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Prevention of immunodeficiency virus induced CD4+ T-cell depletion by prior infection with a non-pathogenic virus

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ABSTRACT

Immune dysregulation initiated by a profound loss of CD4+ T-cells is fundamental to HIV-induced pathogenesis. Infection of domestic cats with a non-pathogenic lentivirus prevalent in the puma (puma lentivirus, PLV or FIV_{PCO}) prevented peripheral blood CD4+ T-cell depletion caused by subsequent virulent FIV infection. Maintenance of this critical population was not associated with a significant decrease in FIV viremia, lending support to the hypothesis that direct viral cytopathic effect is not the primary cause of immunodeficiency. Although this approach was analogous to immunization with a modified live vaccine, correlates of immunity such as a serum-neutralizing antibody or virus-specific T-cell proliferative response were not found in protected animals. Differences in cytokine transcription profile, most notably in interferon gamma, were observed between the protected and unprotected groups. These data provide support for the importance of non-adaptive enhancement of the immune response in the prevention of CD4+ T-cell loss.

Introduction

Dysregulation of the immune response brought on by the loss of CD4+ T-cells is the initiator of progression to Acquired Immunodeficiency Syndrome (AIDS) following infection with human immunodeficiency virus (HIV) (Alimonti et al., 2003; Douek et al., 2003; Lackner and Veazy, 2007), virulent simian immunodeficiency virus (SIV) infection in Asian macaques (Lackner and Veazy, 2007; Viollet et al., 2006) and virulent feline immunodeficiency virus (FIV) infection in domestic cats (Bendinelli et al., 1995; Egberink and Horzinek, 1992). Prevention of CD4+ T-cell loss following HIV infection has not been achieved despite more than 20-years of vaccine research, although multi-drug therapy has proven successful in extending patient survival (Lima et al., 2007; Rogers et al., 2000). Reduction in viral load or preservation of CD4+ T-cells has been noted in a small percentage of animals in individual vaccine studies using SIV or simian/human virus hybrids in non-human primate models (Ahmed et al., 2002; Connor et al., 1998; Stolte-Leeb et al., 2006). Due in part to this variation in response these models have not yet revealed an obvious approach or an immune response to target in order to prevent infection or the development of AIDS (Davenport et al., 2007; Douek et al., 2003; Lackner and Veazy, 2007; McMichael, 2006). Further, the cause of the CD4+ T-cell depletion during HIV infection is controversial (Alimonti et al., 2003; Hel et al., 2006). Viral infection can directly cause the death of these cells (Alimonti et al., 2003); however following recognition that the rate of cell death far exceeds the infection rate, it has been postulated that the chronic nature of the immune response and induction of apoptosis in activated bystander cells are at least in part responsible for the disease state resulting from virulent HIV and SIV infections (Anderson et al., 1998; Douek et al., 2003; Hel et al., 2006), recently it has been recognized that even the chronic state of activation may not explain the kinetics of CD4+ T-Cell depletion (Yates et al., 2007).

FIV infection has many analogies to HIV infection; the virus has emerged relatively recently, targets similar cell types, and the disease state demonstrates a similar time course, clinical signs and outcome (Bendinelli et al., 1995; Overbaugh et al., 1997; Siebelink et al., 1990). Concurrently, FIVs which are endemic to the non-domestic cat population parallel SIVagm and other naturally occurring SIVs in that they are minimally pathogenic in their natural host (VandeWoude and Apetrei, 2006). Our previous work indicates that infection of domestic cats with a lentivirus native to the puma (FIV_{PCO}

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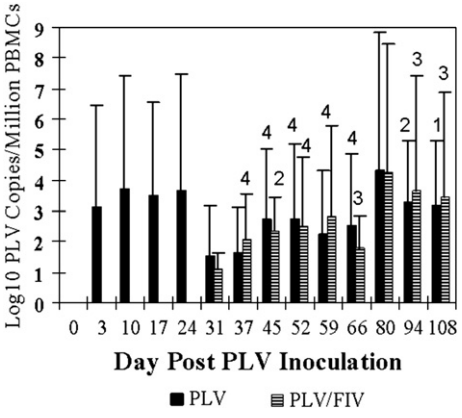

Fig. 1. Mean PLV viral copy number per 1 million PBMCs as measured by real time PCR in groups inoculated with PLV or medium only on day 0. Bars indicate the standard deviation. Five animals were inoculated with FIV on day 28. $N = 10$ for days 3, 10, 17 and 24 and $n = 5$ for day 31 and thereafter. A number less than 5 above the standard deviation bar indicates the number of animals that had copy numbers above the lower limit of detection. Calculation of means used values for only those animals with detectable virus.

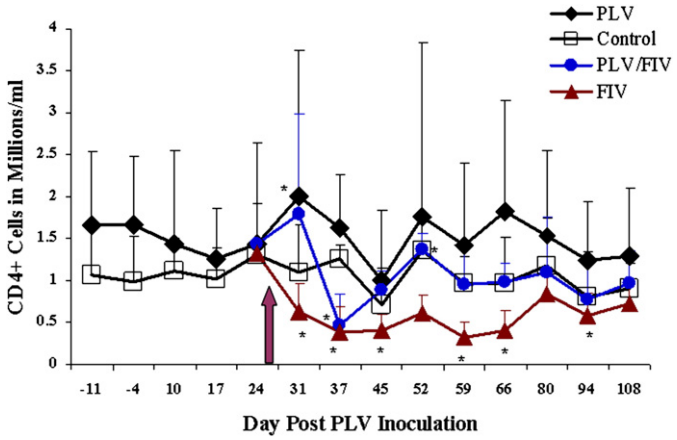

Fig. 2. Mean CD4-positive T-Cell counts in groups inoculated with PLV or medium only on day 0. Solid diamonds represent the PLV-inoculated group and open boxes represent the medium-inoculated controls. Arrow indicates inoculation of 5 animals from each group with FIV or diluent. Solid triangles represent the single-virus inoculated group (FIV only) and shaded circles represent the dual-virus inoculated group. Asterisks indicate a statistically significant difference ($p \leq 0.05$) from baseline values.

Strain PLV-1695, subsequently referred to as PLV) results in mild lymphadenopathy and only a slight, transient decrease in CD4+ T-cell count. PBMC viral burden gradually decreases over the course of several months of infection and is ultimately virtually eliminated by the host without evidence of strong adaptive immune activation. In late infection, proviral burden persisted in gastrointestinal vs lymphoid compartments (TerWee et al., 2005). In this study, domestic cats were inoculated with PLV or sham inoculum ($n = 10$/group). After infection was established, five animals from each group were inoculated with virulent FIV and monitored for clinical signs, peripheral blood cell counts, CD4+ and CD8+ T-Cell counts, cellular and plasma viral loads, humoral and cellular immune response, and PBMC cytokine expression. A highly pathogenic molecular clone of FIV subtype C which induces high viral loads and significant CD4+ T-Cell loss early after infection (De Rozières et al., 2004) was used as the challenge virus. Protection against CD4+ T-cell depletion was afforded by prior infection with a non-pathogenic lentivirus using the domestic cat model of immunodeficiency virus pathogenesis.

Results

PLV infection was uniformly detected by 3 days post inoculation

Fig. 1 demonstrates mean viral copy number in peripheral blood mononuclear cells (PBMC) from cats inoculated with PLV on day 0 as assessed by real time PCR specific for PLV-1695. Provirus was detected in all PLV-inoculated cats by 3 days after inoculation, while virus was not detected in sham-inoculated controls or animals only infected with FIV at any time point. Copy number peaked at an average of 1 copy per 500 cells at day 10 and then declined, becoming no longer regularly detectable in some individual animals beyond day 37. Cellular virus was still detectable in 4 animals at levels of 1 copy per 1000 cells to 1 copy per 100,000 cells at the last time point (day 108).

Prior PLV infection rescued CD4+ T-cell depletion following virulent FIV infection

Complete white blood cell counts with differentials were conducted to determine if the kinetics of CD4+ T-Cell depletion in FIV-C infection was altered by prior PLV infection. As shown in Fig. 2 and consistent with previous observations, CD4+ T-cell count was not significantly decreased by infection with PLV (study days 10, 17 and 24) and in fact there was a significant increase in CD4+ T-cell count in PLV-only inoculated animals at day 31. Lymphocyte and white blood cell (data not shown) counts were also unchanged following PLV inoculation. One-half of the PLV-infected animals and one-half of the sham-inoculated controls were inoculated with FIV on day 28 (indicated by an arrow). The PLV-negative, FIV-inoculated cats (solid triangle) demonstrated a marked decline in CD4+ T-cell count by 3 days after FIV inoculation which persisted for the course of the study. The differences attained statistical significance on days 3, 9, 17, 31, 38 and 66 post FIV (study days 31, 37, 45, 59, 66 and 94). This decline in CD4+ T-cell count was reflected in total lymphocyte count with a statistically significant decrease on days 31, 37, 45, 52 and 59

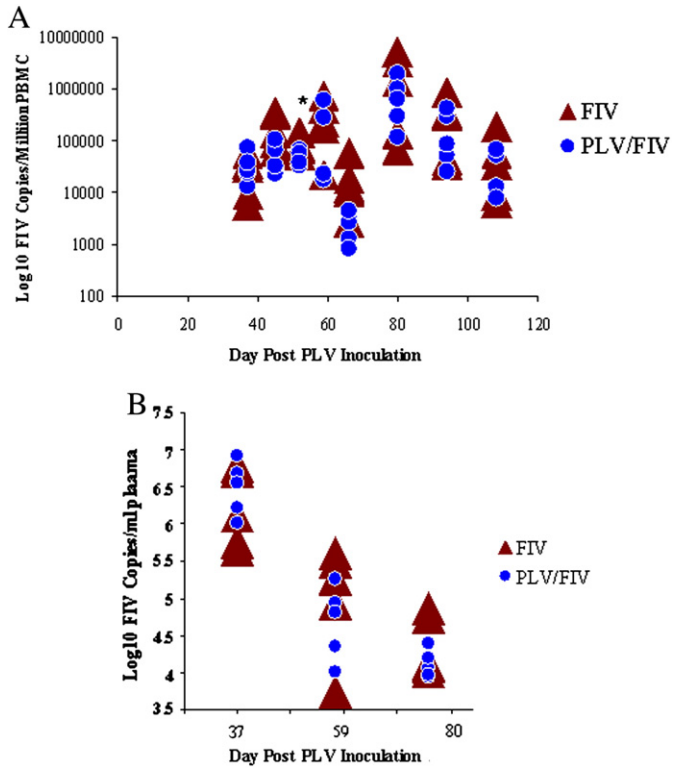

Fig. 3. FIV copy number per 1 million PBMCs (A) and FIV copy number per milliliter of plasma (B) as measured by PCR in individual animals inoculated with PLV on day 0 followed by FIV on day 28 (shaded circles) or medium only on day 0 followed by FIV on day 28 (solid triangles). Asterisk indicate the single time point at which the higher copy number in the FIV-only group were statistically significantly higher than in the group inoculated with PLV prior to FIV.

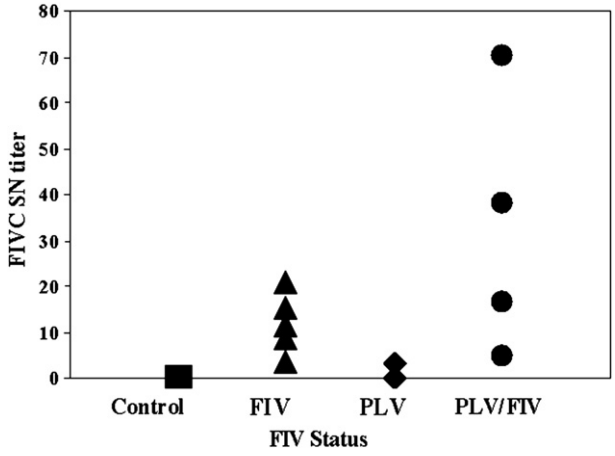

Fig. 4. Serum Neutralizing Antibody (SN) titer measured 59 days post PLV inoculation in individual animals inoculated with PLV on day 0 followed by FIV on day 28 (circles), medium only on day 0 followed by FIV on day 28 (triangles), PLV on day 0 followed by 0.9% NaCl on day 28 (diamonds) or medium only on day 0 followed by 0.9% NaCl on day 28 (squares). There was no statistically significant difference between groups.

(data not shown). In contrast, PLV-infected cats challenged with FIV (solid gray circles) showed a statistically significant decline in CD4+ T-cell count only at day 37 (9 days post FIV inoculation) and a decrease in lymphocyte count on days 37 and 45. Control animals also demonstrated a statistically significant increase in CD4+ T-cell count on study day 52. White blood cell count (data not shown) was significantly higher in PLV-only infected animals on study days 31 and 52, significantly lower in FIV-infected animals on study days 37, 59 and 66 and significantly lower in dual-infected animals on study days 59, 66 and 94. In summary, PLV infection did not result in CD4+ T-Cell depletion, and prevented the sustained loss brought about by FIV-C infection in all animals.

FIV PBMC proviral load is slightly diminished in cats previously inoculated with PLV

FIV-C in PBMCs and in plasma was measured by real time PCR to determine if prior PLV infection inhibited FIV infection. FIV was not detected in either group at day 31 (3 days after FIV inoculation). However, as shown in Fig. 3A, both PLV and sham-inoculated controls had a similar FIV copy number in PBMCs on day 37 (9 days after inoculation). FIV viral copy number in PBMCs was statistically significantly lower in PLV-inoculated animals only on study day 52. However there was a trend for FIV copy number to be lower in PLV-inoculated animals throughout the study, with p values of 0.071 and 0.100 on study days 45 and 66 respectively. FIV plasma viremia was measured on study days 37, 59 and 80. As shown in Fig. 3B, these results support the lack of overall reduction in FIV replication by previous PLV infection as there were no significant differences between the two groups at the time points examined. Sham inoculated animals, and those only infected with PLV, were FIV negative at all time points when evaluated by real time PCR (data not shown).

Protection from CD4 depletion does not correlate with acquired immunity

To determine if prior infection with PLV primed an antibody or T-Cell response to FIV, we examined SN antibody 3 weeks after FIV inoculation. As shown in Fig. 4, FIV SN antibody titer at day 59 (day 31 post FIV) was not significantly different in cats inoculated with FIV compared to those infected with PLV prior to FIV, indicating no anamnestic response to FIV following PLV infection. FIV SN titer ranged from 1:5 to 1:70 in PLV-infected animals and 1:3 to 1:20 in FIV-infected animals. Only 1 PLV single-virus-inoculated animal had a detectable SN response to FIV.

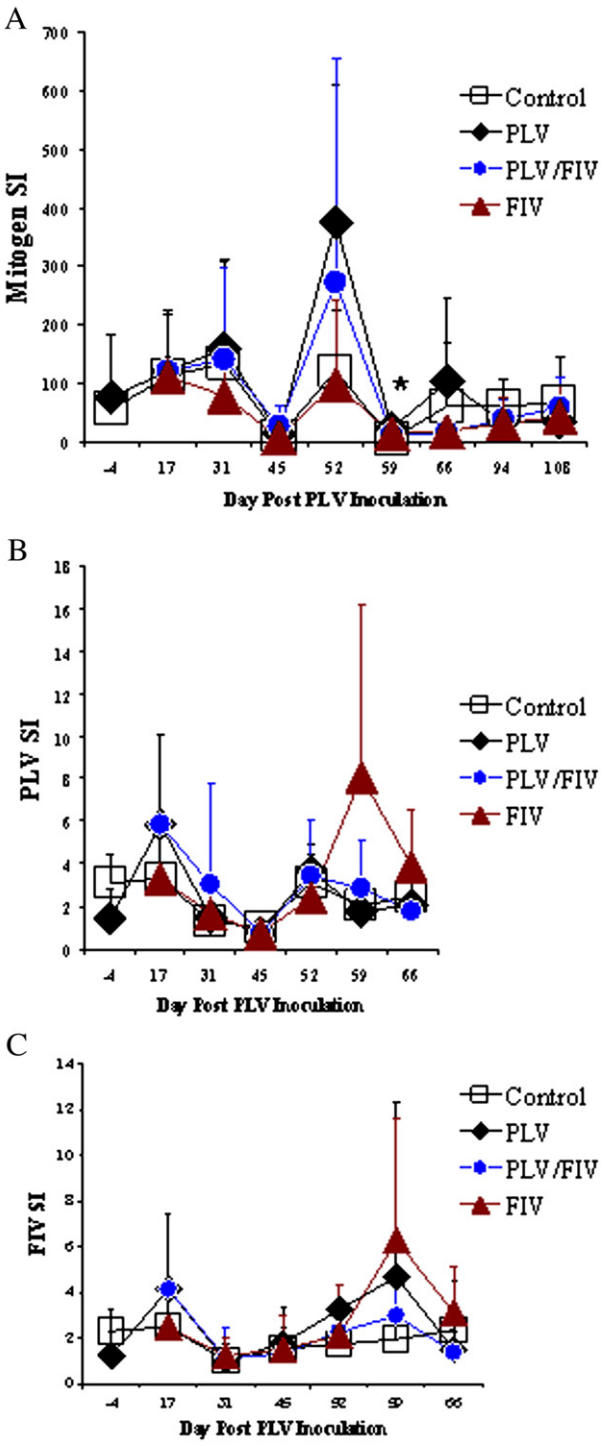

Fig. 5. Lymphocyte blastogenesis response to mitogen or antigen as measured by incorporation of ^{3}H thymidine. PBMCs were stimulated in triplicate with 1 μg of inactivated PLV or FIV for 4 days or with 1.5 μg Con A for 3 or 4 days prior to incubation in the presence of ^{3}H thymidine and harvest. Stimulation indices (SI) were calculated by dividing the average count for each agent by that of the average count for unstimulated cells. Group mean SI are shown for A) mitogen B) PLV or C) FIV in groups inoculated with PLV or medium only on day 0. Solid diamonds represent the PLV-inoculated group and open boxes represent the medium-inoculated controls. Five animals were inoculated with FIV or diluent on day 28. Solid triangles represent the single-virus inoculated group (FIV only) and shaded circles represent the dual-virus inoculated (PLV followed by FIV) group. As indicated by an asterisk, the only statistically significant difference was in Con A response on Day 59 in the PLV-only group (SI of 23) was compared to the control group (SI of 5).

Lymphocyte proliferative response to mitogen or antigen was determined at various study time points (Fig. 5). Although the group infected with FIV had the lowest Concanavalin A (Con A) response at most time points, there were no statistical differences as compared to controls in response in any group in proliferation to Con A (study days −4, 17, 31, 45 and 66). The group inoculated with FIV following PLV infection had a significantly higher Con A response as compared to controls on day 59 (Fig. 5A). PLV-inoculated animals had a higher proliferative response to both FIV and PLV on day 17, but this difference did not achieve statistical significance. Similarly, although animals inoculated with FIV had a high proliferative response to both FIV and PLV on study day 59, this difference did not achieve statistical significance.

CD4+ T-cell maintenance is associated with interferon gamma (IFN-γ) expression

To determine if protection was associated with a strong TH-1 or TH-2 cytokine response, cytokine mRNA (IL-10, IL-4, IL-12, TNF-α, and IFN-γ) was measured using real time PCR from bulk PBMC. In order to normalize for differences in measured cytokine expression between sample days, cytokine levels are expressed as fold increase over values at the same time point for controls. As shown in Fig. 6, cats inoculated with PLV had a statistically significant fold increase from controls in IFN-γ expression on study days 10, 17, 31, 37, 52 and 59. FIV inoculation was also followed by an increase in IFN-γ expression, but not until day 52, (24 days after FIV inoculation). Nine of 10 PLV-inoculated animals exhibited a 2-fold or greater (range=0.8–10.8, mean=4) increase in IFN-γ between days 3 and 31. PLV-inoculated animals thus had significantly higher IFN-γ levels not only at the time of FIV exposure, but for over 3 weeks following FIV inoculation. Though samples were not available from all animals for analysis at day 108, the trend was for IFN-γ mRNA levels to return to baseline for all infected groups (data not shown).

IL-10 (data not shown) was significantly elevated on study day 52 in the FIV-inoculated animals. IL-4 expression (data not shown) was slightly elevated in the groups receiving PLV from day 10 onward, achieving statistical significance on day 31. IL-4 was more variable in the FIV-only group, falling below the control levels on day 52 and above on days 31 and 59, but not significantly so. IL-12 expression (data not shown) was not statistically different from control levels in any group at any time point. TNF expression was also variable demonstrating a statistically significant difference only prior to and day 10 after PLV inoculation.

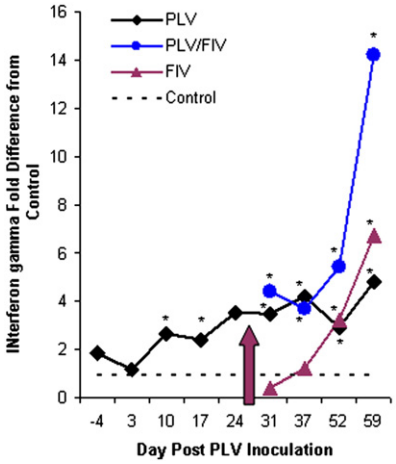

Fig. 6. IFN-γ expression was measured by real time PCR. Level of expression relative to that of the control group is shown as group means for groups inoculated with PLV or medium only on day 0. Solid diamonds represent the PLV-inoculated group. Arrow indicates inoculation of 5 animals from each group with FIV or diluent. Solid triangles represent the single-virus inoculated group (FIV only) and shaded circles represent the dual-virus inoculated (PLV followed by FIV) group. Asterisks indicate statistically significant differences ($p \leq 0.05$) from the control group. The number of samples tested for each group at each time point was 10 (before day 28) or 5 (after day 28) with the following exceptions: day 3 $n=8$ for control, day 10 $n=9$ for control, day 17 $n=8$ for control, day 31 $n=3$ for control and $n=4$ for FIV.

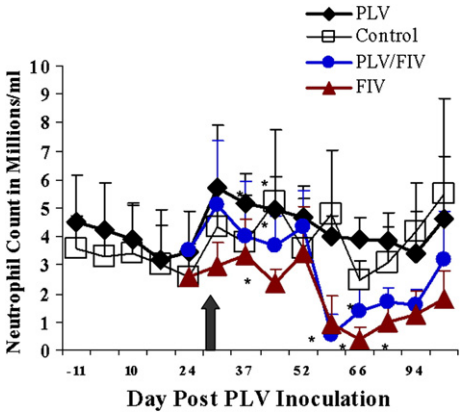

Fig. 7. Mean neutrophil counts in groups inoculated with PLV or medium only on day 0. Solid diamonds represent the PLV-inoculated group and open boxes represent the medium-inoculated controls. Arrow indicates inoculation of 5 animals from each group with FIV or diluent. Solid triangles represent the single-virus inoculated group (FIV only) and shaded circles represent the dual-virus inoculated (PLV followed by FIV) group. Asterisks indicate a statistically significant difference ($p \leq 0.05$) from baseline value.

PLV infection did not prevent neutropenia subsequent to infection with FIV

Neutrophil counts declined precipitously in FIV-inoculated groups with or without prior PLV inoculation (Fig. 7). Neutropenia was significant relative to baseline in PLV-infected, FIV-inoculated animals on days 59 and 66 and in FIV-infected animals on days 66 and 80. The diminished neutrophil count in these groups coincides with a statistically significant decrease in WBC count on days 59 and 66 for the PLV/FIV group and on day 66 for the FIV-only group (data not shown). The control group demonstrated a significant neutrophilia on day 45 while PLV-only animals experienced neutrophilia relative to baseline on post-inoculation days 37 and 45.

Clinical signs were unremarkable

Mild lymphadenopathy was noted following PLV inoculation. FIV inoculation resulted in mild to moderate lymphadenopathy in both PLV-inoculated and sham-inoculated animals.

Discussion

Previous infection with a non-pathogenic lentivirus protected against the significant and prolonged peripheral blood CD4+ T-cell depletion that occurs following inoculation with virulent FIV, consistent with results of previous studies (VandeWoude et al., 2002). Preservation of CD4+ lymphocytes did not correspond to an immediate or sustained significant decrease in peripheral viral load as measured by viremia or intracellular (PBMC) virus and was not dependent upon a high PBMC PLV viral load at the time of FIV inoculation.

This disconnect between pathogenic viral load and CD4+ T-cell depletion is consistent with results for the natural course of HIV infection, in which it has been shown that CD4+ T-cell decline cannot be predicted by viral load in untreated HIV infection (Rodŕiguez et al., 2006); further, although infection with viral strains such as subtype D results in a faster rate of CD4+ T-cell decline and more rapid progression of disease, set-point as well as early viral loads are equivalent

to those in infection with less pathogenic strains (Baeten et al., 2007). Similarly, in non-pathogenic SIV infection, consistently high viral loads are not associated with peripheral blood CD4+ T-cell loss or with the development of AIDS (Broussard et al., 2001; Chakrabarti, 2004; Rey-Cuille et al., 1998). Additionally, HIV-infected patients undergoing treatment with anti-retroviral agents typically experience a reduction of viral load to the limit of detection without complete recovery of CD4+ T-cells (Goicoechea et al., 2006). Data from other studies suggest a correlation between viral load and CD4+ T-cell loss (Goldstein et al., 2005; Mellors et al., 1997), and undeniably viral infection initiates the destruction of this cell population. It is therefore evident that there are factors yet to be discovered to explain the complex association of viral load with CD4+ T-Cell loss.

As might be predicted from our previous observations that PLV does not induce a vigorous immune response during domestic cat infection (TerWee et al., 2005), and that the homology between FIV and PLV *env* is low (Smirnova et al., 2005), prevention of CD4+ cell loss in this feline model was not linked to a cross-neutralizing immune response to FIV in PLV-infected cats. An anamnestic virus neutralizing antibody response was not observed after FIV inoculation in the animals infected with PLV, and enhanced lymphocyte proliferation to virus-specific antigen was not detected. Although additional measures may reveal a role for adaptive immunity in the mechanism of protection against lentivirus-induced immunodeficiency, our results are consistent with those reported in many HIV, SIV and FIV studies that fail to show a clear cause-and-effect relation between protection and a robust immune response (Amara et al., 2005; Hosie et al., 1988; Langlois et al., 1998; Singh et al., 2005; Stebbings et al., 2002). In a recent study, non-progressors and patients treated with HAART actually had lower neutralizing antibody responses to autologous virus than untreated viremic patients, indicating that the viral replication was driving the antibody response rather than being controlled by it (Bailey et al., 2006). This has also been documented in SIV; an antibody response was detected after challenge exposure in non-vaccinated groups and in vaccinated progressors, but not in the vaccinated and protected non-progressors (Kawada et al., 2007). Others however have noted a correlation between induction of a post-vaccinal neutralizing antibody response against the challenge strain and reduction in viral replication (Quinnan et al., 2005), a correlation between antibody avidity and rate of disease progression (Korthels Altes et al., 2006), and greater loss of CD4+ T-cells and increased viral replication after experimental B-cell depletion (Miller et al., 2007), indicating that an antibody response may be effective if it reaches a certain threshold of quality and quantity. In light of the envelope diversity of circulating strains of HIV, the goal of many vaccine research programs is a cytotoxic T-cell response (Davenport et al., 2007; McMichael, 2006). Disparate results for the effectiveness of this type of immune response are also evident. Although depletion of CD8+ T-cells following SIV infection has been shown to cause an increase in virus replication (Schmitz et al., 2005), vaccines which induce high levels of CD8+ T-cells have not been able to prevent infection or disease and CD4+ T-cell loss (McMichael, 2006). Even though there is an overall reduction in chronic viral replication, CTL escape mutants emerge (Loffredo et al., 2007; Mandl et al., 2007) and an effective response is achieved too late (Davenport et al., 2007).

In contrast to the lack of correlation between adaptive immune response induction by PLV and down-regulation of PLV or protection against FIV challenge, we have documented changes in recombination rates, genomic selection, and mutation rate in PLV genomes following passage through domestic cats, notable at key residues in *pol* and signified by G to A conversion (Poss et al., 2006; Poss et al., 2007). This accumulated evidence strongly supports innate and intracellular antiretroviral defenses are enhanced in the face of PLV infection in domestic cats—supporting these host defenses as primary mechanisms preventing pathogen infection of non-target species.

Prevention of superinfection is documented as a means by which lentiviruses increase fidelity. HIV downregulates the expression of its binding receptor, CD4, during its replicative cycle (Lama, 2003; Lindwasser et al., 2007). Although we have observed reduced FIV replication after PLV inoculation *in vitro* (VandeWoude et al., 2002), PLV and FIV appear to use different receptors (Smirnova et al., 2005). Additionally, since the number of PLV-infected cells was low at the time of FIV inoculation a global antiviral state induced by PLV infection seems a more likely explanation for our observations than a direct block to superinfection. In this context the 'non-adapted' pathogen is prevented from replication and/or successful infection because the new host has molecular machinery able to interfere with the lentiviral lifecycle. It is interesting to speculate that these non-specific innate factors, activated during PLV infection because of host:pathogen discordance, modify the host environment to partially cripple subsequent virulent FIV infection. While we did not see an overall significant reduction in FIV viral load in circulation, it is possible that the viruses being produced were less fit due to residual intracellular restriction mechanisms induced by PLV infection. Evaluation of FIV genomes for evidence of hypermutation and recombination will determine this possibility.

It is also plausible that replication of virulent FIV was abrogated at key sites of early replication other than PBMCs. Although high levels of FIV are found in bone marrow (Beebe et al., 1992; Dua et al., 1994; S. Troth, unpublished data), we have previously shown that PLV cannot be detected in samples of bone marrow harvested 6 months after PLV inoculation; however, proviral is maintained in gastrointestinal tract and associated lymph nodes (TerWee et al., 2005). Lack of co-localization of the two viruses in bone marrow may explain the absence of protection from neutropenia in dual-virus infected animals; further, it is possible that FIV replication in FIV-PLV dual-infected cats is limited in sites such as the gastrointestinal tract due to primary interference with PLV, or redistribution of FIV target cells, preserving CD4+ T-cell populations that ultimately circulate peripherally. Quantitation of both PLV and FIV in lymphoid and gastrointestinal tissues during single and dual infection will provide informative clues about mechanisms underlying CD4+ T-Cell loss in FIV-infected animals.

IFN-γ expression was increased in PBMC by both pathogenic and non-pathogenic virus exposure, however animals protected from CD4+ T-cell lymphopenia had a higher level of IFN-γ mRNA prior to exposure to virulent virus. The role of IFN-γ in protection from feline or human AIDS is not clear. Vaccination with a DNA vaccine expressing IFN-γ has been shown not to reduce viremia following exposure to FIV (Gupta et al., 2007). However, effect on CD4+ T-Cell count was not determined since this challenge virus did not induce measurable CD4 +T-cell loss. In HIV-infected individuals, CD4+ T-cell decline was not found to correlate with HIV-specific IFN-γ secretion (Peretz et al., 2005) and interferon gamma is at higher levels in pathogenic cross-species SIV infection than in non-pathogenic infection, indicating that an activated immune system is prognostic of pathogenesis (Kornfeld et al., 2005). Conversely, other studies support our results of an association between IFN-γ and diminished pathogenesis. Vaccine-induced antigen-specific IFN-γ secretion has been shown to correlate with survival, protection and reduced viral loads following challenge with SIV or SHIV (Abel et al., 2003; Boyer et al., 2006; Sun et al., 2006). Given that others have observed induction of cytidine deaminase by treatment with IFN-γ *in vitro* (Jost et al., 2007) it is possible that the protection we observed in conjunction with increased IFN-γ is related to synergy between innate pathways of altered cytokine expression and intracellular restriction in individuals exposed to certain pathogens.

The feline model provides a well controlled system to examine pathogenesis, protection and early effects of infection with viruses which induce immunodeficiency. Prior infection with a minimally pathogenic virus from a related species provided solid protection from

CD4+ T-cell loss. Our data lend support to the body of evidence that decouples viral replication and peripheral blood CD4+ T-cell loss. Our data also support the lack of either an antibody or cell mediated immune response in the responsibility for maintenance of this population of cells. Further studies dissecting potential mechanisms for our observations will provide fundamental knowledge toward the understanding of the pathogenesis of AIDS.

Materials and methods

Viral stocks

Table 1 describes the viral stocks used in this study. The MYA-1 cell line is of domestic cat PBMC origin (Miyazawa et al., 1989). PLV was isolated from a co-culture of puma and domestic cat PBMCs and was expanded in MYA-1 cells. FIV-C was recovered from the retropharyngeal lymph node of a cat inoculated with a molecularly cloned FIV-C by culture with MYA-1 cells. Reverse transcriptase (RT) activity of culture supernatants was monitored and virus was harvested by slow speed (200–500 ×g) centrifugation using a Beckman GPR centrifuge with a GH 37 rotor (Beckman Coulter, Fullerton, CA) to remove cell debris at peak RT activity. The sham inoculum (medium) was similarly prepared from culture supernatant of un-infected MYA-1 cells.

Animals

Twenty specific-pathogen-free (SPF) cats were obtained from a breeding colony at Colorado State University. Animals were randomized by litter and gender and were housed in groups of 5 in pens in isolation rooms in an AAALAC-international accredited animal facility. All procedures were approved by the CSU Institutional Animal Care and Use Committee prior to initiation.

Study design

Blood samples were obtained by venipuncture of the jugular or cephalic vein on study days −11, −4, 3, 10, 17, 24, 31, 37, 45, 52, 59, 66, 80, 94, and 108 (relative to PLV exposure). On Day 0, ten 14–22 week-old cats were inoculated IV with 1 ml of PLV while the remaining 10 cats received culture supernatant from un-infected MYA-1 cells IV. Twenty-eight days later, 5 of the PLV-inoculated animals and 5 of the sham-inoculated controls received 1 ml of FIV stock which had been diluted 1:100 in 0.9% NaCL solution IV and the remaining animals were inoculated by the same route with 0.9% saline. Thus study termination was 108 days post PLV inoculation and 80 days post FIV challenge.

Clinical signs

Animals were observed for clinical signs at least daily throughout the study. Lymph nodes were palpated at least weekly. Body weights were measured weekly from 4 weeks prior to until 12 weeks post PLV exposure.

Viral copy number

A real time PCR standard curve was generated from serial dilutions of feline PBMC from 1000 to 5×10^6 subjected to real time PCR for the cellular house-keeping gene, Glyceraldehyde-3-Phosphate Dehydrogenase (GADPH) as described by Leutenegger et al. (1999). Peripheral blood mononuclear cells (PBMC) from study animals were purified from heparinized whole blood samples from experimental animals using a Histopaque (Sigma, St. Louis, MO) gradient according to the product insert. DNA was extracted from 1 million PBMCs using the Qiamp blood mini DNA kit (Qiagen, Valencia, CA). DNA from each sample was eluted with 50–200 µl of buffer and PLV in 5 µl of sample was quantitated in triplicate using real time PCR with plasmid encoded PLV as a positive control. Primers and probe were designed for the *pol* region of PLV 1695. This assay has a less than 3% within and between sample variance and is sensitive to a minimum of 10 copies. The difference in amplification efficiency for plasmid vs sample DNA was less than 1% (Sondgeroth et al., 2005). Primers and probe for FIV-C gag (Pedersen et al., 2001) were used to quantitate FIV in PBMCs and plasma. The sensitivity of detection is a minimum of 5 copies. Due to substantial heterogeneity between FIV-C and PLV 1695, primers and probes are specific for each virus and do not cross-amplify (reported in results). DNA samples were also subjected to GAPDH real time analysis and values normalized to GAPDH standard curve to determine the number of cell equivalents per DNA sample; proviral copy number per cell was calculated on this basis.

To determine the number of viral copies in plasma, RNA in 140 µl of EDTA anti-coagulated plasma was purified using the Qiamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Following treatment with DNase (DNA-free, Ambion, Austin, TX), RNA was transcribed to DNA using Superscript II (Invitrogen) and then was quantitated using real time PCR. The standard curve was generated using dilutions of a viral stock which was quantitiated against a DNA plasmid standard curve, diluted in negative plasma and then extracted using the same procedure as samples. All samples collected on a given study day were prepared and tested together.

Cytokines

Cytokines were quantitated by real time PCR using the method of Leutenegger et al. (1999). Five to ten million PBMCs (purified as above) were dissolved in Trizol (Sigma, St. Louis, MO) at 10 million cells/ml. cDNA was reverse transcribed from RNA purified by extracted phenol: chloroform extraction and ethanol precipitation. Cytokine expression for IL-10, IL12p40 and interferon gamma (IFN-γ) was quantitated relative to that of the gene, GAPDH using the formula $2^{-\Delta CT}$, where ΔCT represents the cycle at which threshold is reached for the GAPDH is subtracted from the cycle at which threshold is reached for the

Table 1
Description of virus stocks

Stock	$TCID_{50}$/ml[a]	Days in culture[b]	Copies/ml[c]	RT activity[d]	Passage history	In vivo infectivity
PLV-1695	$10^{4.7}$	12	5×10^6	5847	Puma PBMC co-cultivated with domestic cat cells	1 ml infected 14/14 cats IV and 4/4 ON
FIV-C	$10^{7.2}$	17	Not done	19628	Retropharyngeal lymph node from cat inoculated with molecular clone cultured with domestic cat cells	1 ml of 1:100 dilution infected 11/11 cats IV and 2/2 ON

[a] Titer calculated on MYA-1 cells.
[b] Days in culture for final passage.
[c] Calculated by real time PCR.
[d] Calculated by micro-titer reverse transcriptase assay.

cytokine. All samples collected on a given study day were prepared and tested together.

Hematology

Total white and red blood cell counts were measured using a Coulter Z1 (Coulter, Miami, FL). Differential counts were performed manually and the percentages of lymphocytes positive for CD4, CD8 and CD25 were determined by flow cytometry using monoclonal antibodies to feline CD4 and CD8 (Southern Biotech, Birmingham, AL). Two to five $\times 10^5$ PBMCs were incubated for 20–60 min at room temperature in monoclonal antibody at 5 μg/ml in flow buffer (PBS containing 2% FBS and 0.2% sodium azide). Cells were then washed twice in flow buffer, resuspended in 100 μl of fluorescein-labeled sheep anti-mouse IgG (Sigma, St. Louis, MO) at 10 μg/ml in flow buffer and incubated for 20–60 min at room temperature in the dark. Cells were washed once in flow buffer and then analyzed with a Coulter EPICS XL MCL flow cytometer (Beckman Coulter, Miami, FL). List mode files were analyzed using FlowJo (Tree Star Inc., San Carlos, CA). Total cell counts for each phenotype were calculated by multiplying the total white blood cell count by the percentage of lymphocytes in the sample as determined by the differential count and then by the percentage of lymphocytes expressing that phenotype.

Virus-neutralizing antibody (VN)

VN titer was determined by adding a constant amount of virus to serial two fold dilutions of plasma, starting at a 1:5 final dilution. Following a 90-minute incubation at 37 °C with 5% CO_2, 100 μl of the virus/plasma mixture was added in triplicate to MYA-1 cells seeded at 1×10^5 cells/well in 96 well plates. The virus inoculum was titrated in triplicate using 10 fold serial dilutions. Virus was detected by reverse transcriptase assay after 14 days incubation at 37 °C with 5% CO_2 and titers of virus inoculum and antibody were calculated using the Spearman–Kärber method (Blake and O'Connell, 1993).

Lymphocyte blastogenesis assay

One-hundred thousand PBMCs were incubated in triplicate with medium alone, 1 μg of PLV or FIV inactivated using AT-2 (Rossio et al., 1998) and 1.5 μg of Concanavalin A (Con A). ^{3}H at 1 μCi/well was then added 3 (Con A days −4, 17, 37, 45 and 52, 94 and 108) or 4 (antigen, PMA, and days 59 and 66 for Con A) days later and the cells were harvested onto filter paper using a 96 well cell harvester (Wallac, Turku, Finland) 20 h later. Stimulation indices were calculated by dividing the count for each sample by the count with medium alone.

Statistical analysis

Repeated-measures analysis of variance (ANOVA) 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 the Student *t* test or Student *t* test for paired data as appropriate. All analyses were performed using Microsoft Excel (Microsoft Corporation, Redmond WA.)

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