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CD8⁺ clonality is associated with prolonged acute plasma viremia and altered mRNA cytokine profiles during the course of Feline Immunodeficiency Virus infection

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Abstract

Acute lentiviral infection is characterized by early CD8⁺ cytotoxic T cell (CTL) activity and a subsequent decline in plasma viremia. However, CD8⁺ lymphocytes fail to eliminate the virus and a progressive T cell immune dysfunction develops during the course of chronic lentiviral infection. To further define this CD8⁺ immune dysfunction we utilized PARR (PCR for Antigen Receptor Rearrangements), a technique which measures clonally expanded lymphocyte populations by comparison of highly conserved T cell receptor (TCR) regions to identify the prevalence of clonal CD8⁺ T cells following FIV infection. We then compared phenotype, mRNA profiles, CD8⁺ proliferation and plasma viremia during acute and chronic infection for PARR positive (PARR⁺) and PARR negative (PARR⁻) Feline Immunodeficiency virus (FIV) infected cats. We demonstrated that approximately forty percent of the FIV⁺ cats examined exhibit CD8⁺ clonality compared to none of the FIV⁻ control cats. There were no phenotypic differences between PARR⁺ and PARR⁻ CD8⁺ lymphocytes from FIV⁺ cats but retrospective analysis of plasma viremia over the course of infection revealed a delayed peak in plasma viremia and a decline in lymphocyte counts were observed in the PARR⁺ group during acute infection. CD8⁺ lymphocytes isolated from chronically infected PARR⁻ cats exhibited significantly higher mRNA expression of IFN- γ and IL-2 following mitogenic stimulation when compared to PARR⁺ CD8⁺ lymphocytes. These data suggest that clonal CD8⁺ expansion may be related to impaired control of acute viremia and less effective CD8⁺ anti-viral function. Using PARR to assess changes in CD8⁺ clonality during the progression from acute to chronic FIV infection may help to better characterize the factors which contribute to CD8⁺ anergy and lentiviral persistence.

Keywords

FIV; AIDS; CTL; CD8⁺ clonality; PARR

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Introduction

During acute viral infection, T cells undergo proliferation and acquisition of effector function after appropriate TCR stimulation and co-stimulation (Obar et al., 2008). The development of lymphocyte responses under these conditions can be loosely divided into three phases: expansion (with resolution of the infection), contraction, and the development of stable memory lymphocytes (Baumgartner and Malherbe, 2011; Seder and Ahmed, 2003). The local cytokine environment established by the innate immune response favors rapid expansion and activation of antigen-specific CD8⁺ and CD4⁺ T cells. Activated CD8⁺ T cells then disseminate to tissues and target infected cells for killing. As the primary infection is controlled, CD8⁺ lymphocytes undergo apoptosis with contraction of CD8⁺ cell numbers and persistence of a small population of memory cells (Kaeche and Wherry, 2007). The selection and expansion of specific CD8⁺ T cell clones during infection can be monitored by enumeration of CD8⁺ T cells with unique TCR specificities. As reliable feline tetramers still do not exist for use in the feline lentivirus model, we examined T cell receptor diversity in FIV⁻ control cats and in chronically FIV-infected cats using PARR. TCR gene rearrangement is a complex process which results in the generation of a diverse T cell repertoire. Immature T cells rearrange their TCR α , TCR β , TCR γ and TCR δ genes in the thymus to generate $\alpha\beta$ or $\gamma\delta$ T cell TCRs. During this process, most $\alpha\beta$ T cells rearrange their TCR γ segment prior to TCR α and TCR β rearrangement (Theodorou et al., 1994). Taking advantage of this, Moore et al described highly conserved regions within TCR γ which can be targeted in PCR amplification of TCR gene products for the identification of clonal T cell populations during feline intestinal lymphoma (Moore et al., 2005). Weiss et al (Weiss et al., 2008) further refined this technique to describe specific sequences within the feline TCR γ gene and allow detection of specific CD8⁺ T cell clones with identical TCR γ regions. Targeting these CD8⁺ T cell TCR regions then allows for the observation of CD8⁺ T cell dynamics during acute infection.

AIDS lentivirus infections are characterized by an early CD8⁺ T cell lymphocytosis which is concurrent with a decline in plasma viremia; however, recent evidence suggests it is the quality of the CD8⁺ response, not the magnitude, which is responsible for the clearance of virus from circulation while a substandard or absent cytolytic response has been associated with insufficient control of viral replication (Betts et al., 2006; Borrow et al., 1994; Bouscarat et al., 1996; Bucci et al., 1998; De Maria et al., 1994; Koup et al., 1994; Rowland-Jones et al., 1993). Importantly, HIV studies indicate that even in the face of a robust CD8⁺ response there are both viral and host factors that allow the virus to escape elimination and establish chronic infection (Fryer et al., 2010; Lee et al., 2002; Price et al., 1997). As such, emphasis has been placed on understanding what factors contribute to the quality of the CD8⁺ response which may dictate virus set point and disease progression or non-progression. Using the murine LCMV model of infection, Sevilla et al (Sevilla et al., 2000) and Brooks et al (Brooks et al., 2006) have defined subtle changes in the cytokine environment that can alter CD8⁺ clonal responses and ultimately lead to CD8⁺ T cell exhaustion. The LCMV variant Armstrong (Arm) induces a vigorous T lymphocyte response with viral clearance in 8–10 days. Infection with the Arm-derived LCMV Clone 13 (CI 13) which differs by a single amino acid, promotes dendritic cell IL-10 production resulting in a persistent infection (Brooks et al., 2006; Sevilla et al., 2000). These results suggest that early immunosuppression can lead to subversion of the CD8⁺ immune response, particularly in the CD8⁺ effector memory pool, despite initial vigorous CD8⁺ antiviral activity. The results in the murine LCMV model offer some clues to what might be occurring in AIDS lentiviral models. Particularly, early immune dysfunction has been shown to correlate with changes in CD8⁺ immune responses during chronic infection for HIV and SIV (Hong et al., 2009; Kelleher and Rowland-Jones, 2000; McMichael et al., 2000; Tjernlund et al., 2010; Wherry et al., 2006). In agreement with this, evaluation of

lymphocyte phenotypic changes during FIV lentiviral infection in cats indicates that CD8⁺ lymphocytes follow a pathway directed toward anergy (Fogle et al., 2010a). Taken together, these data suggest that many of the events that shape the CD8⁺ immune responses occur during acute lentivirus infection and have lasting effects upon the CD8⁺ repertoire, leading us to ask if early immune dysfunction in FIV-infected cats can be observed by alteration in CD8⁺ T cell clonal expansion. As such, we employed PARR analysis to identify the existence of a clonal expansion of CD8⁺ T cells in FIV⁺ or FIV⁻ cats. We asked whether FIV⁺ cats exhibited clonality and determined if this correlated with differences in CD8⁺ T cell phenotype and function when compared to CD8⁺ cells from PARR⁻ FIV⁺ cats.

Here, we identified clonal populations of CD8⁺ T cells during chronic lentiviral infection. We demonstrated that 37% of the chronic FIV⁺ cats in our study exhibited CD8⁺ clonality as compared to none of the FIV⁻ control cats. We hypothesized that PARR⁺ FIV⁺ cats would differ from PARR⁻ FIV⁺ cats in CTL responses and viral loads, which would ultimately contribute to differences in disease progression. We therefore prospectively evaluated CD8⁺ lymphocytes from PARR⁺ or PARR⁻ cats during chronic infection for any phenotypic or functional differences. We assessed plasma viremia retrospectively during acute infection and retrospectively during chronic infection and evaluated lymphocyte numbers and composition during these times as well. PARR analysis for evaluation of CD8 TCR diversity thus represents a novel tool for understanding T cell responses during lentiviral infection. The presence of clonal CD8⁺ lymphocyte populations in only some of the FIV⁺ cats evaluated here may highlight key differences in early immune responses that shape the outcome of infection.

Materials and Methods

Cats

Specific pathogen-free (SPF) male cats were obtained from Liberty Research, Inc. (Waverly, NY) at 6 months of age, and housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. FIV-infected cats were housed separately from uninfected control cats. FIV⁺ cats were infected shortly after arrival (7 months of age) with 5×10^5 TCID₅₀ of cell-free virus culture prepared as previously described (Davidson et al., 1993). Chronically infected cats were characterized as having been FIV-infected for 1–3 years. Several of the cats were euthanized for unrelated studies during the course of the reported experiments. The number of PARR⁺ or PARR⁻ cats used per experiment is reported in table 1.

Blood and lymph node cell collection

Whole blood was collected by jugular venipuncture into EDTA container tubes. Plasma was separated and frozen for analysis of viral load by RT-PCR. For surface phenotyping, blood was incubated with the indicated antibodies, washed with sterile PBS and red blood cells removed by whole blood lysis prior to analysis. Peripheral blood mononuclear cells (PBMCs) used for CFSE proliferation assays were isolated by Percoll (Sigma-Aldrich, St-Louis, MO, USA) density gradient centrifugation as previously described (Tompkins et al., 1987) and PBMCs for cytokine PCR analysis were isolated by Ficoll-Histopaque-1077 (Sigma-Aldrich, St-Louis, MO, USA) following the manufacturer's guidelines for immediate FACS purification of the CD8⁺ population. Peripheral lymph node (PLN) cells were obtained by lymph node biopsies and single cell suspensions were prepared by gently and repeatedly injecting sterile PBS into the tissue with an 18 gauge needle to release cells. After isolation, cell counts and viability were obtained using Trypan Blue dye exclusion. Cell viability was >90% in all experiments.

PARR analysis

PARR has been well described for the cat by Moore et al (Moore et al., 2005) and purified CD8⁺ T cells were evaluated by following this protocol with PCR primers as listed in Table 1. Cats used for this study were 2–4 years old at the time of PARR testing and the FIV+ subjects were classified as chronically infected as they had been infected for 1–3 years at time of sample collection. No cats confirmed to be PARR negative were shown to become PARR positive at a later time. PBMCs or PLN cells were isolated as described then FACS sorted into the CD8⁺ population by positive selection. PARR analysis was performed by a single investigator (SS) with extensive experience who was blinded to FIV infection status of the CD8⁺ samples.

Flow cytometry analysis

At least 5×10^5 PBMCs or PLN cells were stained for surface expression of various markers using specific antibodies. CD8 α or β chains were detected using mAb 117 (anti-CD8 β chain) and mAb 357 (anti-CD8 α chain) described elsewhere (Bucci et al., 1998). Phycoerythrin (PE)-conjugated anti-TGF β R2 (FAB241P) was purchased from R&D. Anti-human CD49d and CD62L mAbs (BD Biosciences, San Jose, CA, USA) used in this study were previously found to be cross-reactive with feline lymphocytes (Gebhard et al., 1999). Three- or four-color flow cytometry was performed on a FACS Caliber flow cytometer (BD Biosciences, San Jose, CA, USA). Each antibody combination was analyzed as duplicate samples. Lymphocytes were gated based on forward versus side scatter. For analysis of CD8 subsets, lymphocytes were gated based on CD8 α expression and then further gated into CD8 β ^{lo} or CD8 β ^{hi} populations for differential detection of TGF β R2, CD62L and CD49d. Gated events were analyzed using CellQuest software and all gates were determined by isotype controls. For FACS purification of CD8 cells used for in vitro assays, PBMCs were stained using MAb 357 to purify CD8 α ⁺ lymphocytes by positive selection. At the time of analysis, 6 PARR⁺ and 6 PARR⁻ FIV-infected cats were available for surface phenotyping as several cats had been sacrificed for unrelated studies.

Plasma Viremia

Plasma viremia was assessed on each plasma sample using quantitative real-time PCR to determine virus gag-mRNA loads. Primers are listed in Table 2 and have been described elsewhere (Frey et al., 2001). Briefly, 1 ml of plasma was used to extract viral RNA using Qiagen's QIAamp Ultrasense Virus Isolation kits. 10 μ l of the isolated viral RNA was reverse transcribed in a separate reaction using Promega's Reverse Transcription System with random primers. This reaction was followed by a real-time PCR step using specific primers for FIV-gag-mRNA as listed in Table 2. SYBR Green Taqman PCR master mix (Life Technologies Corporation, Carlsbad, CA, USA) was used for quantification according to the manufacturer's guidelines. The reactions were run in duplicate in 96-well plates and incubated at 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min, before returning to 25 °C. A standard curve was run in each reaction using serial dilutions of previously sequenced and quantified FIV-gag-mRNA. The standard curve was used to determine absolute viral mRNA copy numbers per ml of plasma. At the time of this experiment, samples from only 5 PARR⁺ and 5 PARR⁻ FIV-infected cats had been stored for subsequent PCR analysis.

Cell culture and stimulation

Unfractionated PBMCs or purified CD8⁺ T cell subsets were cultured in RPMI medium containing 10% heat inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine and 1 mM HEPES buffer. All cells were cultured at

2×10^6 total cells per mL of culture media. For ConA stimulation, 5 μ g/mL ConA and 100 U/mL rhIL-2 were added to the media for the indicated length of time.

Detection of Cytokines by Real time PCR

FACS purified populations of CD8⁺ PBMCs were either left unstimulated or stimulated in vitro with ConA for 24 hours and used for PCR analysis. Previously described primers for the relative quantification of IL-2 (Fogle et al., 2010b), TNF- α (Avery and Hoover, 2004), IFN- γ (Fogle et al., 2010a) and GAPDH (Fogle et al., 2010b) mRNA by reverse transcription and real-time PCR are listed in Table 2. At least 10^6 cells were used for each experimental group. Total RNA extraction was carried out using Qiagen's RNeasy plus mini kits and eluted in a final volume of 20 μ l per reaction. All of the RNA obtained was used in reverse transcription reactions using Oligo(dT) primers and the Promega Reverse transcription system according to the manufacturer's instructions. Products were assessed in triplicate for the specific mRNA of interest in separate reactions and relative expression was calculated using the $2^{-\Delta\Delta CT}$ method with GAPDH as the control gene (Livak, 2001). Primers used for real-time RT-PCR are displayed in table 2. At time of analysis, 6 PARR⁺ and 6 PARR⁻ cats were available as several cats had been sacrificed for unrelated studies. During statistical analysis, 1 PARR⁺ and 1 PARR⁻ cat were determined to be outliers by statistical evaluation as the values were greater than 4 times the standard deviation from the mean of the remaining samples and thus excluded from the analysis.

Proliferation Assay

Lymphocytes were isolated from blood by Percoll density gradient centrifugation and labeled with CFSE using the CellTrace CFSE Proliferation Kit (Life Technologies Corporation, Carlsbad, CA, USA) according to manufacturer's instructions. Cells were washed and cultured in fresh medium and left unstimulated or stimulated with ConA for 72 hours, labeled with CD8 MAb 357 and analyzed by flow cytometry. Proliferation was analyzed using ModFit *LT* software. At the time of analysis, only 4 PARR⁺ and 6 PARR⁻ cats were available for analysis as several cats had been sacrificed for unrelated studies.

Statistical Analysis

A Fisher's exact test comprised of a 2 $\times$ 2 contingency table (FIV⁺ or FIV⁻ and PARR⁺ or PARR⁻) was used for comparison of CD8⁺ lymphocyte samples analyzed by PARR. Differences were considered to be significant at $p < 0.05$. For all other experiments, the Mann-Whitney *U* test (*t* test-like for nonparametric data) was used for pair wise comparison of different parameters (e.g. surface molecule expression). Differences were considered to be significant at $p < 0.05$.

Results

PARR analysis of FIV-infected or noninfected feline PBMCs and PLN cells

As FIV infection has been shown to alter CD8⁺ T cell development and function, we asked whether these changes could be detected using PCR for Antigen Receptor Rearrangement (PARR). PBMCs or peripheral lymph node (PLN) cells were isolated from chronically infected FIV⁺ ($n = 27$) or FIV⁻ ($n = 13$) cats and analyzed by PARR. Representative PARR data is provided in figure 1 with CD8⁺ PMBCs isolated from Subject A exhibiting PARR⁺ clonality while CD8⁺ PBMCs from subject B are PARR⁻. Results for all PBMC or PLN samples were summarized and, as shown in Table 3, there is a significant difference in the PARR status between FIV⁺ and FIV⁻ cats. 37% (10/27) of the blood or PLN samples from chronically FIV-infected cats examined exhibit CD8⁺ clonality (PARR⁺) compared to none (0/13) from the noninfected control cats ($p = 0.0164$). These data suggest that within a

significant percentage of FIV⁺ cats, infection results in clonal expansion of CD8⁺ T cells which can be detected by PARR analysis.

Surface phenotype of CD8⁺ PBMCs from PARR⁺ and PARR⁻ cats

As shown in table 3, a significant percentage of the peripheral blood samples from chronically infected FIV⁺ cats demonstrated CD8⁺ clonality; therefore, we asked if this reflected changes in peripheral blood CD8⁺ T cell numbers or phenotype. PBMCs from FIV⁺ PARR⁺ and FIV⁺ PARR⁻ cats were isolated and analyzed by flow cytometry. As shown in figure 2A, the percent of CD8⁺ T cells was not significantly different between PARR⁺ and PARR⁻ PBMCs. Previous studies have characterized a unique population of CD8 α ⁺ β ^{lo} T cells that are expanded during infection and demonstrate strong antiviral responses (Bucci et al., 1998). To determine whether the clonally expanded population in PARR⁺ cats was due to an increase in these antiviral CD8⁺ cells, PBMCs were labeled with both CD8 α and CD8 β antibodies for flow cytometry analysis. Lymphocytes were gated on CD8 α expressing cells and then further defined as CD8 β ^{hi}, CD8 β ^{lo} and CD8 β ⁻ (Fig. 2B). As shown in figure 2B, the percent of CD8 α ⁺ β ^{lo} did not significantly differ between the two groups of cats. Additionally, CD8 α ⁺ β ^{lo} PBMCs from PARR⁺ and PARR⁻ cats were analyzed for phenotypic differences by flow cytometry using antibodies specific for TGF β RII, CD62L and CD49d, but no significant differences were found between groups (data not shown).

Plasma viremia

Because CD8⁺ T cell function is related to viral set point and disease progression, we asked if there was a difference in viral loads for PARR⁺ and PARR⁻ FIV⁺ cats. As shown in figure 3A, acute plasma virus levels from cats that had been identified as either PARR⁺ or PARR⁻ during chronic infection were analyzed retrospectively for 0, 2, 4 and 6 weeks post infection (PI). PARR⁺ cats exhibited a delayed peak in viremia compared to PARR⁻ cats. The PARR⁻ peak viremia level at two weeks PI and the PARR⁺ peak viremia level at three weeks PI were not statistically different. To ascertain whether this lengthened period of high viremia during acute infection influenced chronic viral loads, plasma viremia was also analyzed at 1 year PI but no significant differences were found between the PARR⁺ and PARR⁻ groups (Fig. 3B). The protracted peak in viral loads during acute FIV infection in the PARR⁺ cats was further correlated to a decrease in total lymphocyte counts at 6 weeks PI as compared to PARR⁻ cats and although not statistically significant, there was a trend toward a decrease in CD8⁺ lymphocytes in PARR⁺ cats (Fig. 3C–D). Total CD4⁺ T cell counts did exhibit a significant decrease in PARR⁺ cats as compared to PARR⁻ cats (data not shown).

CD8⁺ PBMC Cytokine responses

While overall percentages of CD8⁺ cells in the PARR⁺ and PARR⁻ cats did not differ at any time tested, Betts, et al. have demonstrated that the functionality of CD8⁺ populations is more important than the number of cells in controlling virus load and disease progression (Betts et al., 2006). Based upon these findings and the findings noted in figure 3, we asked if there was a difference in CD8⁺ cytokine mRNA profiles between PARR⁺ and PARR⁻ cats chronically infected with FIV. Sorted CD8⁺ cells were stimulated in vitro with 5 ug/mL ConA for 24 hours or left untreated then analyzed for expression of IL-2, TNF- α and IFN- γ message by real-time RT-PCR. As shown in figure 4, PBMCs from PARR⁺ cats expressed significantly less IL-2 and IFN- γ mRNA in response to mitogenic stimulation as compared to PARR⁻ cats (Fig.4).

CD8⁺ PBMC Proliferative responses

To further investigate the differences in PARR⁺ or PARR⁻ CD8⁺ T cell responses, PBMCs were isolated from the chronically infected cats, labeled with CFSE and incubated as a mixed lymphocyte culture with 5 ug/mL ConA. After 72 hours in culture, cells were washed, surface stained for CD8 expression and then the CD8⁺ population was analyzed for proliferation by flow cytometry. ModFit LT software was used to calculate the proliferation index (PrI) or stimulation index (SI) for each sample, as shown in figure 5. Interestingly, PARR⁺ cats showed a trend toward greater proliferative capacity; however, there were no significant differences in proliferative responses between the PARR⁺ and PARR⁻ groups by either measurement (PI p=0.981; SI p=1.000).

Discussion

Here we demonstrated that almost 40% of the chronic FIV⁺ cats in our study exhibited CD8⁺ clonality as compared to none of the FIV⁻ control cats. This suggests that FIV infection can influence the selection of cells for expansion during an immune response. We therefore hypothesized that the FIV⁺ PARR⁺ cats would exhibit differences in CD8⁺ T cell phenotype, mRNA profile, and proliferative responses as compared to FIV⁺ PARR⁻ cats. Analysis of plasma viremia levels over the course of infection revealed that PARR⁺ cats exhibited a delayed peak in viremia during acute infection as compared to PARR⁻ cats with no statistical difference in maximum viral load between groups. Lower total lymphocyte counts were also observed in the PARR⁺ group at 6 weeks PI. These differences were caused by a decrease in both the CD8⁺ T cell count (Fig. 3D) and CD4⁺ T cell count (not shown). We hypothesized that the differences observed between PARR⁺ and PARR⁻ cats during acute infection would alter chronic infection by influencing the development of the CD8⁺ lymphocyte pool. Specifically, we measured the presence of a CD8⁺ subpopulation (CD8^β^{lo}CD62L^{lo}CD49d^{hi}) previously reported to have strong antiviral activity during FIV infection (Bucci et al., 1998). Evaluation of lymphocytes from PARR⁺ and PARR⁻ FIV⁺ cats during chronic infection revealed no differences in the percentages of CD8⁺ T cells or in the occurrence of the antiviral CD8^β^{lo}TGFβRII^{lo}CD62L^{lo}CD49d^{hi} subpopulation. There was also no difference in viremia between the two groups at the chronic stage of infection. Chronic FIV-infection is generally asymptomatic and characterized by low levels of circulating virus so these results do not preclude differences in disease outcome for PARR⁺ and PARR⁻ cats as the progression to AIDS has not been observed for either group. Although not statistically significant, PARR⁺ cats exhibited slightly greater proliferative capacity in response to mitogenic stimulation while also displaying significantly lower transcription of IL-2 and IFN-γ following mitogenic stimulation. This profile is consistent with a scenario of CD8⁺ anergy, sometimes described as “adaptive tolerance,” which has been observed for chronic lentiviral infection and in other chronic inflammatory conditions (Fogle et al., 2010b; Lim et al., 2000; Schwartz, 2003). While not the focus of this manuscript, the decreased production of IL2 simultaneous to a slight elevation of proliferation indices was an interesting observation. We have noted a loose correlation between the lack of IL2 production and what is likely a compensatory up-regulation of CD25 (Fogle, unpublished data), which might perhaps explain a modest increase in CD8⁺ proliferation with exogenous IL2 added to culture medium as noted in the materials and methods.

These data suggest that FIV infection leads to CD8⁺ clonal expansion in a large subset of cats. Further, these data suggest that a less diverse CD8⁺ repertoire may result in prolonged acute viremia. Higher antigenemia and longer duration of antigen exposure have been correlated with tolerogenic environments which produce clonally expanded, anergized CD8⁺ cells that lack effector function (Hernandez et al., 2001; Schwartz, 2003). Further study is needed but our results suggest early clonal selection leads to impaired CD8⁺ responses, as

demonstrated by prolonged plasma viremia during the acute stage of infection and decreased transcription of IL-2 and IFN- γ during the chronic stage of infection. We believe that the PARR⁻ cats have developed a more substantial pool of effector memory CD8⁺ T cells, as this particular subset has been shown to rapidly produce cytokines in response to stimulation (Williams and Bevan, 2007). As increased prevalence of memory lymphocytes is correlated with improved protection in HIV/AIDs (van Grevenynghe et al., 2008; Wells et al., 2000), future studies utilizing PARR will involve phenotypic characterization of CD8⁺ cells throughout infection and specifically monitor the CD8⁺CCR7⁺CD45RA⁻CD27⁺CD28⁺CD62L⁻ effector memory subset (Appay et al., 2008). Longitudinal studies tracking the induction of anergy, the expansion and contraction of the CTL response and the specificity of these CD8⁺ cells is necessary. The exhaustion associated with chronic lentiviral infection has been shown to be mediated by PD-1 expression and amplified IL-10 production, so evaluation of expression dynamics for these molecules may provide more details for understanding the progression of anergy and how this relates to the PARR⁺ and PARR⁻ cats as well (Brooks et al., 2006; Ejrnaes et al., 2006).

Although some cats have been confirmed PARR⁺ as early as 8 weeks post infection, the exact temporal aspects of clonal expansion were not the focus of this study. Therefore, to ensure that any changes in CD8⁺ clonality during the acute to chronic transition did not influence the data, this study only evaluated cats that were PARR⁺ following at least 6 months of infection. Importantly, none of the cats that were confirmed PARR⁻ converted to PARR⁺ at a later time point. Future studies should incorporate tracking the progression of FIV-infected PARR⁻ cats to PARR⁺ status from acute infection through chronic infection to provide a better model of fluctuations in clonal CD8⁺ populations over the course of infection.

Conclusions

These results lead us to conclude that specific selection of CD8⁺ clones for expansion during virus induced immune activation may correspond with changes in cell-mediated immune responses during chronic infection. Specifically, we have identified that CD8⁺ T cell clonal expansion in the PBMC lymphocyte fraction during chronic FIV infection by PARR analysis corresponds to CD8⁺ T cell populations which are hypo responsive to mitogen stimulation as measured by a difference in IL-2 and IFN- γ cytokine production following stimulation. Retrospective analysis indicated PARR⁺ cats experienced a prolonged peak viremia and a significantly lower total lymphocyte count during the acute stage of infection, supporting a model where virus-induced changes during acute infection can lead to differences in CD8⁺ T cell function during the chronic infection. Fully determining the dynamics of CD8⁺ T cell clonal expansion during the acute stage, including antigen specificity and activation state, and determining correlates with chronic infection is therefore critical not only for understanding viral mechanisms for immune subversion but also for potential therapeutic designs.

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List of Abbreviations

PARR	PCR for antigen receptor rearrangements
FIV	Feline Immunodeficiency Virus

LCMV	Lymphocytic Choriomeningitis Virus
SIV	Simian Immunodeficiency Virus
TGFbRII	TGFb receptor type II
PI	Post-infection
PrI	Proliferation Index
SI	Stimulation Index
PLN	Peripheral Lymph Node

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Figure 1. Representative results for CD8⁺ analyses by PARR

Representative test results for PARR positive FIV⁺ (A) and PARR negative FIV⁺ (B) PBMCs are shown. PBMCs were isolated by Percoll density gradient and PLN cells were isolated by lymph node biopsy. CD8⁺ cells were purified from mixed lymphocyte populations by FACS sorting for CD8 expression. The positive control consisted of cells from a cat with confirmed T cell lymphoma and the negative control consisted of all reagents for the PCR reaction minus the sample DNA.

Figure 2. Surface phenotyping of PBMCs from FIV-infected PARR⁺ or PARR⁻ cats

A. PBMCs from FIV-infected PARR⁺ (N = 6) or PARR⁻ (N = 6) cats were analyzed by flow cytometry for CD8 α expression and total percent expressing CD8 are shown on the right. **B.** PBMCs were also evaluated for CD8 β expression by flow cytometry. Lymphocytes were gated on the CD8 α^{+} population and then grouped into CD8 β^{hi} , CD8 β^{lo} or CD8 β^{-} . The percent of total CD8⁺ cells that were CD8 β^{lo} is shown on the right. Statistics were performed using the Mann-Whitney test with $p < 0.05$ considered significant.

Figure 3. Plasma viremia and lymphocyte counts for acute and chronic FIV infected PARR⁺ or PARR⁻ cats

A and B. Plasma viremia was measured on FIV-infected PARR⁺ (N = 5) or PARR⁻ (N = 5) cats at (A) 2, 4 and 6 weeks and (B) 1 year post infection (PI). **C and D.** (C) Total lymphocyte counts or (D) total percent of lymphocytes expressing CD8 were analyzed for FIV-infected PARR⁺ (N = 6) and PARR⁻ (N = 6) cats at 6 weeks and 1 year PI. No statistical difference was noted in maximum viremia load between groups but PARR⁺ cats exhibited a delay in reaching peak viremia when compared to PARR⁻ cats. Statistics were performed using the Mann-Whitney test with $p < 0.05$ considered significant.

Figure 4. Cytokine response of CD8⁺ T cells to mitogenic stimulation

PBMCs from FIV-infected PARR⁺ (N = 5) or PARR⁻ (N = 5) cats were FACs purified into CD8⁺ T cell populations and either stimulated in vitro with ConA (5 ug/mL) or left untreated for 24 hours. RNA was then isolated from the lymphocytes and analyzed for IL-2, TNF- α or IFN- γ by real-time RT-PCR. Relative expression was determined by using the $2^{-\Delta\Delta C_T}$ method with GAPDH as the control gene. Statistics were performed using the Mann-Whitney test with $p < 0.05$ considered significant.

Figure 5. Proliferative response of CD8⁺ T cells to mitogen stimulation

PBMCs from FIV-infected PARR⁺ (N = 4) or PARR⁻ (N = 6) cats were CFSE stained, incubated for 72 hours with ConA (5 ug/mL) and IL-2 (100 U/mL) stimulation, and analyzed for proliferative response by flow cytometry. ModFit LT software was used to calculate the proliferation index (PrI) and the stimulation index (SI). Statistics were performed using the Mann-Whitney test with $p < 0.05$ considered significant.

Table 1
Number of cats included for each experimental analysis

<u>EXPERIMENT</u>	<u>PARR+ FIV+</u>	<u>PARR- FIV+</u>
CD8 Surface Phenotype	6	6
Plasma Viremia	5	5
CD8 Cytokine PCR Analysis	5	5
CD8 Proliferation Assay	4	6

Table 2
Primers used for PARR and RT-PCR

All primers used during PARR or real time RT-PCR analysis have been previously documented for use in feline lymphocytes as described in the Materials and Methods section and are listed here for ease of access.

<u>Target gene</u>	<u>Forward 5' → 3'</u>	<u>Reverse 5' → 3'</u>
PARR	AAGAGCGAYGAGGGMGTGT	CTGAGCAGTGTGCCAGSACC
FIV-gag	GATTAGGAGGTGAGGAAGTTC	CTTTCATCCAATATTTCTTTA
IL-2	ACAGTGCACCTGCTTCAAGCTCT	CCTGGAGAGTTTGGGGTTCTCA
TNF-α	ATGCCCTCCTGGCCAATGGCG	TAGACCTGCCCCGACTCGGC
IFN-γ	TGGTGGGTCGCTTTTCGTAG	GAAGGAGACAATTGGCCTTGA
GAPDH	GGAGAAGGCTGGGGCTCAC	GGTGCAGGAGGCATTGCTGA

Table 3
PBMC and PLN PARR testing results

PBMCs from FIV-infected (n = 15) or noninfected (n = 8) cats and PLN cells from FIV-infected (n = 12) or noninfected (n = 5) cats were subjected to PARR analysis. The percent positive for PARR is given for each group and statistics were performed using the Fisher's exact test with $p < 0.05$ considered significant.

FIV+	Blood	PLN	All samples
PARR+	7	3	10
PARR–	8	9	17
Positive (%)	47%	25%	37%
FIV–	Blood	PLN	All samples
PARR+	0	0	0
PARR–	8	5	13
Positive (%)	0%	0%	0%
P value	ns	ns	*0.0164