective baseline viruses and showed that the treatment had only produced a perturbation of the preexisting viral quasispecies, as revealed by changes in dominant viral variants. Given the time frame used, the possible appearance of CTL escape mutants at later times cannot, however, be excluded.

The period of clearly unbalanced FIV infection was relatively brief and was followed by a return of viral set points to pretreatment levels. This implies that, whatever their nature, the mechanisms involved had been short lived. Nevertheless, the imbalance was not insignificant in terms of immunological status since most of the cats who went through it also showed a concomitant, often very dramatic, decrease in circulating $CD4^+$ T cells, a well-recognized marker of increased FIV infection severity. This effect may have been contributed to by enhanced lymphocyte destruction due to increased viral replication or increased recognition and killing by FIV-specific T cells. However, it seems likely that altered recirculation of lymphocytes and possibly other mechanisms also concurred. Of note, $CD8^+$ T cells also underwent a parallel decline, so even when $CD4^+$ cell counts were lowest the CD4/CD8 ratios remained essentially unchanged. It is interesting that late during the follow-up this ratio actually showed a substantial improvement in some cats and that this occurred in the same animals who showed increased numbers of circulating FIV-specific IFN-γ-secreting cells as well.

In conclusion, the present study has shown that FIV-specific T-cell stimulation can be achieved ex vivo but that reinfusion of the in vitro-stimulated cells into their donors, in the absence of any concomitant antiviral drug treatment, produced no beneficial effects and was actually detrimental in ongoing FIV infections. However, the findings suggested that adoptive immunotherapy had exerted both favorable and harmful consequences on viral loads, although the latter had apparently been more important at least as judged from parallel effects on $CD4^+$-T-cell counts. It seems therefore possible that, if the recipients were concomitantly given antiviral drugs to prevent the stimulation of virus replication produced by the cells infusions, the favorable effects would have outweighed the detrimental ones. Further studies are clearly warranted.

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