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CD4⁺CD25⁺ REGULATORY T CELLS ARE INFECTED AND ACTIVATED DURING ACUTE FIV INFECTION

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Abstract

HIV-induced AIDS may be mediated by the activation of immunosuppressive CD4⁺CD25⁺ T regulatory cells (Treg cells). Treg cells have been shown to regulate CD4⁺ and CD8⁺ immune responses to HIV and FIV antigens in vitro. We tested the hypothesis that Treg cells become infected and activated during the acute infection with FIV leading to the suppression of CD4⁺ T helper cell responses. Cats were experimentally infected with FIV-NCSU₁ and blood and lymph node cells were collected at weekly intervals following inoculation. Real-Time RT-PCR was used to determine plasma viremia and the relative expression of FIV, FoxP3, TGF- β , and GAPDH mRNA copies in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell subsets. Flow cytometry was used to assess the absolute numbers of each cell type and the expression of surface TGF- β and intracellular FoxP3 in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells at each time point. Treg suppression of IL-2 production in CD4⁺ T helper cells was assessed by ELISPOT assays. Our results showed that peak viremia occurred at 2 weeks post infection and correlated with maximal infectivity in CD4⁺CD25⁺ T cell populations. FIV-gag-mRNA levels were higher in CD4⁺CD25⁺ T cells than CD4⁺CD25⁻ T cells throughout the acute phase of infection. Induction of FoxP3 and TGF- β indicated activation of Treg cells during the acute stage infection, which was confirmed by Treg cell suppression of IL-2 production by CD4⁺ Th cells in an ELISPOT assay. Our findings support the hypothesis that early activation of Treg immunosuppressor function may limit an effective anti-FIV response, contributing to the establishment of chronic infection and the immunodeficiency caused by this virus.

Keywords

FIV; HIV; Regulatory T cells; Acute infection

1. Introduction

The feline immunodeficiency virus (FIV) infection of domestic cats is a well established model for the study of infections with human immunodeficiency virus (HIV) and the related immunodeficiency syndrome (AIDS) (Vahlenkamp et al., 2006). Both HIV and FIV infections are characterized by a short acute phase with high but self-limiting viremia, followed by a long latent subclinical phase with low viremia. Most infected humans and felines subsequently

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progress into a phase of severe immunodeficiency, which is manifested in opportunistic infections or lympho-proliferative diseases. Immunologically, the acute phase of infection is characterized by humoral and T cell-mediated anti-viral immune responses that correlate with a sharp decrease in plasma viremia. However, evidence suggests that the immune response to the virus is lost during the acute phase of infection. For example, CD4⁺ and CD8⁺ T cells fail to produce IL2 and IFN γ and to proliferate in response to stimulation by viral antigens. The mechanism(s) regulating T cell hypo-responsiveness to viral antigen stimulation have not been fully explained. Several mechanisms have been proposed including: cytokine dysregulation (decreased IL-2, increased IL-10 and IFN- γ production), activation-induced cell death (apoptosis) and clonal deletion or anergy (Clerici et al., 1989; Reddy et al., 1987; Wahren et al., 1987). The observation that CD4⁺ T cells produce IFN- γ , yet do not produce IL-2 or proliferate in response to HIV gag stimulation supports the theory of clonal anergy (Arrode et al., 2005). Recent studies in our laboratory have focused on a population of CD4⁺CD25⁺ T regulatory (Treg) cells as possible mediators of T cell clonal anergy in FIV infection.

Treg cells were first identified as a distinct thymus-derived T cell population that suppress autoimmune T cells and maintain peripheral self tolerance (natural Treg cells). In addition, we now recognize that peripherally activated Treg cells (adaptive Treg cells or pathogen-induced Treg cells) modulate CD4⁺ and CD8⁺ immune responses to microbial pathogens, including bacteria, viruses, fungi, and intracellular parasites (Vahlenkamp et al., 2005). While the origin of microbial-activated Treg cells has yet to be established, data suggest that these cells can be derived from either existing CD4⁺CD25⁺ cells or by recruitment from CD4⁺CD25⁻ T cells in the periphery. These Treg cells are indistinguishable from natural Treg cells phenotypically in that they up-regulate CTLA4, GITR, certain Toll-like receptors, CD62L, and membrane associated TGF- β , and most importantly, they express the repressive intranuclear transcription factor FoxP3 that is required for Treg homeostasis and function (Chen et al., 2003; Fantini et al., 2004; Walker et al., 2003). Pathogen-induced regulatory T cells are thought to play an important role in preventing excessive inflammation associated with the acute immune responses to infectious agents (Rouse and Suvas, 2004). However, several chronic viral infections, including HBV, HCV, HIV, HTLV-1, FIV, Friend virus and the murine form of AIDS, have additionally been linked to unregulated Treg cell activation and suppressor function (Vahlenkamp et al., 2005).

Previous work in our laboratory has established that feline regulatory T cells share phenotypic and functional characteristics with their human and murine counterparts (Vahlenkamp et al., 2004). Here, we were the first to report in the FIV model for human HIV infection that CD4⁺CD25⁺ Treg cells are chronically activated and immunosuppressive for CD4⁺ T cells in asymptomatic infected cats. We, therefore, speculated that Treg cells may play a role in the T cell immunodeficiency that is the hallmark of AIDS-related lentivirus infections. Subsequent to our studies, several investigators reported activation of Treg cells in HIV-infected individuals and SIV-infected monkeys (Aandahl et al., 2004; Carbonneil et al., 2004; Kinter et al., 2004). Kornfeld et al. (Kornfeld et al., 2005) reported an early increase in CD4⁺CD25⁺ and CD8⁺CD25⁺ T cells following the SIV infection of African green monkeys that correlated with an early TGF- β and IL-10 response. Interestingly, in the more pathogenic SIV-Macaque infection, there were no early CD4⁺CD25⁺ and TGF- β responses, which correlated with greater inflammation and disease, leading to the speculation that the greater pathogenicity in the SIV-Macaques infection is due to failure to activate Treg cells and control acute stage inflammation. In contrast to Kornfeld et al., Estes et al. (Estes et al., 2006) reported that SIV infection of Macaques induced an early acute stage immunosuppressive response which correlated with a marked increase in the frequency of CD4⁺CD25⁺Foxp3⁺ Treg cells and TGF- β and IL-10 positive T cells, suggesting that activated Treg cells may contribute to viral persistence by prematurely limiting the antiviral immune response. These contrasting results in the SIV-Macaque infection model underscore the importance of utilizing other

lentivirus animal models, such as FIV, to address the complex Treg/Th immunoregulation that may determine the course of the disease.

Based on our previous results from chronically infected, long-term, asymptomatic cats, we were interested in determining how early after FIV infection the CD4⁺CD25⁺ Treg cells become infected and express characteristics of activated Treg cells. We report here that CD4⁺CD25⁺ T cells are infected with FIV and express phenotypic and functional characteristics of activated Treg cells early during the acute stage of FIV infection. Specifically, we show that as early as one week post infection, CD4⁺CD25⁺ T cells have higher viral mRNA levels than CD4⁺CD25⁻ T cells and cellular infectivity correlates with plasma viremia levels. FoxP3 and TGF- β , markers of Treg activity, are increased in CD4⁺CD25⁺ T cells during the first eight weeks of infection. In addition, CD4⁺CD25⁺ Treg suppression of autologous mitogen-stimulated CD4⁺ Th responses, as measured by IL-2 ELISPOT assays confirmed the activation of regulatory T cells in acutely FIV-infected cats.

2. Materials and Methods

2.1. Cats

Specific pathogen free (SPF) cats were acquired from Liberty Research, Inc. (Waverly, NY) or Harlan Sprague Dawley (Madison, WI) at 6 to 12 months of age. They were housed in groups according to guidelines from the IACUC at NCSU's laboratory animal resources facilities. All of the experiments described herein were performed on the cats at 2–3 years of age. FIV-infected cats were housed separately from negative controls.

2.2 Infection with FIV

The NCSU₁ isolate of FIV was originally obtained from a naturally infected cat at the North Carolina State University College of Veterinary Medicine and has been described in detail elsewhere (Davidson et al., 1993; English et al., 1993; English et al., 1994). Virus inoculum was grown as a single cycle infection of an IL-2-dependent feline CD4⁺ cell line (FCD4-Ecells) as previously described (Davidson et al., 1993). Twenty-eight cats were inoculated intravenously with 1×10^5 TCID₅₀ of cell-free virus culture supernatant and seven control cats were sham inoculated with equal volumes of sterile FCD4-E cell culture medium.

2.3. Lymph node biopsies

The cats were divided into 8 different groups, such that popliteal lymph nodes were biopsied from eight infected and two control cats at each time interval (1, 2, 3, 4, 5, 6, and 8 weeks) post inoculation. Thus, each cat had two lymph nodes biopsied during the course of the study. Cats were anesthetized with intravenous ketamine and valium and anesthesia was maintained with isoflurane gas. Buprenorphine was administered intra-operatively to control postoperative discomfort. Popliteal lymph nodes were excised through a small incision in the caudal aspect of the stifle. The incision was sutured with monofilament sutures which were removed 7–10 days post-operatively. Lymph node cells were processed into single cell suspensions by methods previously described (Levy et al., 1998) and used for phenotype analysis by flow cytometry or MoFlo purification of lymphocyte subsets.

2.4. Purification of peripheral blood mononuclear cells

Prior to virus inoculation and at the time of each lymph node biopsy, 20 mls of blood were collected by jugular venipuncture into vacutainer tubes containing EDTA anticoagulant. Plasma was separated and frozen for analysis of viral load by RT-PCR. The cells were resuspended to twice the original volume in PBSS with 2% EDTA and 8 mls of blood were layered over 3 mls of Ficoll-Histopaque-1077 (Sigma-Aldrich). Following centrifugation at

300×G for 20 minutes, cells at the interface were collected, washed, and re-suspended in cell culture medium. Cell counts were performed on a hemocytometer and viability was assessed by Trypan Blue dye exclusion at 40X magnification. Cells were immediately used for multi-color staining and flow cytometry or MoFlo purification of lymphocyte subsets.

2.5. Plasma viremia

Evidence of infection was assessed on each plasma sample using a commercially available snap test ELISA (IDEXX laboratories) to detect antibodies against FIV. In addition, quantitative real time PCR was used to determine virus gag-mRNA loads in each plasma sample. Briefly, 1ml of plasma was used to extract viral RNA using Qiagen's QIAamp Ultrasense Virus Isolation kits. 10 µl 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: 491f (5'-GAT TAG GAG GTG AGG AAG TTC AGC T-3') and 617r (5'-CTT TCA TCC AAT ATT TCT TTA TCT GCA-3'), universal Taqman PCR Mastermix (Applied Biosystems) and the FIV-specific probe: FIVNC555P (5'-56FAM/CAT GGC CAC ATT AAT AAT GGC CGC A/36-TAMSP/-3') in the relative concentrations specified by the manufacturer. The reactions were run in duplicates in 96 well plates and incubated at 50° C for 2 min, 95° C for 10 minutes, followed by 45 cycles of 95° C for 15 seconds and 60° C for 1 minute, 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.

2.6 Phenotypic analysis

The surface phenotype of mononuclear cells from blood and lymph nodes was determined by two and three-color flow cytometric analysis. At least 5×10^5 PBMC or LN cells were stained with biotin-conjugated anti-CD4 (mAb 30A), PE-conjugated anti-CD8, (mAb 3.357) and FITC-conjugated anti-CD25 (mAb 9F23) (Vahlenkamp et al., 2004). Membrane TGF-β expression was determined using anti-human TGF-β (MAB240, R&D Systems, Minneapolis MN). Each monoclonal antibody combination was analyzed in duplicate samples. Flow cytometric analysis was performed using a FACSCalibur machine and 20,000 gated events were acquired from each tube. Lymphocytes were gated according to their characteristic forward and side scatter parameters and the data was analyzed for percent positive cells.

2.7. Four way cell sorting

The remaining mononuclear cells isolated from blood and lymph nodes were stained with anti-CD4 biotin-PerCP, anti-CD8-PE and anti-CD25-FITC and sorted into four distinct populations (CD4⁺CD25⁺, CD4⁺CD25⁻, CD8⁺, and CD4⁻CD8⁻) using a high speed Moflo cell sorter. Each population was more than 90% pure and the number of viable cells acquired was confirmed by manual counts using trypan blue dye exclusion. Cells were either used immediately for suppression assays as described below or maintained in RNA later (Qiagen) at -20°C for RNA extraction and PCR analysis.

2.8. Reverse transcription and real-time PCR

Purified populations of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from lymph nodes and peripheral blood were used for relative quantification of FIV-gag, FoxP3, TGF-beta, and GAPDH mRNA by reverse transcription and real-time PCR. Total RNA extraction was carried out in each case using Qiagen's RNeasy mini-prep kits and eluted in a final volume of 60 µl per reaction. 10 µl of the RNA obtained were used in reverse transcription reactions using random primers and the Promega Reverse transcription system according to the manufacturer's instructions. From each reverse transcription reaction, 5µl of the product were used to assess relative quantities

of each specific mRNA of interest in separate reactions in duplicate wells. The SYBR Green Taqman PCR master mix (Applied Biosystems) was used for FIV, FoxP3, TGF- β and GAPDH quantification according to the manufacturer's guidelines. All reactions were carried out using the same temperature programs in an ICycler PCR machine. Quantification was carried out using the delta delta CT method (Winer et al., 1999) using GAPDH mRNA amounts as the housekeeping gene. In each reaction the same calibrator sample was used to compare relative values within and between reactions. Similar Ct values were obtained for each repeat of the calibrator sample, but the specific values obtained in each reaction were used to calibrate relative quantities of the unknown samples.

2.9. Detection of intracellular FoxP3 by flow cytometry

Intracellular immunofluorescent staining for flow cytometric analysis of FoxP3 protein expression was performed on mononuclear cells obtained from peripheral blood and lymph nodes using a commercially available kit (eBiosciences, FoxP3 Staining Buffer set) and an anti-mouse FoxP3 antibody conjugated to APC (eBiosciences, clone: FJK-16s) that had been shown to cross-react with the feline protein (Smithberg et al., 2008). 4×10^6 unsorted cells were placed in each tube and surface stained for CD4 and CD25 prior to fixing and permeabilization. Following intracellular protein staining, cells were washed in staining buffer overnight and analyzed by flow cytometry the following day.

2.10. IL-2 ELISPOT assays

Inhibition of target cell IL-2 production by CD4⁺CD25⁺ T cells as a measure of their suppressive function was determined using lymph node cells from nine control samples and 3 or 4 samples from FIV-infected cats at 1,2,3,8, and 12 weeks post infection using a commercially available, validated ELISPOT assay for feline IL-2 secreting cells at the single cell level (R&D Systems). CD4⁺CD25⁻ (target cells) were mixed with an equal number of CD4⁻CD8⁻ (APC) cells and stimulated with Concanavalin A (10 μ g/million cells) for 2–3 hours, washed once and plated at 4×10^5 cells/well in the ELISPOT 96-well plate. Preliminary studies demonstrated that CD4⁺CD25⁻ T cells alone and CD4⁻CD8⁻ T cells alone did not produce IL-2 when stimulated with Con A independently (data not shown). However, IL-2 was produced when the two populations were mixed at a ratio of 1:1 prior to stimulation. CD4⁺CD25⁺ Treg cells (effectors) were added at specific effector to target ratios of 0.1 or 0.5. Controls consisted of wells containing target cells alone (positive control), no cells (negative control), human recombinant IL-2 (positive control), or CD4⁺CD25⁺ T cells only. Each assay was run in duplicate wells. The cells were incubated overnight and spots were developed following the manufacturer's protocol. Quantification of spot forming cells was carried out using an automated spot counter. Very few or no spots were detected when target cells were not stimulated with ConA prior to incubation. The percent suppression was calculated as the average number of spot forming cells (SFC)/well in target only wells minus the average number of SFC/well at each E:T ratio divided by the average number of SFC/well in the target only wells.

2.11. Statistical analysis

To determine statistical significance between data points from FIV-infected and non-infected controls we performed Mann-Whitney U tests (non-parametric analysis). In experiments comparing data from the same group of cats at different time points we used student t tests to determine significant variation for each parameter analyzed. Individual p values are given for each significant result in the figure captions.

3. Results

3.1. Plasma and cell associated viremia

To confirm infection of the cats, plasma samples obtained starting at 4 weeks post inoculation were evaluated for the presence of anti-FIV antibodies using a commercially available ELISA snap test (IDEXX). All of the plasma samples from sham inoculated control cats tested negative at all time points. The FIV-infected cats tested at 4 weeks post inoculation also tested negative for anti-FIV antibodies but were sero-positive at 5 weeks post infection and at all time points thereafter. Quantitative RT-PCR, used to evaluate plasma viremia, revealed a peak in the mean number of FIV-gag mRNA copies per ml of plasma at week 2 post infection. This was followed by a decline to low but positive levels thereafter (Figure 1). The plasma viremia values obtained from individual cats listed in Table 1 demonstrate a wide range in values throughout the study. To determine if CD4⁺CD25⁺ Treg cells were infected with FIV during the acute stage of infection, relative quantities of viral mRNA were compared in FACS purified sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ lymphocytes from FIV-infected cats using reverse transcription and real time PCR. Peak virus levels were found in CD4⁺CD25⁺ T cell subsets from the blood and lymph nodes at week 2 post infection (Figure 1), which closely paralleled the plasma viremia pattern described above. The relative quantities of viral mRNA were greater in CD4⁺CD25⁺ than in CD4⁺CD25⁻ cells, resulting in ratios of FIV-mRNA in CD25⁺ vs CD25⁻ T cells greater than one (Figure 2) in all but one sample (week 1, LN).

3.2. Lymphocyte changes in acutely FIV-infected cats

Consistent with previous reports (English et al., 1994; Jeng et al., 1996; Novotney et al., 1990) infection of cats with the NCSU⁻¹ isolate of FIV caused a T cell lymphopenia as early as 7–14 days post inoculation in both the blood and lymph nodes, which was followed by a CD8⁺ lymphocytosis (data not shown). This resulted in a decline in the CD4:CD8 T cell ratios in peripheral blood which, although not significant ($p>0.05$) in this case, is typical of FIV infection (Fig. 3).

To determine if acute FIV infection altered Treg cell numbers, we compared the percent and absolute numbers of CD4⁺CD25⁺ T cells in FIV and sham-inoculated controls during the first twelve weeks post inoculation. There was no difference between FIV+ and control cats in the percent of CD4⁺CD25⁺ T cells in the peripheral blood or lymph nodes during the course of this study. Figure 4 shows slightly higher, but not statistically significant differences in percent CD4⁺CD25⁺ T cells in the blood of FIV-infected cats compared to control cats at weeks 2 and 3 post infection. There was also no difference in the absolute CD4⁺CD25⁺ T cells counts in the blood and lymph nodes between infected and control cats (data not shown).

3.3. Treg cells become phenotypically activated during the acute phase of FIV infection

Consistent with our results, Vahlenkamp et al (Vahlenkamp et al., 2004) reported that although the number of Treg cells in asymptomatic chronically FIV-infected cats did not differ from control cats, these cells were phenotypically and functionally activated. To determine the activation status of CD4⁺CD25⁺ Treg cells in acutely FIV-infected cats, FoxP3 and TGF- β expression were analyzed as indicators of Treg cell activation (Huber et al., 2004; Zheng et al., 2006). Real time RT-PCR analysis for FoxP3 mRNA levels in CD4⁺CD25⁺ cells from the blood of FIV-infected cats, revealed an increase in FoxP3 transcription starting at one week post infection (Figure 5). The levels of FoxP3 mRNA expressed in peripheral blood cells reached statistically significant differences at weeks 5 and 6 post infection. There was no change in the expression of FoxP3 mRNA in CD4⁺CD25⁻ T cell subsets from both groups over time (data not shown). These results suggest that Treg cells are activated and transcriptionally up-regulate FoxP3 during the acute phase of FIV infection.

In addition to the detection of FoxP3 mRNA by RT-PCR, intracellular staining for FoxP3 protein was performed to detect changes at the protein level. There was no change in the percent CD4⁺CD25⁺ T cells expressing FoxP3 protein in the lymph nodes following FIV infection (Fig. 6a). However, the mean fluorescence intensity for FoxP3 staining was significantly higher in the CD4⁺CD25⁺ T cells from the lymph nodes at weeks 3, 4, and 8 post inoculations when compared to week 1 (Fig. 6b), suggesting an increase in FoxP3. A similar trend was noted in the CD4⁺CD25⁺ cells from the blood; however, these numbers did not reach statistical significance. Interestingly, there is an increase over time in the percent CD4⁺CD25⁺ and CD4⁺CD25⁻ PBMCs, and CD4⁺CD25⁻ lymph node cells that express FoxP3 (Fig. 6a), but these increases are not significant ($p < 0.05$). The histograms in Figure 6c show representative samples for all T cell subsets at weeks 1, 2, and 5 post inoculation.

TGF- β , another recognized marker for activated Treg cells was analyzed in T cell subsets of FIV-infected and control cats throughout the acute phase of infection. Analysis of TGF- β expression on the surface of CD4⁺CD25⁺ Treg cells by flow cytometry revealed increased expression of mTGF- β on CD4⁺CD25⁺ T cells in the lymph nodes of FIV-infected cats at 2 and 3 weeks post infection (Figure 7). The percent of cells expressing mTGF- β in the blood, however, was low at all time points and in all T cell subsets (data not shown). These data suggest there is an early and transient increase in mTGF- β ⁺ Treg cells in the lymph nodes early in the course of infection, which correlates with the early peak viremia. Analysis of TGF- β mRNA transcription, as measured by RT-PCR, showed essentially no difference in mRNA levels between CD4⁺CD25⁺ and CD4⁺CD25⁻ cells. However, there was a greater than 5-fold increase in mRNA expression in the CD4⁺CD25⁺ cells of the blood at 3–8 weeks post infection (Figure 8a). In contrast, there was a 2-fold or less increase in the CD4⁺CD25⁻ T cells from infected cats in the blood and lymph nodes (Figure 8b).

3.4. Suppressor function of Treg cells during acute phase FIV infection

Having shown that Treg cells are infected and phenotypically activated during the acute phase of FIV infection, we next tested their ability to suppress the effector function of target CD4⁺CD25⁻ T cells. The addition of FACS purified Treg cells from FIV-infected cats to stimulated CD4⁺CD25⁻ target cells suppressed IL-2 production by the Th target cells (Figure 9). The mean percent suppression of IL-2 production by Treg cells from FIV-infected cats was higher at all time points as compared to Treg cells from the control cats ($p \leq 0.05$).

4. Discussion

Activation of CD4⁺CD25⁺ regulatory T cells has recently been proposed as a mechanism for the immuno-suppression associated with a number of chronic infectious diseases including HIV and FIV (Vahlenkamp et al., 2005). That Treg cells might influence functional anti-viral immunity may have serious implications for the prevention of chronic infections with HIV and other viruses (Vahlenkamp et al., 2005). In this study, we have shown that Treg cells are infected, activated, and functionally immunosuppressive *in vivo* during the acute phase of FIV infection. Peak plasma viremia levels correlated with maximal infectivity in blood and lymph node cell populations and were highest at two weeks post infection. CD4⁺CD25⁺ T cells had higher viral mRNA levels than their CD4⁺CD25⁻ counterparts, suggesting either preferential infectivity or increased viral replication in these cells early after infection with FIV. The induction of FoxP3 and an increase in expression of TGF- β is evidence of Treg cell activation in the first eight weeks post infection. In support of this, high levels of Treg-mediated suppressor function could be measured in cats during the acute phase of FIV infection. These results support previous observations of Treg cell activation in long-term, chronically infected cats (Vahlenkamp et al., 2004) and suggest that Treg cells are activated early after FIV infection and maintain this activation state throughout the course of infection.

Plasma viremia studies by RT-PCR also indicated a peak viremia 2 weeks post inoculation, which correlated with previously published studies (Liu et al., 2006). PCR results also indicated that there was a higher mRNA viral load in the CD4⁺CD25⁺ compartment compared to CD4⁺CD25⁻ cells. This is consistent with the observation of Joshi et al. (Joshi et al., 2004), who reported that CD4⁺CD25⁺ Treg cells from asymptomatic FIV-infected cats preferentially supported a productive FIV infection. Further, Joshi et al reported that CD4⁺CD25⁺ Treg cells but not CD4⁺CD25⁻ Th cells supported productive FIV replication when infected in vitro in the presence of exogenous IL-2. These studies collectively suggest that early and persistent activation of Treg cells may provide a reservoir of FIV replication, as well as a mechanism for immune dysregulation, both of which persist throughout the course of infection. In support of this, we showed herein that CD4⁺CD25⁺ Treg cells in acutely FIV infected cats up-regulated FoxP3 and mTGF- β , suggesting that Treg cells are activated as a result of FIV infection. The increased viral load within the CD4⁺CD25⁺ T cells could be due to preferential infectivity, meaning more of these cells are initially and persistently infected, or it could be due to increased viral replication within a small proportion of CD4⁺CD25⁺ T cells. In support of the former, CD4⁺CD25⁺ T cells have been shown to be more susceptible to FIV binding than CD4⁺CD25⁻ cells, which correlates with an increase in expression of CXCR4, the co-receptor for FIV (de Parseval et al., 2006; Joshi et al., 2005).

FoxP3, a member of the forkhead/winged-helix family of transcriptional regulators is essential for the development and function of Treg cells (Walker et al., 2003). Unlike other markers (such as GITR, CTLA4, and OX40) that can also be up-regulated on non-regulatory T cells after activation, FoxP3 is specifically expressed in regulatory T cells, making it the most valid marker for Treg cells known to date. Our previous studies have shown that FoxP3 mRNA expression is significantly greater in CD4⁺CD25⁺ than CD4⁺CD25⁻ feline T cells (Petty et al., 2008), and that the CD4⁺CD25⁺ T cells possess the key characteristics of Treg cells (Vahlenkamp et al., 2005). Here we have shown that the level of FoxP3 mRNA was significantly increased in CD4⁺CD25⁺ T cells during acute infection with FIV, which correlated with other measures of activity, including expression of mTGF- β and expression of suppressor function. In support of our results, other investigators have reported that FoxP3 mRNA expression was increased in the T cells from the lymphoid organs of untreated HIV+ patients when compared to patients on HAART, and it correlated with an increase in viral load (Andersson et al., 2005). However, the expression of FoxP3 mRNA was not assessed in the lymph nodes of uninfected patients in this study, making it difficult to compare these results with ours. In addition to detecting increases in FoxP3 mRNA production in Treg cells from FIV-infected cats, we detected increased expression of intracellular FoxP3 protein expression using flow cytometry, which supports the conclusion that FoxP3 was up-regulated in the CD4⁺CD25⁺ T cell compartment of cats acutely infected with FIV. In support of our studies, immunohistochemistry and confocal microscopy detected increased expression of FoxP3 in CD4⁺CD25⁺ T cells from acutely SIV-infected Rhesus Macaques (Estes et al., 2006). These authors also reported that the increased rate of viral replication at these early time-points correlated with an increase in Ki67 expression, which is an indicator of immune activation and cell proliferation. Similar to our results and those of Estes et al, others found significant increases in FoxP3 expression following infection of African green monkeys with SIV (Kornfeld et al., 2005). Here, however the increases in FoxP3 were noted at days 1 and 6 post infection, which is sooner than we could detect. However, it is noteworthy that peak viremia levels were also appeared earlier (days 3–10) in these infected primates. These authors suggested a protective role for Treg cells in their model, as infection was associated with the production of anti-inflammatory cytokines.

TGF- β is another factor that has been associated with regulatory function and modulation of the immune response. Recent studies indicate that TGF- β is expressed on the surface of activated Treg cells and may play a role in both Treg cell homeostasis and suppressor function

(Nakamura et al., 2001). More recently, we reported that ConA and TGF- β treatment of CD4⁺CD25⁻ T cells converted them into CD25⁺mTGF- β ⁺FoxP3⁺ Treg cells with potent suppressor function (Petty et al., 2008). In addition, we have demonstrated by flow cytometry that CD4⁺CD25⁺ Treg cells from FIV-infected cats but not control cats express TGF- β on their surface while CD4⁺CD25⁻ T cells from infected and control cats do not, suggesting cell surface TGF- β is up-regulated on activated Treg cells and can be used to distinguish activated Treg cells in FIV infected cats (Petty et al., 2008). Other researchers have also reported that TGF- β is up-regulated on the surface of activated Treg cells and may mediate regulatory function (Chen et al., 2003; Nakamura et al., 2004; Nakamura et al., 2001). Here we showed a transient increase in the surface expression of TGF- β (mTGF- β) on CD4⁺CD25⁺ T cells in the lymph nodes following infection with FIV. Membrane TGF- β expression on CD4⁺CD25⁺ T cells could mediate the subsequent induction of transcription of FoxP3 mRNA (weeks 3 and later above) and the increased suppressor function that we suggest is associated with decreased anti-viral effector responses. In the study of African green monkeys infected with SIV (Kornfeld et al., 2005), an early increase in TGF- β gene expression in the infected macaques at days 1, 3, and 13–16 post-infection was also demonstrated by RT-PCR in the total population of PBMCs, again suggesting that TGF- β may induce FoxP3 transcription in response to retroviral infections. However, changes in mRNA levels are difficult to interpret for TGF- β , as post-transcriptional and post-translational regulatory events can greatly influence the levels and function of this protein in vivo (Li et al., 2006). Interestingly, Petty et al. (Petty et al., 2008) also reported that treatment of converted Treg cells or activated natural Treg cells from FIV+ cats with anti-TGF β antibodies abrogated their suppressor function, showing that mTGF- β is instrumental in mediating Treg suppressor function. The source of TGF- β that is responsible for the functional activation of Treg cells, however, has not been identified. In support of these findings, Estes et al. (Estes et al., 2006), used immunohistochemistry techniques to detect early increases (day 7–12) in the number of TGF- β positive cells in the lymph nodes following SIV infection. Additionally, using confocal microscopy, they found that most of the TGF- β + cells co-expressed FoxP3 and were CD4⁺CD25⁺ Treg cells. In support of our studies with AIDS related lentiviruses, investigators have shown in murine autoimmune disease models that TGF- β can induce FoxP3 gene expression and up-regulate CD4⁺CD25⁺ suppressor function (Chen et al., 2003). The functional suppressor capacity of CD4⁺CD25⁺ Treg cells was evaluated in our study using a commercially available ELISPOT assay to detect changes in IL-2 production by ex vivo, Con A stimulated target cells. The inhibition of IL-2 production is a well established mechanism by which CD4⁺CD25⁺ Treg cells are thought to modulate immune responses (Muralidhar et al., 1992; Thornton et al., 2004; Vahlenkamp et al., 2004), and the ELISPOT assay has recently been used and validated as a way to assess IL-2 production in the presence or absence of regulatory T cells (thereby measuring their regulatory function) by several investigators (Chichester et al., 2006; Sojka et al., 2005). Using this assay, we were able to detect suppressor function from in vivo activated CD4⁺CD25⁺ Treg cells in FIV infected cats as early as day 3 following infection. However, a high degree of variability was noted between cats at all time-points. Some variability was also noted in the control group of cats at days 35 and 56 post-sham inoculation. Elucidating the reasons for some cats to show more suppressor function than others in response to the same viral stimulus may have important implications regarding virus burden and the pathophysiology of lentivirus infections in general.

In conclusion, data from this acute phase FIV infection suggest that regulatory T cells may be productively infected, phenotypically activated (FoxP3⁺, TGF- β ⁺), and functionally able to suppress early anti-viral CD4⁺ T helper cell responses, which may contribute to the establishment of a chronic viral infection. In addition, the anergic properties of this cell subset provide a possible reservoir of infection, where the virus can survive and replicate in low levels without becoming a target of the immune response. Future studies are needed to determine the effects of depleting this cell subset before, during and after infection with FIV. Better ways of

differentiating activated T helper cells from regulatory T cells during the early phase of infection will also need to be explored.

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Figure 1. Relative quantification of viral load in subsets of feline lymphocytes

Sorted populations of $0.5-3 \times 10^6$ CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from blood (BL) and lymph nodes (LN) were maintained in RNA later (Qiagen) and evaluated for relative quantities of FIV-gag mRNA using the delta delta CT method of quantification in relation to the amount of GAPDH measured. Equal amounts of RNA were used in each test. The calibrator sample consisted of a mixture of RNA samples obtained from all groups of cells so that the same reference sample could be used in all of the assays. At each time point, cells from 2–8 cats were evaluated individually, and the bar represents the mean FIV mRNA fold increase of all samples tested. Each sample was run in duplicate wells of a 96-well plate and the entire assay was repeated twice using the same purified RNA samples. The overlaid line graph represents the mean plasma viremia levels from each group of cats at each time.

Figure 2. Ratio of FIV mRNA copies in CD4⁺CD25⁺ to CD4⁺CD25⁻ T cells

Relative amounts of FIV mRNA copies were measured in T cell subsets from 2–8 cats as presented in Figure 1. The mean of these values was tabulated for each subset (CD25⁺ or CD25⁻ T cells in blood or lymph nodes) for each time point and the ratio of FIV copies in CD4⁺CD25⁺ vs. CD4⁺CD25⁻ cells over time is represented.

Figure 3. CD4:CD8 T cell ratios decrease in the blood of FIV infected cats

The numbers and ratios of CD4⁺ to CD8⁺ T lymphocytes were calculated for each cat evaluated at each time-point during the first 8 weeks following FIV or sham inoculation. Blood and lymph node cells were isolated and evaluated by flow cytometry as described in the methods. Each bar represents the mean and standard deviation of values obtained from 8 FIV-infected cats at each time point.

Figure 4. Infection of cats with FIV has no affect on the percent of CD4⁺CD25⁺ T cells over time
 The percentages of CD4⁺CD25⁺ T cells from the PBMC samples were determined for each cat by flow cytometric analysis. Each point represents the mean and standard deviation of values for 8 cats in each FIV-infected group and 2 cats in each control group.

Figure 5. Relative quantification of FoxP3 mRNA in feline lymphocyte subsets

Sorted subsets of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells isolated from FIV-infected and control cats were maintained in RNA Later (Qiagen) for analysis of FoxP3 mRNA levels by reverse transcription and quantitative real-time PCR. Equal amounts of RNA were used in each test. The relative levels of FoxP3 mRNA in the CD4⁺CD25⁺ T cells from FIV-infected cats changed significantly over time. In the blood, these cells exhibited a significant increase in FoxP3 transcription at 5 and 6 weeks post infection ($p < 0.1$). Bars represent the mean and standard deviation of 1–8 cats.

Figure 6. Intracellular expression of FoxP3 in feline lymphocytes during acute FIV infection

Total cell samples from blood (BL) and lymph nodes (LN) were routinely stained for surface expression of CD4 and CD25 molecules, fixed, permeabilized, and incubated with anti-FoxP3 antibodies (1 μ l/tube). The cells were analyzed by flow cytometry and the mean percent and standard deviation of FoxP3+ cells from 4 cats is represented for each T cell subset at each time point (a).

The mean fluorescence intensity of FoxP3 staining in CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell subsets from the blood and lymph nodes of FIV-infected cats was analyzed. The bars represent the mean and standard deviations for 4 cats at each time point. FoxP3 expression was significantly increased in CD4⁺CD25⁺ T cells from the lymph nodes at weeks 3, 4, and 8 post infection ($p < 0.05$) (b).

Histogram overlays of representative samples for weeks 1, 2, and 5 (c).

Figure 7. Surface expression of TGF-β on feline CD4⁺CD25⁺ lymphocytes

The surface expression of TGF-β on feline CD4+CD25+ lymphocytes from the lymph nodes of FIV-infected and control cats was evaluated by flow cytometry in duplicate samples. Each data point represents the mean and standard deviations obtained in each group of cats (n=8 FIV-infected and n=2 control cats) at each time point.

Figure 8. Relative quantification of TGF-β mRNA in feline lymphocyte subsets

The relative levels of TGF-β mRNA were measured in sorted blood and lymph node cells from each cat by reverse transcription and RT-PCR. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cell subsets from FIV-infected cats are depicted separately in each graph. Each bar represents the mean relative fold increase using the delta delta CT method of quantification for 1–8 samples obtained at each time point.

Figure 9. CD4⁺CD25⁺ Mediated Suppression of IL-2 by ELISPOT assays

2×10^5 sorted CD4⁺CD25⁻ (target cells) and 2×10^5 sorted CD4⁺CD8⁻ (APC) were stimulated with Con A ($5 \mu\text{g}/10^6$ cells) overnight at 37° C and 5% CO₂, washed and plated in each well of the 96-well feline IL-2 ELISPOT plate provided by the manufacturer (R&D Systems). 2×10^4 sorted, un-stimulated CD4⁺CD25⁺ (Treg cells) were added to some wells to obtain a E:T cell ratio of 0.1. The plate was incubated at 37° C and 5% CO₂ for 20 hours, washed and developed according to the manufacturer's instructions. Human recombinant IL-2 and media only wells were used as positive and negative controls in each plate. Samples were tested in duplicates and the average number of spot forming cells per test condition was recorded. Percent suppression was calculated as the average number of SFC/well in target only wells minus the average number of SFC/well at the E:T=0.1 ratio divided by the average number of SFC/well in the target only wells for each cat. Each marker represents the percent of suppression from Treg cell function from an individual cat, at each time-point. The box and whisker plots show the median, 25 and 75 percentile values for a group of cats at each time-point.

Table 1
Quantification of FIV-gag mRNA in plasma samples by real-time RT-PCR.

Copies of FIV-gag mRNA per ml of plasma								
Cat number	Weeks post inoculation							
	0	1	2	3	4	5	8	
1	0 ^a	33	570	42	952	142	720	
2	0	43	1184	110	44	13	164	
3	2	13	569	47	0	32	113	
4	0	27	4584	13	142	32	335	
5	NT	4	12	3	0	10	541	
6	NT	9	2	16	21	4	557	
7	NT	5	33	1974	11	1	158	
8	NT	0	6378	1	4	21	99	
Mean plasma viremia	1.1	16.7	1666.6	275.8	146.7	31.9	338.38	

^a values less than 5 are below the test's accurate level of detection