ABSTRACT

MEXAS, ANGELA MARIE. $CD4^+CD25^+$ Regulatory T Cells Are Infected and Activated Phenotypically and Functionally During Acute Infection with Feline Immunodeficiency Virus. (Under the direction of Drs. Wayne and Mary Tompkins.)

HIV-induced immunodeficiency may be mediated by the activation of immunosuppressive $CD4^+CD25^+$ T regulatory cells (Tregs). Tregs have been shown to regulate $CD4^+$ and $CD8^+$ immune responses to HIV and FIV antigens in vitro. We used the feline model to test the hypothesis that Tregs become infected and activated during the acute stage of FIV infection leading to the suppression of $CD4^+$ T helper cell responses. Cats were experimentally infected with FIV-NCSU^{-1} and blood and lymph nodes were collected at 1 week intervals following inoculation. Real-Time RT-PCR was used to determine plasma viremia and the relative number of FIV copies in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets. Flow cytometry was used to assess the percent and absolute numbers of each cell type and the expression of activation markers on T cell subsets. Real-time RT-PCR was also used to assess relative increases in FoxP3 and TGF-β mRNA levels over time. Treg suppression of IL-2 production in $CD4^+$ T helper cells was assessed by ELISPOT assays and inhibition of cellular proliferation was assessed by tritiated thymidine and CFSE assays. Our results show that peak plasma viremia levels correlate with maximal infectivity in lymph node $CD4^+CD25^+$ populations. FIV-gag-mRNA levels are higher in $CD4^+CD25^+$ T cells than $CD4^+CD25^-$ lymph node T cells. Induction of FoxP3 and TGF-β expression in $CD4^+CD25^+$ cells correlates with peak plasma viremia and FIV-gag-mRNA levels in $CD4^+CD25^+$ T cells. Increased expression of FoxP3 and TGF-β suggests Tregs are activated during acute infection with FIV.

Tregs activated in vivo were capable of suppressing IL-2 production and cell proliferation of CD4+ T helper cells during the acute phase of FIV infection. Our findings support the hypothesis that early activation of immunosuppressor function in Treg cells may limit an effective anti-FIV response contributing to the establishment of a chronic infection and the immunodeficiency caused by this virus.

CD4^{+}CD25^{+} Regulatory T Cells Are Infected and Activated Phenotypically and Functionally During Acute Infection with Feline Immunodeficiency Virus.

by

Angela Marie Mexas

A dissertation submitted to the Graduate Faculty of

North Carolina State University

In partial fulfillment of the

Requirements for the degree of

Doctor of Philosophy

Immunology

Raleigh, NC

2007

Approved by:

Mary B. Tompkins, DVM, PhD Chair of Committee	Wayne A. Tompkins, PhD Co-chair of Committee
Gregg Dean, DVM, PhD	Linda Martin, PhD

Robert Franks, PhD
University Representative

DEDICATION

To life,

The good, the bad, and whatever is still to come…

BIOGRAPHY

Angela Marie Mexas Roush was born and raised in Mexico City, Mexico. At the age of 14 years she moved to the United States with her parents and attended high school in Scottsdale, Arizona. She obtained a B.S. in Microbiology in 1995 and a Doctorate in Veterinary Medicine in 1999 from Colorado State University in Fort Collins, Colorado. She completed two internships in small animal internal medicine and surgery.

Doctor Mexas was admitted into the Clinician Investigator Program at North Carolina State University in 2001. She completed a residency and is a board certified specialist in Small Animal Internal Medicine. She joined the Tompkins lab in 2005, where she began studying the immunology of FIV as a model for HIV and AIDS. She plans to continue this work and pursue a career in research and medicine in academia.

ACKNOWLEDGMENTS

I would like to thank Drs. Wayne and Mary Tompkins for giving me the opportunity to join their laboratory, for having confidence in my abilities, and for teaching me how to be a scientist. To everyone in the Tompkins, Dean, and Martin laboratories; thank you for your knowledge, guidance, technical support and friendship.

I would also like to thank the Faculty and Staff of Small Animal Internal Medicine at North Carolina State University's College of Veterinary Medicine for their support, knowledge, patience.

Thank you:

To my father, for teaching me nothing is ever good enough.

To my mother, for her unconditional love and support.

To my brother, Christopher, for teaching me how to apologize for my mistakes, and how to laugh uncontrollably.

To my best friend, Pilar, for teaching me how to be a good and happy person.

To Shane, for everything.

To the cats at home, in the hospital, and in the lab; for their innocence, patience, and joy.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

Chapter 2

INTRODUCTION

The Human Immunodeficiency Virus (HIV) is the primary cause of acquired immunodeficiency syndrome (AIDS), which currently affects approximately 40 million people worldwide. The Feline Immunodeficiency Virus (FIV) is a well established animal model of HIV with morphologic, physiologic and pathologic similarities to both HIV and AIDS. The ability of both of these retroviruses to irreversibly damage the hosts' acquired immune system shortly after infection is established likely contributes to their pathogenesis. Therefore, studying the early phases of infection (i.e. when or why a host allows the virus to establish a productive infection within the cells of the immune system) should give us insight into possible ways of eliminating the virus early on or making more effective vaccinations. $CD4^{+}CD25^{+}$ regulatory T cells (Tregs), a unique subset of T lymphocytes may play a significant role during the early effects on the immune response and the establishment of a persistent infection. Tregs are anergic and resistant to activation induced cell death, constituting a potential reservoir for HIV and FIV. Tregs have already been shown to play a role in the immune dysregulation that is associated with HIV and FIV infections. However, it is currently not known if or at what time following the host's infection these cells become infected with the virus. Furthermore, it is currently not known what effects viral infection would have on the activation and function of Tregs as suppressors of anti-viral T cell responses. In order to determine the role of regulatory T cells in the acute phases of HIV/FIV infection we have used the FIV model to study the hypothesis that $CD4^+CD25^+$ Regulatory T cells become infected with FIV within the first 12 weeks after inoculation and subsequently express altered phenotypes which correlate with the activation of regulatory function and lead to changes in $CD4^+$ T helper cell effector functions. Peripheral lymph nodes and blood samples were collected from experimentally infected cats and these samples were used in experiments designed to determine the viral levels, activation status, and function of Treg cells. The results of these experiments have potential implications in future vaccination and therapeutic strategies against HIV and FIV and contribute to our current understanding of regulatory T cell function as modulators of an immune response.

BACKGROUND AND SIGNIFICANCE

A. LENTIVIRUS INFECTIONS

The lentivirus genus of human and animal retroviruses includes a group of RNA encoded viruses characterized by the induction of slow, progressive degenerative diseases associated with severe immune suppression. Table 1 provides a comprehensive list of retroviruses and their classification, including seven species-specific lentiviruses. It is now recognized that there are important differences in cell tropism, disease progression, and immune dysregulation within and between specific types of these important lentiviruses. Their similarities, however, provide the basis for both their classification into one genus and their use as animal models of human disease.

Clinically, the lentiviruses are associated with long incubation periods, persistence in the face of vigorous immune responses, multi-organ disease and a fatal outcome (1). Transmission occurs through direct contact of body fluids containing infectious virus particles (2). Infection is followed by a transient period of malaise, fever and a variable lymphadenopathy that may pass unrecognized. Acute infection is followed by a prolonged but variable period of latency characterized by lower viral burdens, slowed viral replication and a progressive immune dysfunction (3). Interestingly, during this phase there is evidence of innate, humoral, and cell-mediated anti-viral immune responses. In fact, there is a rise in virus specific $CD8^+$ T cell numbers that corresponds with the sharp decrease in plasma viremia, which suggests a potential mechanism to clear the virus early after infection (4). However, this process is interrupted early on, and in most cases the virus is able to persist at low levels in quiescent cell populations (5).

1. The Human Immunodeficiency Virus (HIV)

The Human Immunodeficiency Virus (HIV) is the primary cause of the acquired immunodeficiency syndrome (AIDS) in people, a slow, progressive disease of the immune system, which despite national and international efforts is currently estimated to infect more than 40 million people worldwide(6). Research efforts during the last 20 years have contributed greatly to our understanding of the virus, its pathophysiology, transmission and therapy. Nevertheless, while current therapies (HAART) delay the time of progression from HIV infection to AIDS and reduce the viral load to undetectable levels for variable periods of time, HAART is still unable to cure the infection or prevent the ultimate outcome (7). Furthermore, while we know more about the ways in which the virus can evade the attack of the immune system we have yet to successfully counteract these viral strategies to eliminate infection. The reasons for our failure to cure or prevent the infection include the ability of HIV to constantly alter its immunogenic proteins after it infects cells of the immune system, and the ability of HIV to irreversibly damage the acquired immune system shortly after infection is established. Therefore, a better understanding of the early viral-host interactions, including how and where the virus establishes an early persistent infection should give us much needed insight into possible ways of eliminating the infection and preventing the disease.

HIV is a lentivirus that carries two copies of its genetic material in two strands of RNA that encode for a total of 9 genes. The virus has been shown to infect primarily T lymphocytes, but can also be found in monocytes/macrophages, dendritic cells and certain cells of the central nervous system (8). The most commonly recognized mode of transmission in humans is through sexual contact, but inoculation through infected needles, blood products and in-utero are also commonly reported (6).

Acute infection in humans is characterized by a sharp rise in plasma viremia, which elicits strong, but usually short-lived or ineffective $CD4^+$ $T_{(h)}$ and $CD8^+$ $T_{(CTL)}$ cell immune responses (9). A decrease in acute-stage viremia correlates with a $CD8^+$ anti-HIV response, and a strong $CD8^+$ response is important in the control and possible elimination of virus following infection (10). The acute phase is followed by an established long-term asymptomatic infection during which the virus replicates at low levels in quiescent cell populations. During this asymptomatic phase the patient develops the hallmark lesion of HIV infection which is an inversion of the $CD4^+$ to $CD8^+$ T cell ratio due to an early expansion of $CD8^+$ T cells and a progressive loss of $CD4^+$ T cells (11). This progressive $CD4^+$ T cell loss is in great part responsible for the late stage immunodeficiency that causes the patient to succumb to opportunistic infections and the development of AIDS. However, T cell immune dysfunction is also evident even before a marked loss in $CD4^+$ T cells occurs, suggesting that other immunological mechanisms may be in play. There are at least two aspects of immune dysregulation associated with HIV infection that are unrelated to the low $CD4^+$ cell count; a chronic, global T cell immune hyper-activation, and paradoxically a hypo-responsiveness to HIV antigens (12, 13). The T helper cell immune hyper-activation as manifested by loss of CD62L expression and up-regulation of cell surface activation markers (e.g. CD49d and CD44) progresses through the course of the disease, and is positively associated with viral load and negatively associated with $CD4^+$ T cell counts. In fact, T cell activation has been suggested to be at least as powerful a predictor for disease progression and death as either $CD4^+$ count or viral load alone (14). Mechanisms leading to $CD4^+$ and $CD8^+$ T cell hypo-responsiveness to HIV epitopes have not been fully elucidated (13). However, it is known that these HIV-specific $CD4^+$ and $CD8^+$ clones are not deleted but appear to be anergic to immune activation, as T cells produce IFN-? in response to HIV epitope stimulation (15, 16). Importantly, however, these cells do not produce IL-2 or proliferate under the same stimulatory conditions.

One cell population recently implicated in mediating virus specific T cell anergy is the immunosuppressive $CD4^+CD25^+$ Treg cell. The hallmark characteristic of $CD4^+CD25^+$ Treg cells is their ability, once activated via TCR stimulation, to interact with other antigen-activated $CD4^+$ T_h and $CD8^+$ T cells and prematurely abort their expansion by inducing anergy, thus preventing an effective immune response (17). There is evidence that $CD4^+CD25^+$ T reg cells support a productive HIV infection and may be functionally activated (18-20). If Treg cells are infected and chronically activated in these lentivirus infections, it is possible that there is a cause and effect relationship between the virus infection of Treg cells, expression of the immunosuppressor phenotype and the resulting T cell immune dysfunction.

However, the relationship between virus infection and Treg cell activation has not been established. Therefore, our current research efforts are aimed specifically at determining the role of these cells in harboring virus and mediating immune dysfunction during the acute phase of infection, when T cell immune dysfunction is first evident.

HIV infection is not associated with specific clinical signs at the time of transmission. In fact, antibody seroconversion, which is routinely used to detect evidence of infection, may not appear positive in humans for up to six months following infection. Most people with HIV carry the virus for prolonged and highly variable periods of time before it is detected. In addition, people are reluctant to seek medical attention immediately following exposure to the virus. For these reasons, as well as the obvious ethical obstacles, it is impossible to study the early phases of infection in humans. Several animal models for the study of HIV have been established in recent years. One such model, FIV infection of domestic cats has been shown to mimic many characteristics of HIV and AIDS in humans.

2. The Feline Immunodeficiency Virus (FIV)

The feline immunodeficiency virus (FIV) of domestic cats is a well established model for the study of infections with human immunodeficiency virus (HIV) and the related immunodeficiency syndrome (AIDS) because HIV and FIV share morphologic, physiologic and pathologic characteristics (21, 22). FIV is also a retrovirus, lentivirus which causes a wide range of diseases associated with immune deficiency in cats (23). The virus has been detected in domestic and wild feline species worldwide. It has been estimated to have a prevalence of anywhere from 1-12% in domestic cats, but the prevalence in sick cats may be between 21 and 44% according to some estimates (24). Male, intact, outdoor-indoor cats are at greatest risk of infection as it is believed that transmission is primarily through bite wounds (21). Infected cats develop an acute infection syndrome which includes high viremia, a low-grade fever and transient lymphadenopathy that is followed by a long asymptomatic period in which CD4:CD8 T cell ratios decline due to an expansion of CD8+ T cells and a progressive loss of $CD4^+$ T cells (3). Many of the subsequent consequences that claim the cats' lives are also similar to those in HIV positive people progressing to full-blown AIDS and include opportunistic infections, lymphoproliferative diseases and neoplastic disorders (25). Similar to HIV in humans, FIV infects T lymphocytes, monocytes/macrophages, and CNS glial cells (26).

The feline immune responses and immune dysregulation are also similar as what is seen with HIV in humans. The feline hosts' immune response to FIV has been shown to include both humoral and cell-mediated aspects of defense. Sero-conversion has been reported at one to five weeks following infection, but neutralizing antibodies are generally not produced (27, 28). Furthermore, when detected, the presence of neutralizing antibodies has been shown not to be associated with virus clearance or lack of disease progression (29-31). In these studies, symptomatic and asymptomatic cats were shown to have similar virus titers. In other studies, however, a more rapid disease progression was noted in cats lacking neutralizing antibodies, and sera transferred from vaccinated or FIV-infected cats did convey some degree of protection to cats experimentally challenged with FIV (32-34). A cell-mediated response is also evident as CD8$^+$ T cells proliferate and produce IFN-γ in response to FIV (35, 36), and cytotoxic FIV-specific T lymphocytes can be induced following immunization with synthetic and constructed peptides (37, 38). Following infection with FIV, there is an increase in circulating CD8$^+$ T cells that is sustained throughout the asymptomatic phase of disease (28, 39). Cytotoxic (38, 40, 41) and non-cytotoxic (37, 42-44) cell mediated mechanisms have been demonstrated and linked to decreases in plasma viremia (42, 45, 46). First, it was demonstrated that FIV is more readily isolated from cultures depleted of CD8$^+$ T cells, and the addition of anti-FIV CD8$^+$ T cells inhibited FIV infection in FCD4E cells (a feline T lymphocyte cell line) in vitro (47). Anti-FIV activity was detected by 6 weeks post-inoculation and correlated with decreasing virus loads at 12, 16, and 32 weeks post infection (42). Anti-FIV activity resided principally in CD8β^{low} populations during both acute and chronic infections (45). These cells were later shown to be L-selectin (CD69L) negative and CD44,CD49, and CD18 positive consistent with a unique activation phenotype (43) and responsible for IFN-γ production in response to FIV antigens (35).

Importantly, recent efforts in our laboratory have shown that cats, similar to humans, also have a CD4$^+$CD25$^+$ population of lymphocytes which suppress T cell proliferative and effector functions (14). These Treg cells are activated in vivo in cats chronically infected with FIV and are capable of suppressing IL-2 production and proliferation of stimulated T_h lymphocytes (14). Feline Treg cells are also capable of being infected with and replicating FIV in vitro and in vivo (48). Given that FIV has been shown to have morphologic, physiologic and pathologic similarities to HIV, and that cats have Treg cell subsets that can harbor FIV, this well established model provided an outstanding system to study the role of Treg cells on $CD4^+$ T_h immune responses during acute phase lentivirus infection.

3. Other Lentiviruses as Models for the Study of HIV

Several published works have reviewed and contrasted the different animal models that have been used to study HIV pathogenesis, prevention, and treatment (49-51). While the ideal animal model may be considered one that can be infected by the same human virus, HIV-1, the virus that most commonly infects humans worldwide does not cause a persistent infection or disease in any other species (24). The most commonly utilized animal models for the study of HIV include rodents, primates and cats. Primate models include infections with HIV and SIV (Simian Immunodeficiency Virus) isolates in either, naturally or experimentally infected species. African green monkeys, chimpanzees, mandrills and mangabeys are known carriers of SIV, but infection does not induce disease in these species (51). This suggests a history of co-evolution between host and virus that has not taken place for HIV-1 in humans or FIV in domestic cats. Infection with SIV in the macaque model, perhaps the most commonly used to study HIV and AIDS, does not represent a naturally occurring infection, but mimics many characteristics of HIV and AIDS, including an acute high viremia period, followed by a sharp decline, seroconversion, and a $CD8^+$ T cell response (52, 53). Certain strains of HIV-2 have also been used to study lentivirus infections in macaques and baboons (54), (55), but this model has fallen out of favor because SIV is genetically more closely related to HIV-1 (51). Recent advances in the use of rodent-human chimeras have made the SCID mouse model another very useful tool in the study of HIV pathogenesis that continues to evolve as new techniques develop (56). FIV is the only naturally occurring infection that mimics the pathology, time course and outcome of HIV and AIDS in people (39).

B. MECHANISMS OF IMMUNE SUPPRESSION

The ability of lentiviruses to establish a persistent infection requires early evasion and alteration of the T cell immune system. Indeed, evidence of immune dysregulation is present before a significant decline in the number of $CD4^+$ T cells occurs (11). Aspects of this immune dysregulation include both a global T cell immune hyper-activation, and a paradoxical hypo-responsiveness to virus antigens in both HIV and FIV infections (12, 13). While the chronic low grade antigenemia that is characteristic of these lentivirus infections could explain progressive T cell immune hyperactivation, the mechanism(s) regulating T cell hypo-responsiveness to antigen stimulation have not been fully explained. Numerous mechanisms responsible for this T cell immune hypo-responsiveness have been proposed, including cytokine dysregulation, activation-induced cell death (apoptosis), and anergy (57-59). Studies indicate these immune suppression mechanisms develop before a significant decrease in $CD4^+$ T cell numbers (59, 60). However, it is clear that loss of $CD4^+$ T cells also contributes to susceptibility to secondary infections (25, 60, 61) and there is evidence of direct killing of $CD4^+$ cells by infection with these lentiviruses (62).

However, direct killing of infected lymphocytes cannot account for the total loss of cells because of the low number of infected cells in vivo and the relatively high degree of immunosuppression.

1. Cytokine Dysregulation

Reduced expression of the so-called Th1 cytokines (IL-2, IL-12, IFN-γ) has been reported in association with HIV infection (59, 63, 64). However, a clear shift in T_h1 vs. T_h2 cytokine predominance has been refuted by several investigators (65-67). In 1997, Klein et al, reported IL-2 and IFN-γ producing T cells decreased in relation to disease progression and the number of IL-4 and IL-10 producing T cells was increased in HIV-infected individuals without AIDS compared to healthy blood donors (64). This, they believed, demonstrated a shift from T_h1 to T_h2 cytokines early in the course of disease. They also showed this early shift is followed by reduced T_h2 cytokine production in later stages of disease progression. In another study evaluating cytokine balance in association with protective vaccine-induced immunity, it was demonstrated that protected individuals mounted both T_h1 and T_h2 immune responses (68).

Evidence for cytokine dysregulation as a possible mechanism in FIV related immunodeficiency includes differences in cytokine mRNA expression in lymph node cells from infected cats compared to negative controls. A reduction of IL-12 and IL-2 and increases in IFN-γ, IL-10 and TNF-α alpha were associated with FIV infections in early studies (10). The TNF-α increase was later found to be associated with chronic activation of $CD8\beta^{low}CD62L^{-}$ T cells (36). In another study, IFN-γ and IL-10 mRNA were up-regulated in both the thymus and lymph nodes of FIV infected cats (69). Because pulmonary immunodeficiency is evidenced in FIV-infected cats by an increased incidence of respiratory tract infections, cytokine changes were also measured in fluid obtained by broncho-alveolar lavage. In these studies, increases in IFN-γ and decreases in IL-12 production from alveolar macrophages were detected in association with FIV infection (70). Cytokine dysregulation is believed to contribute to the suppression of innate and cell-mediated immune responses, resulting in the inability of FIV infected cats to mount normal immune responses to secondary pathogens. This has been demonstrated experimentally in FIV-infected cats by secondary challenge experiments with *Toxoplasma gondii* and *Listeria monocytogenes*. FIV-infected cats challenged with *T. gondii* developed severe and generalized clinical signs and displayed suppressed levels of IL2, IL6 and IL12 mRNA in lymph node cells (10, 71). In a more recent study FIV$^+$ cats infected with *L. monocytogenes* were unable to control bacterial numbers, and addition of IL15 increased NK cell activity and dramatically reduced bacterial cell counts in co-infected cats (61, 72, 73). Differences in cytokine activity levels clearly influence the hosts' immune response to lentiviruses and secondary pathogens; however, they are more likely an effect rather than a cause of chronic antigenic stimulation, immune deficiency and persistent viral infections. For example, there is some evidence that cytokine dysregulation may be a consequence of CD4$^+$CD25$^+$ T cell activation, which potently inhibits the ability of other T cells to produce IFN-γ and IL-2 (74).

2. Apoptosis

Another mechanism of immune dysregulation, increased apoptosis, was found to be selectively affecting cells in a chronic state of hyper-activation in HIV and FIV infections. Peripheral blood lymphocytes from HIV-infected individuals were shown to be primed for apoptosis or activation induced cell death (75, 76), suggesting this mechanism contributes to HIV-induced immune deficiency. Similarly, cells from FIV-infected cats demonstrated increased rates of apoptosis in culture, when compared to cells from non-infected controls, and the frequency of apoptosis correlated with $CD4^+$ T cell counts in infected cats (77). More specifically, apoptosis was seen to affect primarily cells expressing B7.1 (CD80), B7.2 (CD86), and CTLA4 (CD152) molecules on their surface in lymph node cells from FIV-infected cats (4) and could be inhibited in vitro by anti-B7 antibodies and exogenous IL-2 (78, 79). Increases in expression of B7.1 and B7.2 molecules on infected cells, also known to be expressed on activated T cells, could explain in great part how infected cells are able to interact with and affect other non-infected cells resulting in non-antigen-specific T cell effects. This, however, did not answer the original question of why there is chronic T cell activation in the first place but, as is the case in HIV and AIDS, it demonstrates that cats with FIV also suffer from a generalized immune activation that most closely correlates with disease progression.

3. Anergy

The concept of immunologic anergy as a mechanism of immune dysregulation has recently taken center stage, as it was found that $CD4^+$ T cells responded to chronic antigenic stimulation with decreased levels of IL-2 production and proliferation. Anergy is defined in immunological terms as an impaired ability to produce effector responses such as cytokine production and proliferation in response to adequate T cell receptor stimulation. Evidence for anergy as an important mechanism of immune dysfunction includes the fact that there is an early response to HIV and FIV in both $CD4^+$ and $CD8^+$ T cell compartments that appears to be lost following the acute stages of infection. It has long been noted, that one of the cytokines which is unregulated in patients with HIV/AIDS is IL-2 (80, 81), thus cells from HIV/FIV infected individuals appear to be chronically activated (reviewed in (12, 82) and unable to produce cytokines or proliferate normally (i.e. anergic by definition) (9, 59, 83). This loss of immunologic responses is long-term and does not recover in the face of increased viral replication and higher antigen levels in the later stages of disease. However, as previously stated, it has been observed that FIV specific clones are not deleted, as IFN-γ is produced when these lymphocytes are cultured with FIV antigens (84). Furthermore, a decrease in IL-2 production was noticed not only in response to FIV, but also as a potential mechanism of increased susceptibility to secondary infections such as toxoplasmosis in FIV-infected cats (71).

Anergy was also demonstrated in the case of murine acquired immunodeficiency caused experimentally by infection with LP-BM5 (a virus which does not directly infect $CD4^+$ T cells). These investigators demonstrated that an anergic response was induced polyclonaly, because IL2 production and proliferation decreased in a non-antigen specific fashion following lentivirus infection. This anergic transformation was associated with a phenotypic shift in $CD4^+$ T cells from a resting state to a unique phenotype with characteristics of both memory and activated cells that failed to proliferate or produce IL-2 (85).

So, what is responsible for this apparent anergy? One proposed mechanism is that immunosuppressive $CD4^+CD25^+$ regulatory T cells may play a role in the T cell immunodeficiency which is the hallmark of AIDS-related lentiviral infections by contributing to this generalized anergy. It had been noted that a certain T cell subset expressing CD4 and high levels of CD25 on their surface specifically blunted the response of other T cells to antigenic stimulation. Concurrently, efforts in our laboratory showed that cats, like humans, have a population of lymphocytes which suppress effector functions and proliferation of neighboring lymphocytes and preferentially replicate FIV (14). These cells, then defined as $CD4^+CD25^+$ Treg cells, were seen to represent the same percentage of T cells in the cats as had been reported in humans and rodents, and appeared to be activated by FIV in vivo. That is, freshly isolated $CD4^+CD25^+$ T cells from FIV infected cats were shown to suppress proliferation and IL-2 production in target lymphocytes without requiring ex-vivo stimulation, compared to those from non-infected cats (14). They were also shown to be capable of being infected with and replicating FIV in vitro and in vivo (48). Because these cells also harbor the characteristics of an activation phenotype, long half-life, and anergy, they are likely to function as virus reservoirs and play a role in early immune defects associated with lentivirus infections (5, 48, 86). Subsequent to our studies, several investigators reported activation of Treg cells in

HIV infected individuals(15, 16, 87). It is not known from these studies however, at what time post lentivirus infection Treg cells become activated.

C. $CD4^+CD25^+$ REGULATORY T CELLS (Treg cells)

Treg cells, although first identified as a distinct thymus derived T cell population that suppresses autoimmune T cells and maintains peripheral self tolerance (natural Treg cells), are now recognized to also be peripherally activated (adaptive Treg cells or pathogen-induced Treg cells), and modulate $CD4^+$ and $CD8^+$ immune responses to microbial pathogens, including bacteria, viruses, fungi, and intracellular parasites (13). Pathogen-induced $CD4^+CD25^+$ Treg cells can be derived from either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells in the periphery and are indistinguishable from the natural Treg cells phenotypically in that they up-regulate CTLA4, GITR, certain Toll-like receptors, CD62L, surface TGF-β, and most importantly express the repressive transcription factor FoxP3, which is required for Treg homeostasis and function (88-90). In addition, pathogen-induced regulatory T cells have been shown to be important in preventing excessive inflammation associated with the acute immune response to infectious agents (91). Recent work showed the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ Regulatory T cells in vivo requires co-stimulation from B7 molecules, as cells from B7 knockout mice failed to convert to the regulatory phenotype (Liang, 2005). This is interesting because high levels of expression of these co-stimulatory molecules have been associated with lentivirus infections, linking the activation phenotype associated with infection to the recruitment of Treg cell function. Several chronic viral infections have been linked to unregulated Treg cell activation and suppressor function including, HBV, HCV, HIV, HTLV-1, FIV, Friend virus and murine adapted HIV strains (13).

While researchers have shown a link between unregulated Treg cell function and the course of infection with these pathogens (92), the mechanism(s) of Treg activation and their ultimate effects on $CD4^+$ T_h and $CD8^+$ T_{CTL} immune responses have not been well described. In broad terms, Treg cells may play a role in the successful establishment of an HIV/FIV infection by prematurely suppressing an otherwise effective $CD4^+$ T_h and $CD8^+$ T_{CTL} immune response against the virus. On the other hand, by dampening immune responses to pathogens, activated Treg cells may limit tissue damage associated with inflammation, as has been suggested by Kornfeld et al. (93) in the case of SIV infection of Macaques. It is currently not known whether eliminating Treg cells or their function during the acute phase of HIV/FIV infection would result in a more robust and protective immune response(15, 16, 94) or whether an unregulated inflammatory response would contribute to T cell immune hyper-activation, greater viral replication and severe inflammation. The study described herein should shed some light on this dilemma by determining if Tregs are activated to suppress early in course of FIV infection. Future studies should be aimed at determining the effects of altering Treg activation and function during the acute phase of FIV infections.

1. Role of CD25 and IL-2 in Treg Function

Tregs are phenotypically characterized by high levels of expression of CD25, the IL-2 receptor (IL-2R) alpha chain, on their surface (Sakaguchi 1995, 2004). However, CD25 is also expressed on the surface of other, non-regulatory, activated $CD4^+$ T helper cells making it difficult to distinguish regulatory T cells from activated helper T cells by surface phenotype alone (95). The presence of high levels of CD25 on the cell's surface suggests a possible role for IL-2 in Treg function. It is now known that IL2-IL-2R interactions are necessary during thymic development and peripheral expansion of Treg cells and IL2 is involved in regulating the number of Tregs in vivo (96, 97). However, with respect to their function, studies had shown that adding high levels of IL-2 to in-vitro suppressor assays containing $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ target cells abrogates the inhibitory function of Tregs, and transfecting CD25 into $CD4^+CD25^-$ T cells does not convey regulatory function (98). Nevertheless, it was later found by the same investigators that IL-2 is essential in the activation of $CD4^+CD25^+$ Treg cell suppressor function (99). Another group concurred that IL-2 is required for the activation of suppressor function of Tregs in vitro and added that both recombinant IL-2 and Tregs cells individually resulted in the same effects on target $CD4^+CD25^-$ T cells, namely the inhibition of IL-2 production from these cells (100). Lastly, it was suggested that as $CD4^+CD25^+$ Tregs do not entirely eliminate IL-2 production from target cells, and they are unable to produce IL-2 themselves, $CD4^+CD25^+$ Treg cells are in fact competing with their target cells for IL-2 (101).

The suppression of IL2 production by target cells is an important mechanism of Treg function and blocking CD25 signaling on Treg cells using monoclonal antibodies (102), or IL2 binding proteins (103) results in their functional inactivation. Therefore, IL2 and its cell receptor (which includes CD25) are key players in the identification, activation, and regulatory effects of Treg cells. It is also now known that HIV infection increases the binding of NFkB to the viral genome, resulting in increased viral replication and the expression of elevated CD25 levels on T cells, suggesting another mechanism that links lentiviral infection with the expansion of Treg cells (20). These findings suggest that IL-2 signaling through CD25 is important in regulatory function, and changes in the number or function of $CD4^{+}CD25^{+}$ T cells may influence an immune response to infectious pathogens. In addition, if lentivirus infection directly affects the expression of CD25 in infected T cells, these infections may be complicated by changes in regulatory function.

2. Role of FoxP3 in Treg Function

The intracellular fork-head family transcription factor (FoxP3) is the most specific marker of regulatory cells that has been identified to date (104, 105). Naturally occurring mutations of this gene in humans and mice (106) were known to result in well-characterized auto-immune disorders, suggesting that the protein is important in the regulation of immune responses to self antigens. It is now well recognized that FoxP3 is important in the development, maintenance, and function of $CD4^{+}CD25^{+}$ T cells with regulatory functions (107, 108). Briefly stated, signals in the thymus (from stromal or dendritic cells) are probably responsible for determining the regulatory lineage of a subset of $CD4^+$ T cells during development (109, 110). At later stages of differentiation, FoxP3 gets induced by a signal that includes IL-2 and TGF-β participation (111). The expression of FoxP3 in these cells then results in the downregulation of the IL-2 promoter and the upregulation of other Treg-associated surface proteins such as CTLA4 and GITR (112). Then, contact between these molecules on FoxP3 expressing Treg cells and their receptors on target $CD4^+$ effector cells signals them to reduce IL-2 production inhibiting target cell proliferation (113). The mechanisms involved in each step are beginning to be elucidated, but are beyond the scope of this work (110, 112-114).

In the periphery, certain feline and human $CD4^+CD25^-$ T cells can be converted into Treg cells in the presence of IL-2 and TGFβ (115, 116). These induced Treg cells also upregulate FoxP3 expression and develop suppressor functions (116). In fact, $CD4^+CD25^-$ T cells experimentally transfected with FoxP3 genes will incur regulatory function and have decreased IL-2 production and mitogenesis in response to TCR stimulation. While the expression of FoxP3 is not confined to $CD4^+CD25^+$ T cells in humans (117), it is believed that FoxP3 expressing $CD4^+CD25^-$ T cells constitute a reservoir of committed regulatory cells in the periphery (118). In addition, it has been shown that genetically reprogrammed FoxP3 expressing lymphocytes become increasingly susceptible to HIV infection via an unpregulation of CCR5, which follows FoxP3 induction (119). CCR5 is one of the primary virus binding proteins on the surface of T cells. Therefore, an increase in FoxP3 expression has been associated with increased regulatory function and has been used to demonstrate the activation of Treg cells.

3. Role of TGF-β in Treg Function

Transforming growth factor-β is a large dimeric protein that is produced by many cell types in a latent form and, once activated, is known to have important functions in developmental and immunologic processes (120, 121). Its role in preventing autoimmunity was made evident by the observation of TGF-β knockout mice, which consistently develop autoimmune disorders (122). It has long been recognized as a potently suppressive cytokine, and therefore, has been widely studied for a possible role in Treg function. While soluble factors have generally not been thought to be sufficient for Treg function, TGF-β exists as a secreted protein as well as on the cell surface of a variety of cell types, including $CD4^+$ lymphocytes, suggesting a possible mechanism for cell contact mediated suppression through TGF-β signaling. Several investigators have shown that TGF-β plays and important role in the development and induction of Treg cells in both the thymus and periphery. However, the cellular source of TGF-β in such environments has not been clearly defined and whether TGF-β is required for regulation of effector cells is still up for debate. What is widely known is that addition of TGF-β to in vitro systems leads to the induction of FoxP3, which subsequently correlates with increased regulatory function (89, 90, 123). Therefore, changes in TGF-β expression, production, or activity are likely to reflect changes in regulatory function.

4. Regulatory T Cells in Lentivirus Infections

The potential role of Treg cells in HIV and FIV infection has recently been explored by several independent groups (15). Vahlenkamp et al (14) were the first to describe $CD4^+CD25^+$ Treg cells in the cat, and the first to report that Treg cells are chronically, phenotypically, and functionally activated in FIV-infected cats. Vahlenkamp et al. demonstrated that unstimulated, purified $CD4^+CD25^+$ Treg cells from FIV-infected but not control cats expressed cell surface activation markers and are capable of suppressing IL-2 production and proliferation of stimulated $CD4^+CD25^-$ Th cells.

In the case of HIV infection, it has been demonstrated that ex-vivo depletion of $CD4^+CD25^+$ T cells from PBMC's from HIV infected individuals results in an increased percentage of IFN-? and TNF-a producing cells in response to HIV peptide stimulation in vitro (16), suggesting $CD25^+$ T cells suppress the T cell immune response to viral antigens. Others have demonstrated that $CD4^+CD25^+$ T cell depletion increased HIV-p24-specific CD4 T cell proliferation induced by IFN-?-generated dendritic cells in vitro (87). In addition, $CD4^+CD25^+$ T cells suppress cellular proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells stimulated with HIV peptides in vitro; and these effects were cell-contact dependent and IL-10/TGFß independent (15). Another group determined that there is a differential distribution of Treg cells in HIV infected patients, with increased numbers of Treg cells in the lymphoid tissues compared to peripheral blood (92). Here, a positive correlation between FoxP3 expression (increased Treg activity) in lymphoid tissue and plasma HIV viral load was demonstrated.

Using a simian model for human AIDS, Kornfeld et al (93) reported an early increase in $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells following SIV infection of African Green Monkeys that correlated with an early TGFß and IL-10 response. Interestingly, in the more pathogenic SIV-Macaque infections, there was no early $CD4^+CD25^+$ and TGFß responses, which correlated with greater inflammation and disease, leading to the speculation that the greater pathogenicity in the SIV-Macaque infection is due to the failure to activate Treg cells and control acute stage inflammation. In contrast to Kornfeld at al, Estes et al. (124) reported that SIV infection of Macaques induced an early immunosuppressive response that correlated with a marked increase in the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells and TGFß1 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 during the acute stage of infection. For example, it is currently not known what effect lentivirus infection of Treg cells has on their function and/or survival. Also unknown are the consequences of impairment or, in contrast, activation/expansion of these cells in number or function on the establishment of asymptomatic or clinical disease.

D. SPECIFIC AIMS AND SIGNIFICANCE OF THE STUDY

Recent studies suggest that $CD4^{+}CD25^{+}$ Treg cells provide a reservoir for FIV (48) and HIV (20) replication. Evidence also suggests that Treg cells are activated and fully primed for immunosuppressive functions in FIV-infected cats (14). Whether there is a cause and effect relationship between the FIV infection of Treg cells and their activation state is not known. However, as T cell immune dysfunction is manifested during the acute stages of FIV/HIV infection, it is possible that FIV infects and activates Treg cells that in turn prematurely abort protective $CD4^{+}$ T_{h} and $CD8^{+}$ T_{CTL} immune responses, contributing to the immune dysfunction. Thus, FIV-infected and activated $CD4^{+}CD25^{+}$ Treg cells could participate not only in the establishment of a reservoir of chronic infection, but also provide a mechanism for early and persistent T cell immune suppression. This study utilized the FIV/feline model of acute infection to test the hypothesis that $CD4^{+}CD25^{+}$ Treg cells become infected with FIV and activated during the acute stage of infection, and subsequently inhibit $CD4^{+}$ T helper cell immune responses. We believe these activated immunosuppressive cells contribute to the early immune dysfunction that is associated with FIV/HIV infections prior to significant decreases in $CD4^{+}$ T cell numbers.

Specific Aim #1. $CD4^{+}CD25^{+}$ Treg cells become infected with FIV within the first 12 weeks after inoculation of cats with FIV-NCSU^{-1}.

To test the hypothesis that $CD4^{+}CD25^{+}$ T cells become infected with FIV during the acute phase of infection, PBMC's and LN cells will be collected at one week intervals from cats before and after FIV-NCSU^{-1} or sham inoculation, stained, and sorted for $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations using a high speed cell sorter (Mo-Flo). Distinct populations will be analyzed for viral FIV-gag mRNA using real time RT-PCR to detect infection at each time interval. Three and four-color flow cytometry will be used to correlate FIV-gag mRNA expression with cell surface phenotype as described in SA2. Plasma viremia will also be measured by RT-PCR at each time interval.

<u>Specific Aim #2.</u> $CD4^+CD25^+$ Treg cells become phenotypically activated during the acute stage of FIV infection.

This aim will determine phenotypic and functional changes in PBMC and LN $CD4^+$ populations before and after FIV-NCSU^{-1} or sham inoculation. Three- and four-color flow cytometry will be used to assess surface phenotype (CD25, CTLA4, B7.1, and CD62L) of PBMC and LN $CD4^+CD25^-$ (Th) and $CD4^+CD25^+$ (Treg) cells during the course of acute stage FIV infection. To further assess their activation status, known functional markers for Treg cells (i.e. FOXP3 and surface TGFß1) will be assessed by PCR and flow cytometry.

<u>Specific Aim #3.</u> Infection of $CD4^+CD25^+$ Treg cells with the NCSU^{-1} isolate of FIV results in functional activation and suppression of $CD4^+$ Th immune responses to FIV antigens.

This aim will evaluate the suppressor functions (cytokine production and proliferation) of $CD4^+CD25^+$ Treg cells from acutely infected cats on stimulated $CD4^+CD25^-$ responder cell populations. The production of IL-2 by responder cells in the presence or absence of different numbers of in vivo or in vitro activated Treg cells will be assessed using a commercially available ELISPOT assay. Proliferative responses to Con A in $CD4^{+}CD25^{-}$ T_h cells in the presence or absence of in vivo activated $CD4^{+}CD25^{+}$ Treg cells will also be assessed by flow cytometry using CFSE staining and tritiated thymidine incorporation assays.

Our studies are specifically aimed at determining the role of regulatory T Cells (Tregs) in the acute phase of FIV infection. We hope the proposed experiments will not only help develop vaccination and/or therapeutic strategies for HIV and FIV but also, contribute to our understanding of Treg cell function as immune response modulators in infectious diseases.

<u>TABLE 1</u>. Human and animal retroviruses.

VIRUS TYPE	EXAMPLES
Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV) Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (MPMV) Jaagsiekie sheep retrovirus (JSRV)
Gammaretrovirus	Murine leukemia virus (MuLV) Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GaLV) Reticuloendotheliosis virus (ReV)
Deltaretrovirus	Human T-lymphotropic viruses (HTLV 1.2) Bovine leukemia virus (BLV) Simian T-lymohotropic viruses (STLV 1,2,3)
Epsilonretrovirus	Walleye dermal sarcoma virus Walleye epidermal hyperplasia virus
Lentivirus	Human Immunodeficiency virus (HIV-1) Human Immunodeficiency virus (HIV-2) Simian Immunodeficiency virus (SIV) Equine infectious anemia virus (EIAV) Feline Immunodeficiency virus (FIV) Caprine arthritis encephalitis virus (CAEV) Visna/maedi virus
Spumavirus	Human foamy virus (HFV)

MATERIALS AND METHODS

A. Acute Infection Study Experimental Design

To determine the role of Treg cells on the immunopathogenesis of AIDS during the acute stage of FIV infection, the following experiments were designed to evaluate the temporal relationship between Treg infection, activation, and immune dysregulation that are characteristic of FIV/HIV infections. The figures illustrate the design and timeline of the 2006 (Fig. 1) and 2007 (Fig. 2) acute infection studies, detailing the experiments performed on each set of samples acquired.

In order to validate our methods prior to the infection of newly acquired cats, samples were first collected from chronically infected, asymptomatic cats that had been used in previous studies. Data collected from these samples is also presented here. These studies describe the effects of the FIV infection of Treg cells on the phenotype, survivability and suppressor function of Treg cells, including changes in $CD4^+$ T cell cytokine production and inhibition of proliferation.

In addition, we monitored the effects of Treg-FIV infection and activation on the plasma viral load during the acute phase of FIV infection.

B. Cats

Specific pathogen free (spf) cats were acquired from Liberty Research, Inc. (Waverly, NY) or Harlan Sprague Dawley (Madison, WI) at 6 months of age. They were housed in groups according to guidelines from the IACUC at NCSU's laboratory animal resources facilities. All of the acute phase experiments described herein were performed on the cats at 1-2 years of age. These cats had samples collected immediately following infection as described by the timelines in figure 1.

The experiments performed to validate our methods used chronically infected, asymptomatic cats that ranged in age from 3-6 years old. These cats had been infected with FIV-NCSU^{-1} for at least two years at the time the samples for these studies were collected.

C. Infection with FIV

The NCSU^{-1} 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 (26, 28, 60). Virus stock is maintained in liquid nitrogen and a new inoculum was grown as a single cycle infection in an IL-2-dependent feline CD4^{+} cell line (FCD4 E-cells) as described by Davidson et al (60) for these experiments. For the acute infection studies, 28 cats were inoculated intravenously with $1x10^5$ $TCID_{50}$ of cell free virus culture supernatant. During the 2006 acute infection study, seven additional control cats were sham inoculated with an equal volume (100 µl) of sterile FCD4 E-cell media.

D. Lymph Node Biopsies

Acutely infected and sham-inoculated cats were divided into different groups (see Tables 1 and 2), such that popliteal lymph nodes were biopsied from four infected cats at each time interval 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 isofluorane 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 (10) and used for phenotype analysis by flow cytometry or MoFlo purification of lymphocyte subsets.

E. Purification of Peripheral Blood Mononuclear Cells

Prior to viral inoculation and at the time of each lymph node biopsy, 20 mls of blood was 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 was layered over 3 mls of Ficoll-Histopaque-1077 (Sigma-Aldrich). Following centrifugation at 300xG for 20 minutes, cells at the interface were collected, washed, and re-suspended in cell culture media. Cell counts were performed on a hemocytometer and viability was assessed by Trypan Blue exclusion at 40X magnification. Cells were immediately used for multi-color staining and flow cytometry or MoFlo purification of lymphocyte subsets.

F. Plasma Viremia

Evidence of infection was assessed on each plasma sample using a commercially available snap test ELISA’s (IDEXX laboratories) to detect feline antibodies against FIV. Quantitative real time PCR was used to determine viral 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, universal Taqman PCR Mastermix (Applied Biosystems) and the FIV-specific probe in the relative concentrations specified by the manufacturer. Primers used are listed in Table 3. 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 15seconds 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 feline plasma.

G. Phenotypic Analysis

The surface phenotype of mononuclear cells from blood and lymph nodes was determined by two and three-color flow cytometric analysis. Cells were incubated with the antibody combinations shown in Table 4. Each combination was analyzed in duplicate. 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. Absolute cell numbers were determined in some cases by multiplying the percent positive cells by the total lymphocyte counts obtained from manual differential counts from each lymph node sample.

H. Four-Way Flow Cytometry 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, Dako) 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 at -20°C for RNA extraction and PCR analysis.

I. Reverse Transcription and Real-Time PCR of T cell subsets

Purified populations of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells 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.

During the first acute phase infection study (2006) the universal Taqman PCR Mastermix (Applied Biosystems) was used for FIV, FoxP3, and GAPDH quantification according to the manufacturer's guidelines and SYBR Green Taqman PCR master mix was used for TGF-beta quantification, which does not require the use of a sequence specific probe. To confirm primer specificity prior to their use in these experiments, the PCR products from each reaction had been isolated and sequenced independently (data not shown). The specific primers used for each reaction are listed in table 3. All reactions were carried out using the same temperature programs in an ICycler PCR machine.

During the second acute phase infection study (2007) the QuantiTect SYBR Green PCR master mix (Qiagen) was used for FIV, FoxP3, TGF-β and GAPDH quantification according to the manufacturer's guidelines, which does not require the use of a sequence specific probe. To confirm primer specificity prior to their use in these experiments, the PCR products from each reaction had been isolated and sequenced by other investigators in our lab. The specific primers used for each reaction were the same as those listed in table 3, but no probes were used in this cycle.

Quantification was carried out using the delta delta CT method using GAPDH mRNA amounts as the housekeeping gene. In each acute phase infection study a calibrator sample was obtained using a mixture of products from test samples. The same calibrator samples were used in all reactions within each study in orderd 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. Due to the lag in time and the need for a large amount of calibrator sample in the second acute infection study, a new calibrator sample was formulated and used in these experiments. This reflects the differences in fold increases noted in the results for each study. Therefore, no direct comparison can be made for samples between studies, but relative quantification for each cell subset within a study can accurately be assessed.

J. 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 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 (Con A, 10 μg/million cells), a recombinant FIV-gag peptide (50 μg/million cells), or anti-CD3 antibody-coated beads (prepared according to the manufacturer's instructions) for 2-3 hours, washed once and plated at 4x10^5 cells/well in the ELISPOT 96-well plate. Earlier 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. 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. No spots were detected when target cells were not stimulated with Con A 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.

K. Staining for Intracellular FoxP3

Intracellular immunofluorescent staining for flow cytometric analysis of FoxP3 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 (Dean's lab data). $4x10^{\wedge 6}$ unsorted cells were placed in each tube and surface stained for CD4-PE and CD25-FITC prior to fixing and permeabilization. Following intracellular protein staining, cells were washed in Staining Buffer over-night and analyzed by flow cytometry the following day. Staining for FoxP3 was compared to tubes without antibody during each analysis.

L. Tritiated thymidine incorporation Assays

Inhibition of target cell proliferation by $CD4^+CD25^+$ T cells as a measure of their suppressive function was determined using a well established method that utilizes the incorporation of tritiated thymidine during cell replication to assess proliferation in a cell population. $CD4^+CD25^-$ (target cells) were stimulated with Concanavalin A (10 μg/million cells) for 2-3 hours, washed once and plated at 4x10^5 cells/well in a 96-well plate. No IL-2 was added to the media because these studies were to be compared with the ELISPOT experiments described in Chapter 1. No APC's were used in these experiments because previously published methods showed evidence of proliferation in the absence of antigen presenting cells(14). Controls consisted of wells containing target cells alone (positive control), no cells (negative control), or $CD4^+CD25^+$ T cells only. Each assay was run in duplicate wells. The cells were incubated overnight and tritiated thymidine (1μCi/well) was added for the last 18 hours of culture. Quantification of thymidine incorporation was performed using routine methods as described in the appendix. The percent suppression was calculated as the average number of counts from wells without $CD4^+CD25^+$ T cells minus the counts from those were Tregs were added, all divided by the counts obtained from $CD4^+CD25^-$ cells alone.

M. CFSE Cell Proliferation Assays

Freshly isolated $CD4^+CD25^-$ T cells were treated with CFSE prior to stimulation with Con A. Cells were centrifuged and resuspended in 1 ml of a 1μmol/L solution of CFSE per 5 million cells. The cells were incubated at room temperature in the dark for 10 minutes then iced for one minute with one ml of cold FBS. The cells were then washed three times in PBS, stimulated with Con A and plated with $CD4^+CD25^+$ Treg cells as described for the previous assay. All target cells were stained with CFSE and plated in 96-well plates. Only half of the wells were used in this assay and the other half were used for H3-Thymidine incorporation assays described above. After 4-6 days of incubation at 37° C cells were collected into tubes for FACS analysis.

N. Statistical Analysis

The data generated in experiments comparing FIV-infected to non-infected cats is considered nonparametric. Therefore, we performed Mann-Whitney U tests (t test-like analysis for nonparametric data) to determine significance between results from infected and control cats for each parameter analyzed. Differences were determined to be significant when the p value was less than or equal to 0.05.

Experiments comparing data from T cell subsets that were obtained from the same groups of cats are parametric comparisons and for these, student t tests were used to determine significant variance for each parameter analyzed. Individual paired t tests were performed in these cases and significance was determined when the p value was less than or equal to 0.05.

Tests used for statistical analysis are described in more detail within the result sections of each chapter, only for significant findings.

<u>TABLE 1.</u> Groups of cats used in 2006 acute infection study (Study #1).

SUBGROUP	TYPE	NUMBER	NAME	DATE INFECTED	DAYS OF LN BIOPSY	
A1	FIV+	C308	THEODORE	31-Jan	21	84
A1	FIV+	C302	EDGAR	31-Jan	21	84
A2	FIV+	C082	DANIEL	3-Feb	21	84
A2	FIV+	C040	ARCHIE	3-Feb	21	84
B1	FIV+	C150	DOUG	6-Feb	7	42
B1	FIV+	C024	LITTLE-TOE	6-Feb	7	42
B2	FIV+	C102	HERBIE	8-Feb	7	42
B2	FIV+	C022	OZZIE	8-Feb	7	42
C1	FIV+	C028	CLIFF	6-Feb	14	56
C1	FIV+	C016	CLYDE	6-Feb	14	56
C2	FIV+	C080	NORM	8-Feb	14	56
C2	FIV+	C018	DAVID	8-Feb	14	56
D1	FIV+	C100	JUSTIN	6-Feb	3	35
D1	FIV+	C036	TOMMY	6-Feb	3	35
D2	FIV+	C042	ROBBIE	8-Feb	3	35
D2	FIV+	C278	BILLY	8-Feb	3	35
A3	SHAM	C192	MAURICE	6-Feb	3	35
A3	SHAM	C230	BEN	6-Feb	3	35
B3	SHAM	C144	SIMON	10-Feb	7	42
B3	SHAM	C038	NICK	10-Feb	7	42
C3	SHAM	C152	HEATH	10-Feb	2	56
C3	SHAM	C034	JARROD	10-Feb	2	56
D3	SHAM	AHL1	APOLLO	10-Feb	3	84

TABLE 2. **Groups of cats used in 2007 acute infection study (Study #2).**

SUBGROUP	NUMBER	NAME	DATE INFECTED	WEEK OF LN BIOPSY	
A1	BHE3	BOBBY	23-Apr	1	4
A1	JHV3	BILL	23-Apr	1	4
A2	RGK4	BOOMHAUER	25-Apr	1	4
A2	RGL3	BARNEY	25-Apr	1	4
B1	RGL4	FLOYD	23-Apr	2	5
B1	RGM1	CHAD	23-Apr	2	5
B2	RGS1	JON	25-Apr	2	5
B2	RGS2	RICK	25-Apr	2	5
C1	RGL5	EMMET	23-Apr	3	8
C1	RGL6	MOBY	23-Apr	3	8
C2	RGS3	CHRISTOPHER	25-Apr	3	8
C2	JIX3	MUDPUPPY	25-Apr	3	8

TABLE 3. **List of Primers Used for RT-PCR in Acute Infection Studies**

	Forward primer	Reverse primer	Probe
FIV-gag	5'-GATTAGGA GGTGAGGAA GTTCAGCT-3'	5'-CTTTCATC CAATATTTCT TTATCTGCA-3'	5'-56-FAM/CATGG CCACATTAATAA TGGCCGCA/36-TAMSp/-3'
TGF-β	5'-GAGGTCAC CCGCGTGCTA ATG-3'	5' –TCTTCTCCG TGGAGCTGA AGCAAT- 3'	No probe (sybr-green)
FOXP3	5'-GCCTGCCAC CTGGAATCAAC-3'	5'-GTGTGCTGG GGCTTGGGA-3'	5'-56-FAM/CAGTGCTGG CTCCCTGGACACCCA/3BHQ-1/-3'
GAPDH	5'-GCCGTGAA TTGCCGT-3'	5'-GCCATCAAT GACCCCTTCAT-3'	5'-56-FAM/CTCAACTACATGG TCTACATGTTCCAGTAT GATTCCA/36-TAMSp/-3'

TABLE 4. Lists of Antibodies, Fluorochromes, and Sources Used

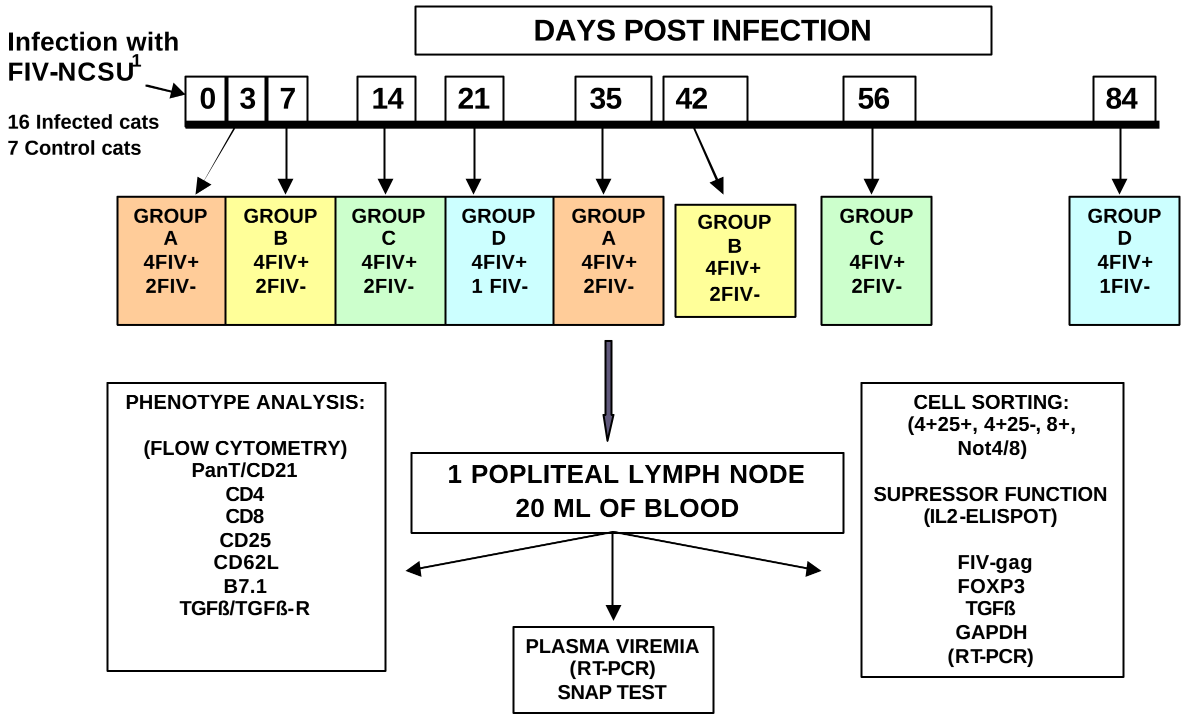
a)Panel for phenotypic analysis in 2006 acute infection study

1.Cells only/neg control
2.PanT-FITC/ CD21 PE
3.CD4biotin-PerCP/CD8-PE/CD25-FITC
4.CD8-FITC/CD62L-PE
5.CD4biotin-PerCP/CD25-FITC/CD62L-PE
6.CD8-APC/B7.1-PE
7.CD4biotin-PerCP/CD25-FITC/B7.1-PE
8.CD4biotin-PerCP/CD25-FITC/TGFβ-APC
9.CD4biotin-PerCP/CD25-FITC/TGFβRI-PE
10.CD8biotin-PerCP/CD25-FITC/TGFβ-APC
11.CD8biotin-PerCP/CD25-FITC/TGFβRI-PE

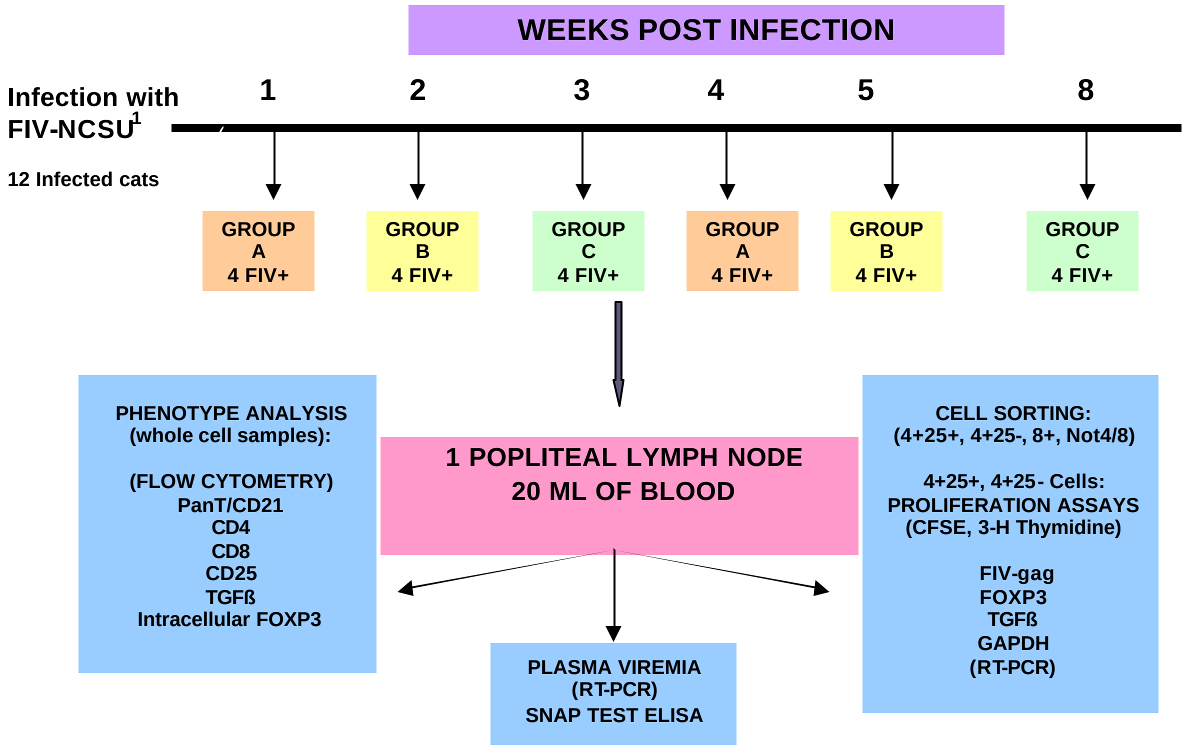
b)Panel for phenotypic analysis in 2007 acute infection study

1. Cells only/neg control
2. PanT-FITC/ CD21 PE
3. CD4biotin-PerCP/CD8-PE/CD25-FITC
4. CD4-PE/CD25-FITC/LAP-biotinPerCP
5. CD4biotin-PerCP/CD25-FITC/TGFβ-APC
6. CD4biotin-PerCP/CD25-FITC/Thrombospondin-PE

c)Antibody specificity and source		
1. PanT	anti-cat	Tompkins lab
2. CD4	anti-cat	Tompkins lab
3. CD8	anti-cat	Tompkins lab
4. CD25	anti-cat	Tompkins lab
5. CD21	anti-canine	Serotec
6. CD62L	anti-human	BD Bioscience
7. TGFβ	anti-human	R&D Systems
8. TGFβRI	anti-human	R&D Systems
9. LAP	anti-human	R&D Systems
10. Thrombospondin	anti-human	Calbiochem



<u>Figure 1.</u> Experimental design of 2006 Acute Phase Infection.
Sixteen one year old spf cats were experimentally infected with FIV-NCSU-1 and 7 spf cats inoculated with sterile cell medium as described in the methods. One popliteal lymph node and 20 mls of blood were extracted from 5 or 6 cats at each time point. Samples were used as indicated in the diagram for flow cytometry, PCR or suppressor function assays.



<u>Figure 2.</u> Experimental design of 2007 Acute Phase Infection
Twelve one year old spf cats were experimentally infected with FIV-NCSU-1 as described in the methods. One popliteal lymph node and 20 mls of blood were extracted from 4 cats at each time point. Samples were used as indicated in the diagram for flow cytometry, RT-PCR, or cell proliferation assays.

CHAPTER 1

2006

ACUTE PHASE OF FIV INFECTION STUDY

(STUDY #1)

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 acute infection with FIV, leading to the suppression of $CD4^+$ T helper cell responses. Cats were experimentally infected with FIV-NCSU^{-1} and blood and lymph node biopsies were collected at 1 week intervals following inoculation. Real time RT-PCR was used to determine relative quantities of FIV mRNA, FoxP3 and TGF-β in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets. Flow cytometry was used to assess the percent and numbers of each cell type and the expression of activation markers on T cell subsets at each time point. Treg suppression of IL-2 production in $CD4^+$ T helper cells was assessed by ELISPOT assays. The results show that peak viremia levels correlate with maximal infectivity in lymph node $CD4^+CD25^+$ and $CD4^+CD25^-$ populations. FIV-gag-mRNA levels were higher in $CD4^+CD25^+$ T cells than $CD4^+CD25^-$ lymph node T cells. Activation of FoxP3 and increased expression of TGF-ß in $CD4^+CD25^+$ cells correlated with peak plasma viremia and FIV-gag-mRNA levels in $CD4^+CD25^+$ T cells. And, the $CD4^+CD25^+$ T cells from some infected cats functionally suppress IL-2 production from stimulated $CD4^+CD25^-$ target cells. Our findings support the hypothesis that early activation of immune suppressor function in Treg cells may limit an effective anti-FIV response contributing to the establishment of chronic infection and immunodeficiency caused by this virus.

INTRODUCTION

The feline immunodeficiency virus (FIV) causes an important natural disease in domestic cats, and is a well established model for the study of infections with human immunodeficiency virus (HIV) and the related immunodeficiency syndrome (AIDS) because HIV and FIV share morphologic, physiologic and pathologic characteristics (21, 22). Both HIV and FIV infections are characterized by progression from a short acute phase with high but self-limiting viremia to a long latent, subclinical phase with low viremia, followed by severe immunodeficiency, a subsequent rise in virus levels and eventually, death from opportunistic infections or lympho-proliferative diseases. In addition to high plasma viremia, the acute stage of infection in humans and cats is characterized by low-grade fevers and transient lymphadenopathy (3). Interestingly, during this phase there is evidence of innate, humoral, and cell-mediated anti-viral immune responses. In fact, there is a rise in virus specific $CD8^+$ T cell numbers that corresponds with the sharp decrease in plasma viremia, which suggests a potential mechanism to clear the virus early after infection (4). However, this process is interrupted early on, and in most cases the virus is able to persist at low levels in quiescent cell populations (5).

The ability to establish a persistent infection requires early evasion and alteration of the immune response. Indeed, evidence of immune dysregulation is present before a significant decline in the number of $CD4^+$ T cells appears. Aspects of this immune dysregulation include both a global T cell immune hyper-activation, and a paradoxical hypo-responsiveness to virus antigens (12, 13). While the chronic low grade antigenemia that is characteristic of HIV/FIV infections could explain progressive T cell immune hyperactivation, the mechanism(s) regulating the T cell hypo-responsiveness to antigen stimulation have not been fully explained. Numerous mechanisms responsible for this T cell immune hypo-responsiveness have been proposed, including cytokine dysregulation (decreased IL2, increased IL10 and IFNγ production), activation-induced cell death (apoptosis) and clonal deletion or clonal anergy (57-59). The observation that $CD4^+$ T cells produce IFN-?, yet do not produce IL-2 or proliferate in response to HIV gag stimulation supports the evidence for clonal anergy (9). 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, although first identified as a distinct thymus derived T cell population that suppresses autoimmune T cells and maintains peripheral self tolerance (natural Treg cells), are now recognized to also be peripherally activated (adaptive Treg cells or pathogen-induced Treg cells), and modulate $CD4^+$ and $CD8^+$ immune responses to microbial pathogens, including bacteria, viruses, fungi, and intracellular parasites (13). Pathogen-induced $CD4^+CD25^+$ Treg cells can be derived from either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells in the periphery and are indistinguishable from the natural Treg cells phenotypically in that they up-regulate CTLA4, GITR, certain Toll-like receptors, CD62L, surface TGF-β, and most importantly express the repressive transcription factor FoxP3, which is required for Treg homeostasis and function (88-

90). In addition, pathogen-induced regulatory T cells have been shown to be important in preventing excessive inflammation associated with the acute immune response to infectious agents (91). However, several chronic viral infections have been linked to unregulated Treg cell activation and suppressor function including, HBV, HCV, HIV, HTLV-1, FIV, Friend virus and murine adapted HIV strains (13).

Previous work in our laboratory has established that feline regulatory T cells share phenotypic and functional characteristics of their human and murine counterparts. Vahlenkamp et al (14) was the first to report that $CD4^+CD25^+$ Treg cells are chronically activated and immunosuppressive for $CD4^+$ T cells in asymptomatic chronically FIV-infected cats. They speculated that activated Treg cells may play a role in the T cell immunodeficiency that is the hallmark of AIDS-related lentiviral infections. Subsequent to those studies, several investigators reported activation of Treg cells in HIV-infected individuals(15, 16, 87). It is not known from these studies however, at what time post lentivirus infection Treg cells become activated.

Two acute infection studies have reported using the SIV infection model. Kornfeld et al (93) reported an early increase in $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells following SIV infection of African green monkeys that correlated with an early TGF-ß and IL-10 response. Interestingly, in the more pathogenic SIV-Macaque infection, there was no early $CD4^+CD25^+$ and TGF-ß responses, which correlated with greater inflammation and disease, leading to the speculation that the greater pathogenicity in the SIV-Macaque infection is due to the failure to activate Treg cells and control acute stage inflammation. In contrast to Kornfeld et al, Estes et al.(124) reported that SIV infection of Macaques induced an early immunosuppressive response which correlated with a marked increase in the frequency of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells and TGF-ß 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 complex Treg/Th immunoregulation during the acute stage infection 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 $CD4^{+}CD25^{+}$ Treg cells become infected with FIV and express characteristics of activated Treg cells following the inoculation of cats. We report here that $CD4^{+}CD25^{+}$ cells are productively 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 starting as early as day 14 post infection; $CD4^{+}CD25^{+}$ T cells have higher viral mRNA levels than $CD4^{+}CD25^{-}$ T cells and infectivity in lymph node cells correlates with plasma viremia levels. FoxP3 transcription levels and TGF-β surface expression are also increased in $CD4^{+}CD25^{+}$ T cells by day 14, while TGF-β mRNA increases in $CD4^{+}CD25^{-}$ T cells at earlier time points. In addition, $CD4^{+}CD25^{+}$ Treg suppression of mitogen-stimulated $CD4^{+}$ responses, as measured by IL-2 ELISPOT assays, could be detected as early as day 3 post inoculation with FIV.

RESULTS

A. Chronically infected, asymptomatic cats: validation studies

In order to validate the methods proposed for the acute infection study without using additional newly infected cats, samples from asymptomatic FIV-infected cats used in previous studies were collected and used in the first set of experiments. Here we show that we are able to differentiate FIV-infected from non-infected T cell populations, and can furthermore detect variable levels of virus load in T cell subsets by PCR analysis. We also use sorted cells from chronically infected cats to validate a newly designed PCR assay for the detection of FoxP3 and the use of a feline-specific IL-2 ELISPOT assay used to detect regulatory function in $CD4^{+}CD25^{+}$ T cells. Alternative methods for the measurement of cytokine production and proliferation of T cells subsets were also explored during this phase (data not shown).

<u>1. Detection of FIV Infection in feline lymphocytes by Real-Time PCR:</u> Several PCR protocols for FIV detection have been developed and validated to date. Because our focus was on detecting infected vs. uninfected populations of lymphocytes, we chose a method that uses cellular RNA as a starting template. Using primers that had been previously designed and validated for the detection of FIV-gag mRNA in feline lymphocytes, we were able to differentiate FIV-infected from control cats (Figure 1). A similar assay using viral RNA isolated from plasma was used to assess plasma viremia in FIV-infected and control cats (data not shown).

2. Real-Time RT-PCR Detection and Quantification of FIV-gag mRNA in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell Subsets from FIV-Infected Cats: Having seen that the real-time reaction can differentiate FIV gag expression in lymphocytes from FIV-infected cats as compared to negative controls, we used the same protocol to quantify FIV-gag mRNA in $CD4^+CD25^+$ and $CD4^+CD25^-$ T lymphocytes from asymptomatic chronically FIV-infected cats or uninfected controls. Lymphocytes were isolated from the peripheral blood of an FIV+ cat and $CD4^+CD25^+$ and $CD4^+CD25^-$ cells were purified by cell sorting. As shown in Figure 2, both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells contain FIV-gag mRNA. However, the number of FIV copies was relatively higher in the $CD4^+CD25^+$ T cells than in the $CD4^+CD25^-$ T cells.

3. $CD4^+CD25^+$ T cells are capable of suppressing cytokine production by target CD4+CD25- Th lymphocytes: Treg cells function to regulate the intensity of an immune response by suppressing the effector functions of other lymphocyte populations, including $CD4^+$ Th cells. While their mechanisms are thought to vary by type of regulatory cell and system studied, evidence points towards the impairment of IL-2 production as a potential mechanism of naturally occurring $CD4^+CD25^+$ Treg function. Inhibition of target cell IL-2 production by $CD4^+CD25^+$ T cells is therefore a measure of their suppressive function, and was demonstrated in FIV^+ chronically infected cats prior to our acute infection studies. We used a commercially available, validated ELISPOT assay for the detection of feline IL-2 secreting cells at the single cell level (R&D Systems). Lymph node cells were obtained and processed by methods previously described, stained with antibodies against CD4 and CD25 and

sorted into $CD4^+CD25^+$ and $CD4^+CD25^+$-depleted populations. Concanavalin A (Con A) stimulated target cells consisting of the $CD4^+CD25^+$-depleted lymph node cells were plated with and without different concentrations of $CD4^+CD25^+$ Treg cells to achieve specific effector to target (E:T) cell ratios in different wells. Samples were plated in triplicate. The cells were incubated overnight and spots were developed following the manufacturer's protocol. Quantification of spot forming cells (SFC) was carried out using an automated spot counter. No spots were detected when target cells were not stimulated with Con A prior to the incubation. There was a negative correlation between the number of IL-2 spots and the number of $CD4^+CD25^+$ cells in each well (Figure 3) using linear regression analysis for the mean number of spots at each E:T ratio (r=0.91). Using student's T tests, the number of IL-2 producing cells was significantly different in the wells containing E:T of 0.5, 0.16, and 0.05 when each was compared to the wells without Treg cells (p= 0.00008, 0.00051, and 0.04276, respectively).

<u>4. $CD4^+CD25^+$ T cells from asymptomatic FIV-infected cats are capable of suppressing cell proliferation of target Th lymphocytes:</u> In addition to cytokine production we measured cell proliferation of target $CD4^+$ Th cell populations in response to mitogenic stimulation in the presence of Treg cells in vitro. In these experiments, the target cells were stained with a membrane staining dye (CFSE) prior to stimulation with Con A, and incubated with or without different E:T ratios of $CD4^+CD25^+$ Treg cells for 3-5 days in optimal growing conditions. CFSE staining was assessed on day 0 of culture to obtain baseline measurements. These

measurements were compared with CFSE staining of target cells at days 3-5 of culture. The percent proliferation of target cells was calculated using the following formula: 100- (%CFSE high at day 3, 4,or 5/ %CFSE high at day 0). % suppression is then measured by comparing the proliferation at each E:T to that of the target cell alone wells. Data shown in figure 4 was collected from a chronically infected, asymptomatic FIV+ cat, and demonstrates a dose dependant suppression of proliferation by $CD4^{+}CD25^{+}$ Treg cells.

B. Virological and Immunological Characteristics of Acute Phase FIV Infection.

Having validated the methods proposed for the acute infection study, we show here the results obtained from samples collected following the experimental infection of a new group of cats obtained in the laboratory. Samples from newly infected cats were collected as described in the methods section (Figure 1 in Materials and Methods).

<u>1. Plasma and Cell Associated Viremia</u>

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 (weeks 4-12 post inoculation). The FIV-infected cats tested at 4 weeks post inoculation also tested negative for anti-FIV antibodies. However, all of the cats inoculated with FIV were sero-positive at 5 weeks post infection and at all time points thereafter. Quantitative RT-PCR, used to evaluate plasma viremia revealed peak mean number of FIV-gag mRNA copies per ml of plasma at day 14 post infection. This was followed by a decline to low but positive levels thereafter (Figure 5). Table 4 shows the calculated number of copies of FIV-gag mRNA/ml of plasma from individual cats at each time point.

To determine if $CD4^+CD25^+$ Treg cells become infected with FIV during the acute stage of infection, relative quantities of viral mRNA were compared in FACS sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes from FIV-infected cats using reverse transcription and real time PCR. Peak virus levels were found in $CD4^+CD25^+$ Treg cells from the lymph nodes at 14 days post infection, which closely paralleled the plasma viremia pattern described above. Virus levels in PBMC, however, peaked later than in lymph nodes (at day 35 post inoculation). In cells from the lymph nodes, the relative quantities of virus mRNA were greater in $CD4^+CD25^+$ than in $CD4^+CD25^-$ cells in both lymph node at all times except day 56 post infection. In cells from peripheral blood, virus mRNA levels were greater in $CD4^+CD25^+$ than in $CD4^+CD25^-$ cells only at 35 and 42 days post infection (Figure 6).

<u>2. Lymphocyte Changes in Acutely FIV-Infected Cats</u>

Consistent with previous reports (47, 125), 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 rebound in the $CD8^+$ numbers to pre-infection levels followed by a $CD8^+$ lymphocytosis (Figure 7). This resulted in the characteristic inversion of the CD4:CD8 ratio as seen in Figure 8. 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. First we tabulated the numbers of cells obtained from a whole popliteal lymph node and the lymphocyte counts obtained by CBC analysis. There was a very sharp decline in the number of lymphocytes obtained from lymph nodes at day 7 post inoculation to levels below those of sham-inoculated cats. The number of lymphocytes counted from sham-inoculated cats remained relatively constant throughout the acute phase study, fluctuating between 26 and 99 million cells at different time points. Following the sharp decline in lymphocyte yield from the lymph nodes observed at day 7 in FIV-infected cats, the number of lymphocytes increased significantly (* below) by day 35 post infection, with numbers ranging from 193 to 185 million cells at days 14 and 21 post-inoculation respectively (Figure 9). (*)Using a student t test, the number of cells obtained at day 21 was significantly different than that at day 7 (p=0.032).

There were no differences noted in the number of lymphocytes counted by automated and manual differentials in the peripheral blood of FIV-infected and sham-inoculated cats (Figure 10). While there was an increase in the percent of Treg cells in the peripheral blood of FIV-infected cats at days 3-35 post infection (Figure 11a), there was no difference in absolute $CD4^+CD25^+$ T cell counts between infected and control cats (Figure 11b). There was also no difference in the percent $CD4^+CD25^+$ T cells in the lymph nodes from infected and control cats (Figure 11c).

<u>3. Treg Cells Become Activated During the Acute Phase of FIV Infection</u>

Valenkamp et al (14) reported that although the number of Treg cells in asymptomatic FIV-infected cats did not differ from control cats, they were phenotypically and functionally activated. To determine the activation status of $CD4^{+}CD25^{+}$ Treg cells in acutely FIV-infected cats, FoxP3 and membrane TGF-β expression were analyzed as these two markers have been proposed as indicators of Treg cell activation in auto-immune disease (126, 127). In control cats, $CD4^{+}CD25^{+}$ T cells had a 20 fold higher expression of FoxP3 mRNA when compared to the $CD4^{+}CD25^{-}$ T cells (Figure 12a). There was no change in FoxP3 mRNA expression in these cell subsets over time in the uninfected control group (data not shown). Real time PCR analysis for FoxP3 mRNA levels in $CD4^{+}CD25^{+}$ cells from the blood of FIV infected cats, however, revealed an induction of FoxP3 transcription starting at day 14 post infection (Figure 12b). Using two-tailed student t tests for comparisons of values between time points, the levels of FoxP3 mRNA reached statistically significant differences on days 35 and 42 post infection when compared to levels from day 3 post infection in FIV-infected and control cats ($p = 0.05$). These results suggest Treg cells are activated and transcriptionally upregulate FoxP3 during the acute phase of FIV infection.

Analysis of TGF-β expression on the surface of $CD4^{+}CD25^{+}$ Treg cells by flow cytometry also suggested that Treg cells are activated by FIV infection. Increased expression of mTGF-β was detected on $CD4^{+}CD25^{+}$ T cells in the blood of FIV-infected cats between 7 and 35 days post infection (Figure 13a). In the $CD4^{+}CD25^{-}$ T cells from FIV-infected cats, the mTGF-β level was elevated at days 14 and 21 post inoculation. These data suggest that Treg cells are activated early in the course of infection and then return to their resting status, which correlates with the early peak viremia that is followed by a marked decline. Interestingly, TGF-β mRNA transcription as measured by RT-PCR, is not increased in the $CD4^{+}CD25^{+}$ cells, but is increased in the $CD4^{+}CD25^{-}$ T cells from infected cats prior to increases noted on surface expression in the $CD4^{+}CD25^{+}$ cells (Figure 13b).

At weekly intervals post-FIV infection, PBMC and LN cells from FIV^{+} cats and sham-inoculated cats were also analyzed by multi-color flow cytometry to establish the phenotype of $CD4^{+}CD25^{+}$ T cells by differential expression of other cell surface markers associated with Treg activation, including CD62L, B7.1, and TGFßR1. The antibodies and appropriate combinations of fluorochromes have been verified in our laboratory. There were no consistent or significant differences in the percentages of cells expressing these molecules on their surface by flow cytometry (figures14-16). We then analyzed the absolute numbers of cells expressing these molecules using the total number of lymphocytes obtained from each lymph node or the lymphocyte count obtained by CBC analysis. While these differences failed to reach statistical significance, it was noted that the absolute number of cells expressing CD62L in the blood and lymph node was greater from days 3-35 post-inoculation in FIV-infected cats, compared to sham-inoculated cats. This was true even when the total number of cells was lower in the FIV-infected cats for some of the time-points noted (Figure 17). This result is consistent with the hypothesis that FIV infection results in the up-regulation of activation markers in Treg cells leading to increased regulatory activity.

The absolute number of $CD4^+CD25^+$ cells expressing B7.1 (CD80) on their surface was slightly higher in FIV than sham-inoculated cats for the first 21 days post infection in the lymph node and thereafter in the peripheral blood (Figure 18), also suggesting some degree of activation in these cells at all time-points. Our attempts to measure changes in the expression of TGF-βR proteins however, yielded much more variability in sham inoculated than FIV-infected cats, making these results difficult to interpret (Figure 19).

4. Suppressor Function of Treg Cells During Acute Phase FIV

Having shown that Treg cells are possibly infected and probably activated during the acute phase of FIV infection we next tested their ability to suppress the effector function of target $CD4^+CD25^-$ T cells. One of the mechanisms proposed for Treg function is the suppression of IL-2 production by $CD4^+$ T helper target cells (98, 128). Here we show that the addition of FACS purified Treg cells from FIV-infected cats to stimulated $CD4^+CD25^-$ target cells results in the suppression of IL-2 production by target cells at an effector to target ratio of 0.1 (Figure 20). Suppressor function against Con A-stimulated $CD4^+CD25^-$ T cells could be detected in three of four cell samples from FIV-infected cats at day 3 post infection. However, some of the cell samples from sham-inoculated cats also suppressed IL-2 production at later time points (days 7, 35, and 56 post inoculations).

In vitro suppression requires the activation of Treg cells via the TCR or by a non-specific mitogen, does not involve killing of target cells, and is mediated by a cell contact-dependent mechanism that transduces a signal for down-regulation of IL-2, resulting in anergy. Our hypothesis is that in vivo infection and activation of Treg cells by FIV during the early acute phase of infection converts them to an activation phenotype with the ability to suppress the anti-FIV $CD4^+$ T cell immune response. We propose that these effects result in ineffective anti-FIV immune responses leading to the establishment of a chronic, long-term infection. After showing the FIV mRNA can be found in $CD4^+CD25^+$ cells during the acute phases of infection and that these cells acquire activation markers following infection, we showed how infection with FIV-NCSU^{-1} affects the suppressive function of $CD4^+CD25^+$ by measuring changes in cytokine production (IL2) of non-specifically (Con A) stimulated $CD4^+$ Th cells in the presence of $CD4^+CD25^+$ Treg cells in vitro. Con A is a potent mitogenic stimulator of T cell proliferation and IL2 production, but we also attempted to examine responses to CD3 antibodies and FIV-gag antigen. For these experiments, the target cells were stimulated in vitro with CD3 specific antibodies and FIV recombinant peptides (FIV-gag) as described before in the Con A experiments. Thus, we attempted to evaluate potential temporal changes in suppression function in response to FIV infection during the acute phase of infection. Unfortunately, the number of spots detected in each well was very small in these experiments making these data statistically insignificant. No evidence of virus-specific suppression could be measured at any time (Figure 21). The results show different responses to FIV-gag at the earlier time-points (day 3-35) compared to the later time-points (day 42-84) in the acute phase study using cells from FIV-infected cats. At the early time-points, adding unstimulated $CD4^+CD25^+$ T cells resulted in an increased (rather than decreased) number of spot forming cells per well than the number of spots produced by target cells alone. We suspect these additional spots were formed by in vivo-activated, virus specific $CD4^{+}CD25^{+}$ Thelper cells. This effect, however, was undetectable after day 42, suggesting the percent of suppression noted at early time-points in figure 7 may have in reality been higher than we could show, but was blunted by the additional spots from activated T cells in these assays. This is further evidence that anti-viral responses are eliminated early in the acute phase of FIV infection, and that more specific techniques are needed to differentiate these two cell subsets before day 42 post infection. Being able to differentiate activated $T_{(helper)}$ cells from regulatory T cells early on, using TGF-beta as a marker (for example) should increase our ability to measure higher levels of suppression activity by regulatory T cells before day 42 post inoculation. The data from our experiments using FIV-gag protein to stimulate target cells confirms that while the suppressor function is activated in response to infection with FIV, there is still a number of activated Thelper cells present in our $CD4^{+}CD25^{+}$ T cell population. Interestingly, the response to gag by the added $CD4^{+}CD25^{+}$ T cells seems to disappear at day 42 post inoculation, suggesting suppression levels noted in the ConA response after day 42 do correspond to regulatory T cell activity alone. In fact, at day 84, there are less spot forming cells in the wells containing $CD4^{+}CD25^{+}$ cells than in the well that only have target cells, denoting FIV-specific suppressive function could be measured after this time-point. These data are consistent with the expected hypo-responsiveness to FIV antigens that has been reported by others as part of the early immune dysregulation. The number of cells plated per well was determined in preliminary studies using Con A stimulation to be optimal for that assay, however, more cells would be required to detect significant differences using more specific (antigen) stimulation. The results for experiments using anti-CD3 stimulation alone were similar than those using FIV-gag peptides (data not shown).

DISCUSSION

The activation of $CD4^{+}CD25^{+}$ regulatory T cells has recently been proposed as a mechanism for the immuno-suppression associated with retroviral infections. The idea that Treg cells might influence functional anti-viral immunity may have serious implications for the prevention of chronic infections with HIV and other viruses (13). In this study, we show that Treg cells become infected, activated, and functionally immunosuppressive in vivo during the acute phase of FIV infection. Peak plasma viremia levels correlate with maximal infectivity in lymph node cell populations and are highest at day 14 post infection. $CD4^{+}CD25^{+}$ T cells have higher viral mRNA levels than their $CD4^{+}CD25^{-}$ counterparts, suggesting either preferential infectivity or increased viral replication in activated T cells early after infection with FIV. The induction of FoxP3 mRNA transcription and an increase in surface expression of TGF-β is evidence of Treg cell activation in the first 12 weeks post infection. High levels of suppressor function can be measured in some cats as early as day 3 post

FIV infection. These results are consistent with previous observations of Treg cell function in long-term, chronically FIV-infected cats (14).

Plasma viremia studies by RT-PCR correlate with previously published studies using ELISA techniques, which indicate a peak viremia occurring at 2 weeks post inoculation followed by a sharp decline (47). PCR results also indicate there is a higher mRNA viral load in the $CD4^{+}CD25^{+}$ compartment compared to $CD4^{+}CD25^{-}$ cells. Joshi et al (48) reported that $CD4^{+}CD25^{+}$ Treg cells from asymptomatic FIV-infected cats preferentially supported a productive FIV infection. Further, they reported that $CD4^{+}CD25^{+}$ Treg cells but not $CD4^{+}CD25^{-}$ T_h cells supported productive FIV replication when infected in vitro. These studies collectively suggest that early and persistent activation of Treg cells may provide a long term reservoir of FIV replication, as well as a mechanism for immune dysregulation. As virus activation likely results in increased expression of CD25 molecules on $CD4^{+}$ T helper cells, it is possible that FIV may also be replicating in activated $CD4^{+}$ T helper cells. Also, it has been shown that $CD4^{+}CD25^{+}$ T cells (as opposed to $CD4^{+}CD25^{-}$ Th cells) obtained from chronically FIV-infected cats and in-vitro infected $CD4^{+}CD25^{+}$ T cells remain anergic and unresponsive to apoptotic signaling, qualities that are known to define regulatory T cells in general (5, 48). The increased viral load within the $CD4^{+}CD25^{+}$ T cells could be due to preferential infectivity, meaning more of these cells initially receive the virus and are persistently infected, or it could be due to increased viral replication within a small proportion of $CD4^{+}CD25^{+}$ T cells. In support of this, $CD4^{+}CD25^{+}$ T cells have been shown to be more susceptible to FIV binding than $CD4^+CD25^-$ cells. Expression of CXCR4 and CD134 is important in viral cell entry and Treg cells have increased expression of both (86, 129). However, because we can not detect viral load at the individual cell level using the techniques described herein, this question was not directly addressed in our present studies.

FoxP3, a member of the forkhead/winged-helix family of transcriptional regulators has been shown to be essential for the development and function of Treg cells (88). Unlike other markers that are also upregulated in non-regulatory T cells after activation, such as GITR, CTLA4, and OX40, FoxP3 is specifically expressed in the regulatory T cells of mice, 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 (116) and that the $CD4^+CD25^+$ T cells possess key characteristics of Treg cells (13). Here we show that the level of FoxP3 mRNA is significantly increased in $CD4^+CD25^+$ T cells following infection with FIV, suggesting transcriptional upregulation of FoxP3 occurs in activated Treg cells. In support of our results, other investigators have reported FoxP3 mRNA expression was increased in the T cells from the lymphoid organs of untreated HIV+ patients when compared to patients on HAART, and was correlated with an increase in viral load in one study (92). 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. While a direct correlation between viral load and FoxP3 expression was not evident in our studies over time, an increase in FoxP3 transcription in the $CD4^+CD25^+$ cells of FIV-infected cats was first detected at day 14 post-inoculation, which corresponds to the time of maximal viral load in these infected cats, suggesting increased viral replication may be associated with the induction of FoxP3 transcription. In support of our studies, immunohistochemistry and confocal microscopy detected increased expression of FoxP3 in $CD4^{+}CD25^{+}$ T cells of acutely SIV-infected Rhesus Macaques. These authors also reported that the increased rate of viral replication at these early time-points correlates with an increase in Ki67 expression which is an indicator of immune activation. Similar to our results and those previously cited, others found significant increases in FoxP3 expression following infection of African green monkeys with SIV. Here, however the increases 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 noted 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, but as was the case for us, the levels of FoxP3 expression did not correlate with increased numbers or percentages in $CD4^{+}CD25^{+}$ T cells.

TGF-β is another factor that has been associated with regulatory function and modulation of the immune response. Recently, we have demonstrated by flow cytometry that $CD4^{+}CD25^{+}$ Treg cells from FIV-infected cats but not control cats express TGFβ on their surface while $CD4^{+}CD25^{-}$ T cells from infected and control cats do not, suggesting cell surface TGFβ can be used to distinguish activated Treg cells in FIV infected cats (116). Several other researchers have also reported that TGF-β is up-regulated on the surface of activated Treg cells and may mediate regulatory function (90, 115, 130). Here we show a transient increase in the surface expression of TGF-β (mTGF-β) on $CD4^+CD25^+$ T cells following infection with FIV. $CD4^+CD25^+$ mTGF-β expression is preceded by an increase in TGF-β mRNA transcription in the $CD4^+CD25^-$ compartment, suggesting TGF-β from non-regulatory T cells may bind and activate Treg cells following FIV infection. Membrane TGF-β expression on $CD4^+CD25^+$ T cells could be associated with the subsequent induction of transcription of FoxP3 mRNA (days 14-42 above) and the increased regulatory function that we suggest is associated with decreased anti-viral effector responses. However, we realize changes in mRNA levels are difficult to interpret for TGF-β, as post-transcriptional and post-translational regulatory events can greatly influence the function of this protein in vivo (131). In the study of African green monkeys infected with SIV (93), 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 PBMC's, again suggesting that TGF-β may induce FoxP3 transcription in response to retroviral infections. Ultimately, neither study can definitively identify the source of TGF-β that we believe is responsible for the functional activation of Treg cells. In support of these findings, Estes et al. (124), also found, using immunohistochemistry techniques, early increases (day 7-12) in the number of TGF-β positive cells in the lymph nodes following SIV infection. And, 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 mice autoimmune disease models that TGF-β can induce FoxP3 gene expression and up-regulate $CD4^+CD25^+$ suppressor function (90). TGFβ in this system was shown to convert peripheral $CD4^+CD25^-$ T cells into Treg cells. While we have not shown the conversion of $CD4^+CD25^-$ T cells into Treg cells here, Petty et al. (116) reported that TGF-β was capable of converting Con A stimulated feline $CD4^+CD25^-$ Th cells into phenotypic and functional T reg cells. Studies to determine the presence and function of TGFβ and TGFβ receptor molecules in these cell subsets during acute FIV infection are currently underway in our laboratory.

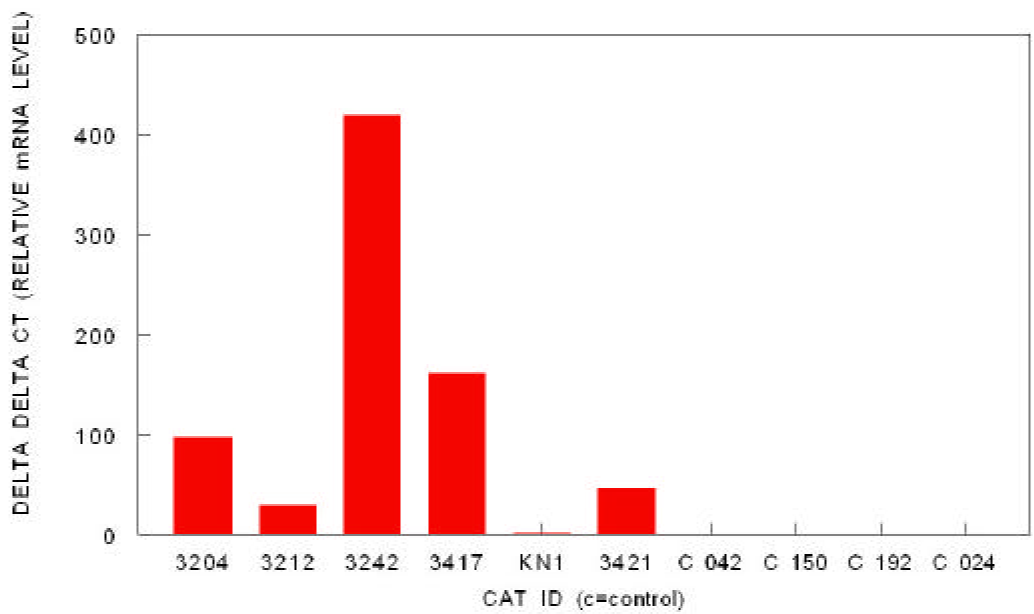
The functional capacity of $CD4^+CD25^+$ Treg cells was evaluated in our study using a commercially available ELISPOT assay to detect changes in IL-2 production by ex-vivo, Con A stimulated target cells. The inhibition of IL-2 production is a well established mechanism by which $CD4^+CD25^+$ Treg cells are thought to modulate immune responses (14, 85, 99), 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 (128, 132). 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 retroviral infections in general.

In conclusion, data from our acute phase FIV infection experiment suggest that regulatory T cells are productively infected, phenotypically activated, and play an important role in suppressing early anti-viral $CD4^{+}$ T helper cell responses, which may consequently allow for 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 (such as TGF-beta expression) during the early phase of infection will also need to be explored.

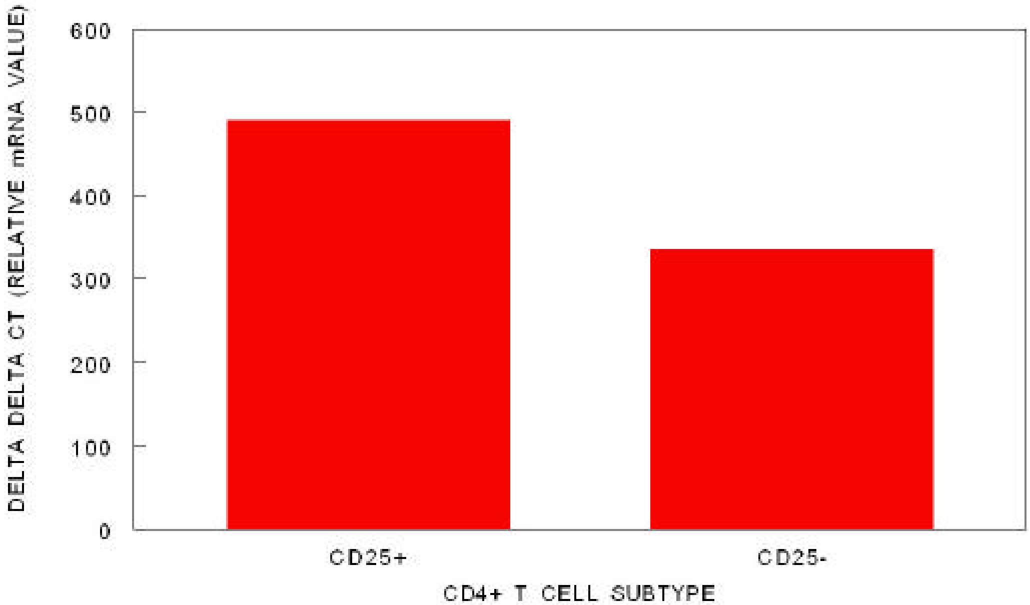
TABLE 1. **Real-time RT-PCR analysis of plasma from FIV-infected cats (copies FIV-gag-mRNA/ml of plasma).**

DAYS PI	3	7	14	21	35	42	56	84
CAT #1	0	33.48	570	42.48	951.6	141.6	630.6	183.48
CAT #2	0	42.6	1184	109.8	43.68	12.96	360.6	22.71
CAT #3	<5*	12.72	568.8	46.56	Not done	32.04	135.48	8.73
CAT #4	0	27.24	4584	12.96	Not done	31.92	227.16	132

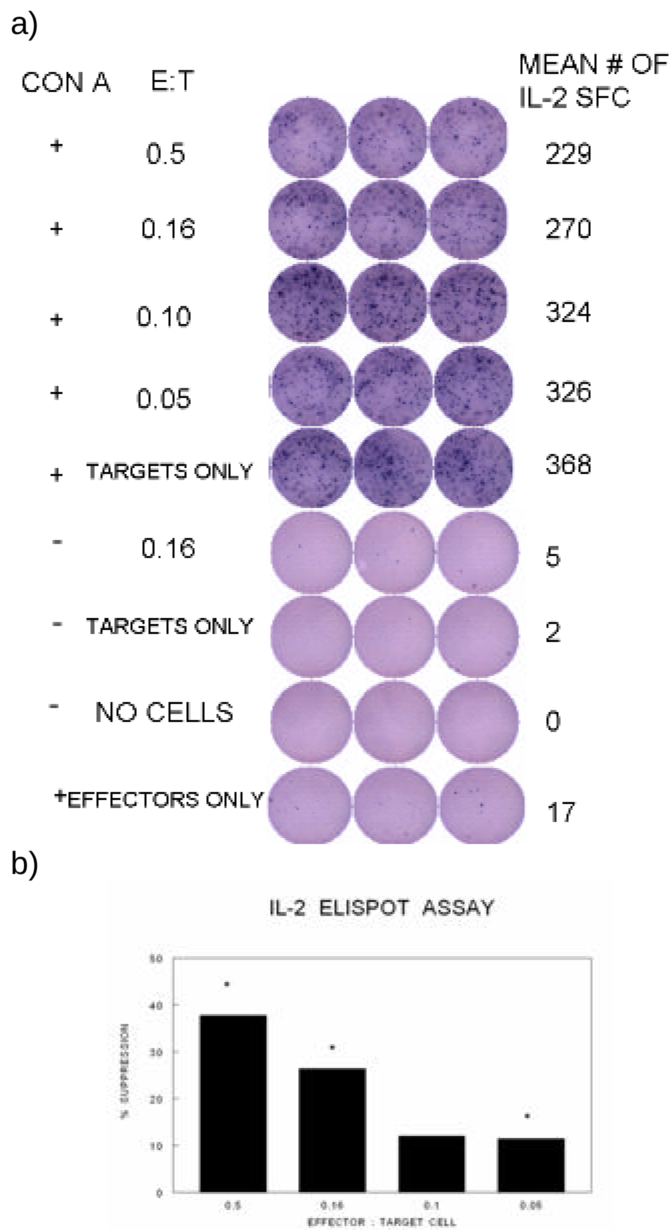
*Least number of copies detectable by this reaction.



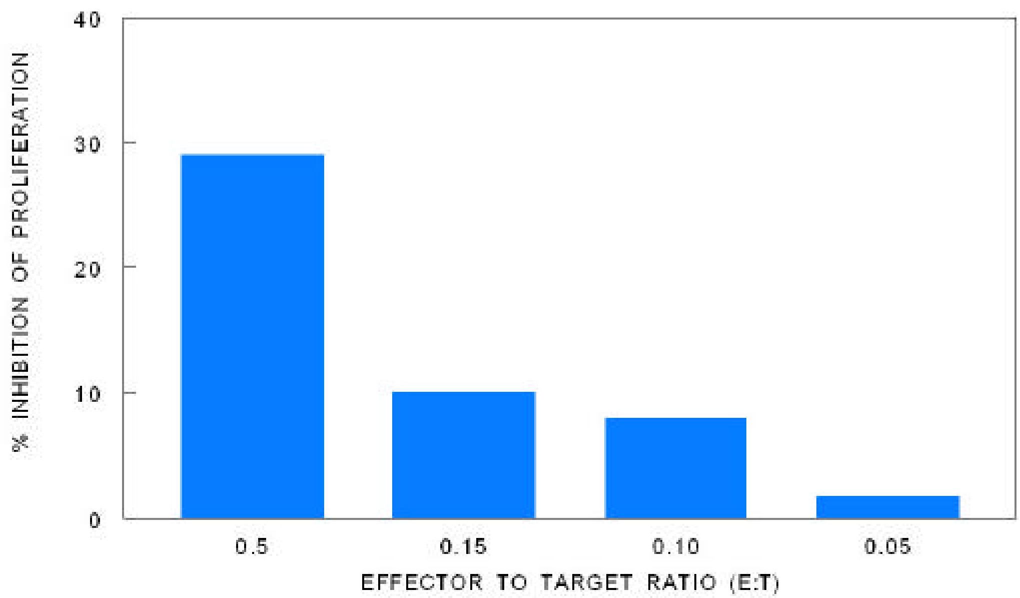
<u>**Figure 1.**</u> **Detection of FIV Infection in feline lymphocytes by Real-Time PCR**.
$5x10^6$ mononuclear cells, purified by Ficoll-Histopaque Centrifugation were isolated from asymptomatic chronically FIV infected cats or uninfected controls (c) for RNA purification using a commercially available RNA purification kit (Qiagen-RNeasy). 10 µl of the RNA product was used in a reverse transcriptase reaction (Promega-reverse transcription system) for conversion into cDNA. 10µl from each of these reactions were then used in a real-time PCR reaction using primers for FIV-gag and GAPDH in duplicate wells along with serial dilutions of FIV-gag plasmid to create a standard curve. The standard curve was then used to estimate the number of copies of FIV-gag per sample of RNA. The housekeeping gene GAPDH was amplified in duplicates for each sample to assure comparable amounts of RNA were being used in each of the FIV reactions.



<u>Figure 2.</u> Detection of FIV-gag mRNA in feline CD4+CD25+ and CD4+CD25- lymphocytes by Real-Time PCR.
28 mls of blood were collected by jugular venipuncture from one cat (ZA6) and PBMC's were isolated by Ficoll-Histopaque centrifugation prior to staining with anti-CD4-Biotin plus Streptavidin PerCP and anti-CD25-FITC antibodies. Cells were sorted into CD4+CD25+ and CD4+CD25- populations by MoFlo high speed, high purity cells sorting (Dako Cytomation). $5x10^6$ sorted cells from each population were used to detect viral mRNA using the real-time RT-PCR technique for FIV-gag mRNA described in the methods.



<u>Figure 3.</u> IL-2 ELISPOT assays.
Lymph nodes cells sorted as described in figure 6 were used in IL-2 suppression assays. $CD4^+CD25^+$-depleted target cells were stimulated with Con A for one hr and washed once in T cell media. $3x10^5$ cells are plated in triplicate wells of the anti-IL-2 coated ELISPOT plates provided by the manufacturer (R&D Systems). Fresh $CD4^+CD25^+$ T cells are immediately added to each well at the numbers indicated by the E:T ratios. Cells are incubated overnight at 37°C. The detection antibody was added for one hour at 37° C. (a) The number to the right of the wells depicts the average number of spots from three wells in each treatment. Targets cells not treated with Con A and $CD4^+CD25^+$ cells treated with Con A are depicted as controls. Human recombinant IL-2 and media only wells were also used as positive and negative controls (not shown). (b) Percent suppression is calculated as the average number of 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 target only-wells.



<u>Figure 4.</u> Suppression of cell proliferation by $CD4^+CD25^+$ Treg cells using CFSE flow cytometry. Sorted lymph node cells from one chronically infected FIV+ cat were used in this proliferation assay. $CD4^+CD25^+$ depleted target cells were stained with CFSE according to the manufacturers' instructions, stimulated with Con A in cell culture medium containing maintenance levels of IL2 for one hour and then washed in media. 3x10^5 cells were plated in triplicate wells of a 96 well plate. Freshly isolated $CD4^+CD25^+$ T cells were added to each well to obtain the indicated E:T cell ratios. Cells were incubated at 37℃ for 5 days prior to analysis by flow cytometry.

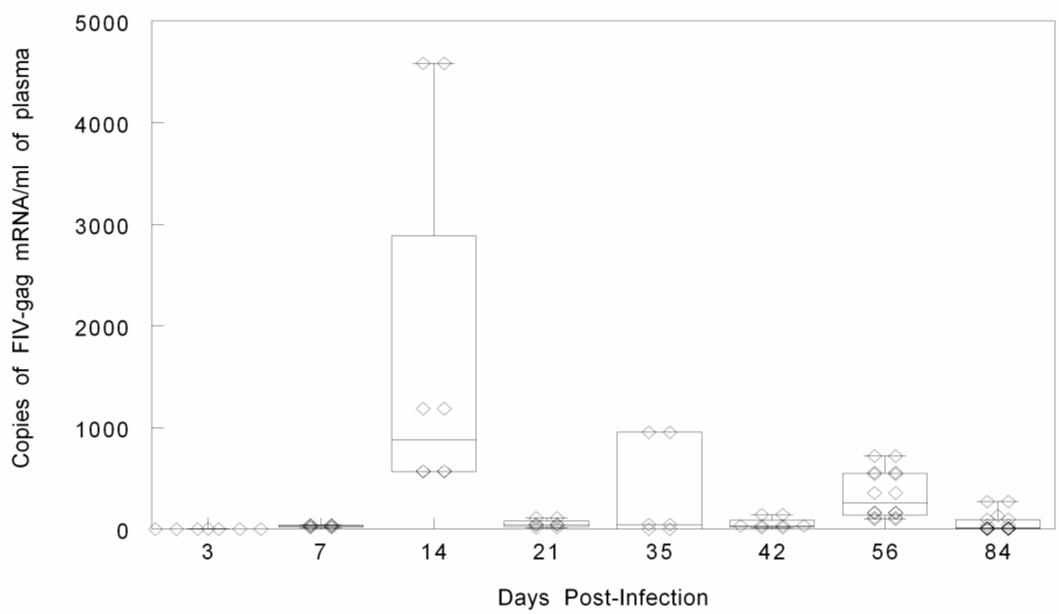


Figure 5. Quantification of FIV-gag mRNA in plasma samples by real-time PCR. Absolute quantification of viral mRNA was evaluated in 1 ml of plasma obtained from FIV-infected cats at different time-points in the study. Serial dilutions of a previously quantified sample were run in parallel in each assay to create a standard curve. The assay can detect as little as five copies of FIV-gag mRNA in a sample. Each of the unknown and standard curve samples were evaluated in duplicate wells and the assay was repeated twice. All of the results for each sample were averaged individually and are tabulated in table 4. Each marker represents the value obtained from one specific cat. The median, 25 and 75 percentiles are represented in the box and whisker plots.

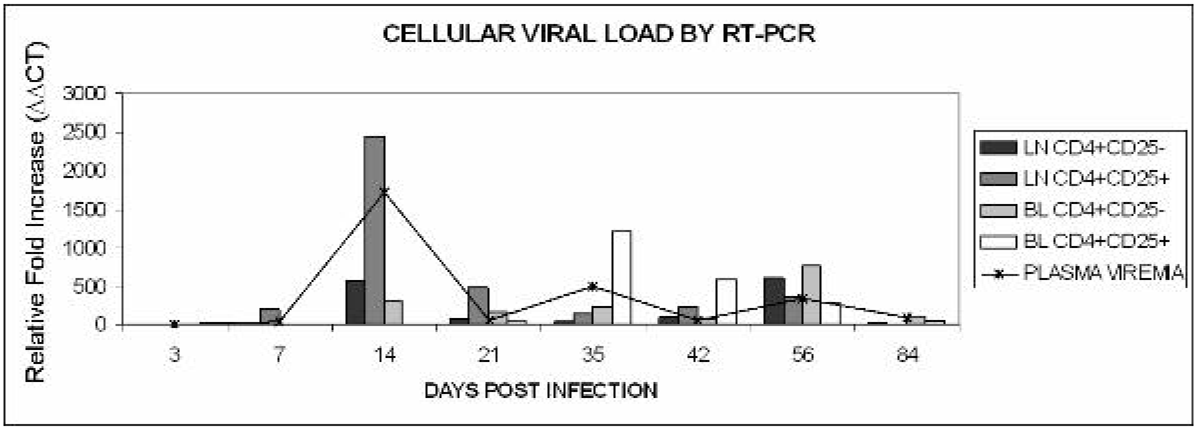
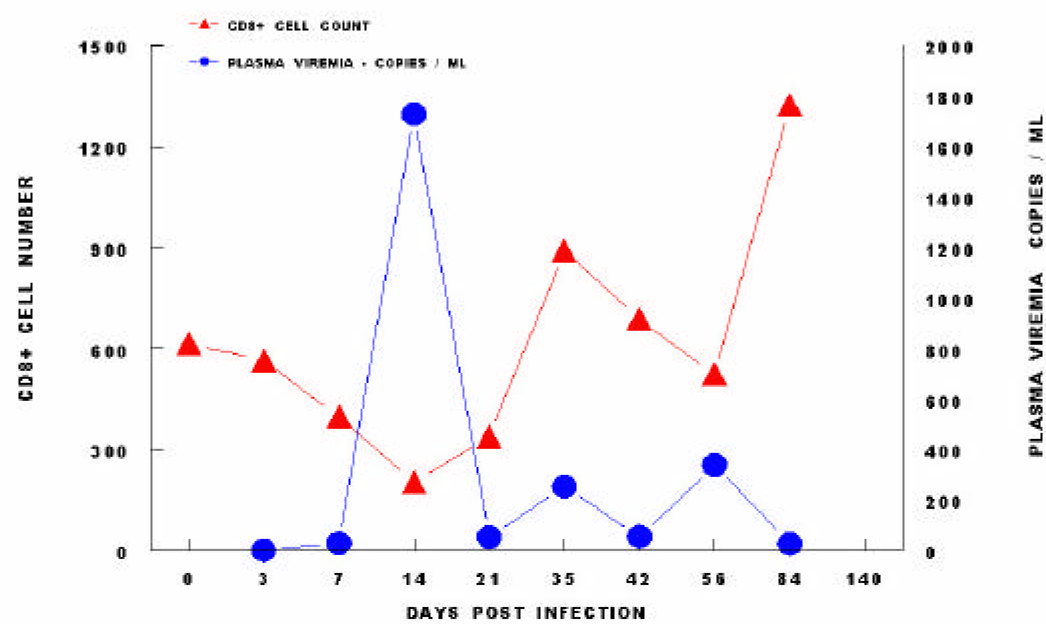
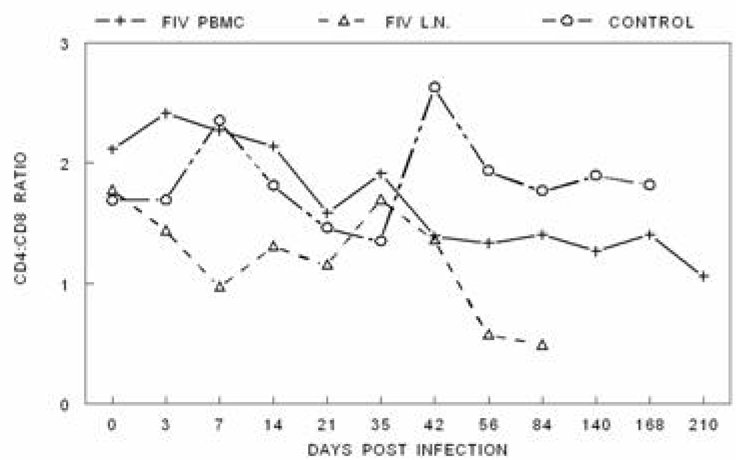


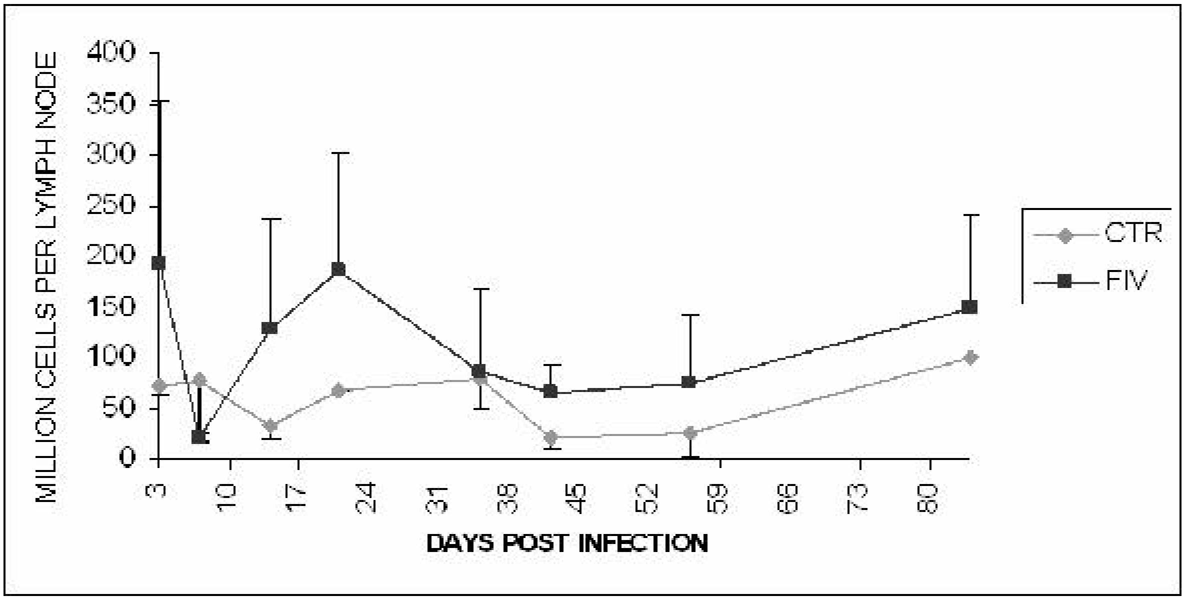
Figure 6. Relative quantification of viral load in subsets of feline lymphocytes by Real-time PCR.
Sorted populations of 0.5-3x10^6 $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from blood and lymph nodes were maintained in RNA later and evaluated for relative quantities of FIV-gag mRNA using the delta delta 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 2-4 cats were evaluated individually. 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 mean plasma viremia levels at each time as described in figure 8.



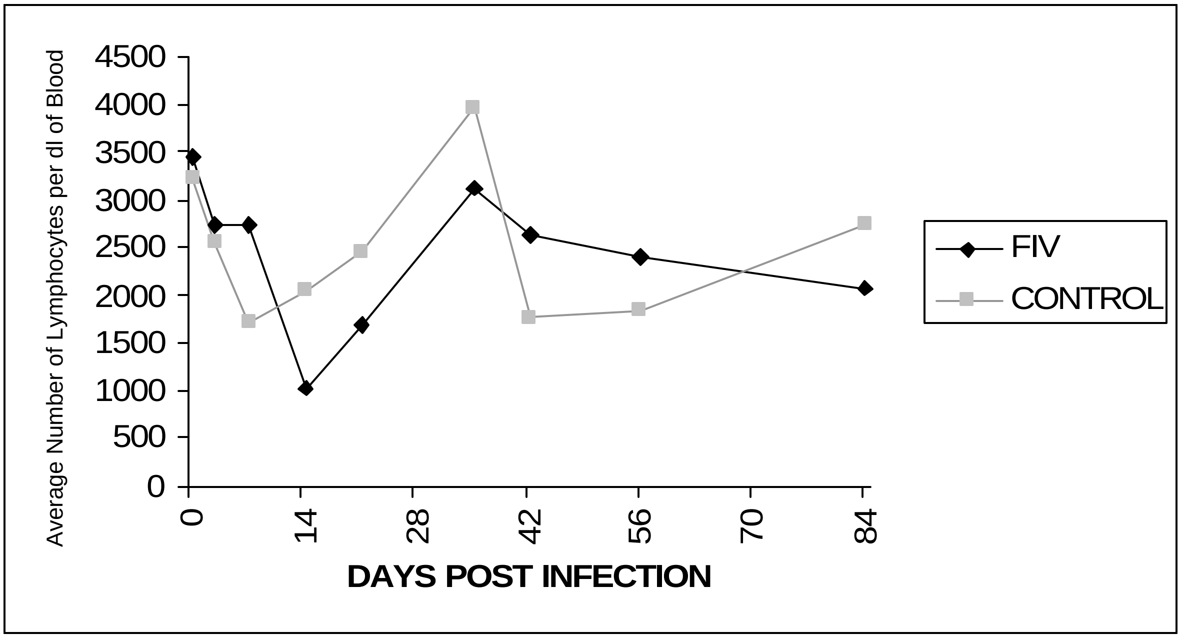
<u>Figure 7.</u> CD8^{+} Lymphopenia and lymphocytosis during acute FIV infection.
The average CD8^{+} T cell count from 1-4 cats was determined at each time point following analysis by flow cytometry. Average CD8^{+} counts are compared to plasma viremia measured by RT-PCR as previously described.



<u>Figure 8.</u> CD4:CD8 ratios decrease in 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 84 days following FIV or sham inoculation by flow cytometry. Each measurement is the average of values obtained from 4 FIV-infected cats, or 2 control cats (except values for the control group at days 21 and 84 which represent only one cat). The average CD4:CD8 ratios at each time-point in the blood and lymph node of FIV-infected cats are compared to CD4:CD8 ratios in the blood of control cats.



<u>Figure 9.</u> Average of total number of cells obtained from each lymph node.
Popliteal lymph nodes were removed as described in the material and methods and maintained on ice in CTL culture media. Connective tissue surrounding each node was gently removed and the node was perforated several times with a 20g hypodermic needle. Media was then injected through the node several times to release the cells contained. The cells were then washed and counted as described in the appendix and the total number of cells was recorded for each lymph node obtained. The graph above represents the mean number of total cells obtained at each time-point x10^6. Each time point represents 1-4 lymph nodes from cats as described in Table 1.



<u>Figure 10</u>. Average of total cell counts in the blood.
The number of lymphocytes in one dl of peripheral blood from each cat at each time point was determined from an automated total cell count and a manual differential count performed by a blinded member of the lab. The lymphocyte count per dl of blood was record for each blood sample obtained and the mean number of cells in each cat group per time point is depicted above. Each point represents 1-4 blood samples from cats as described for each group in Table 1.

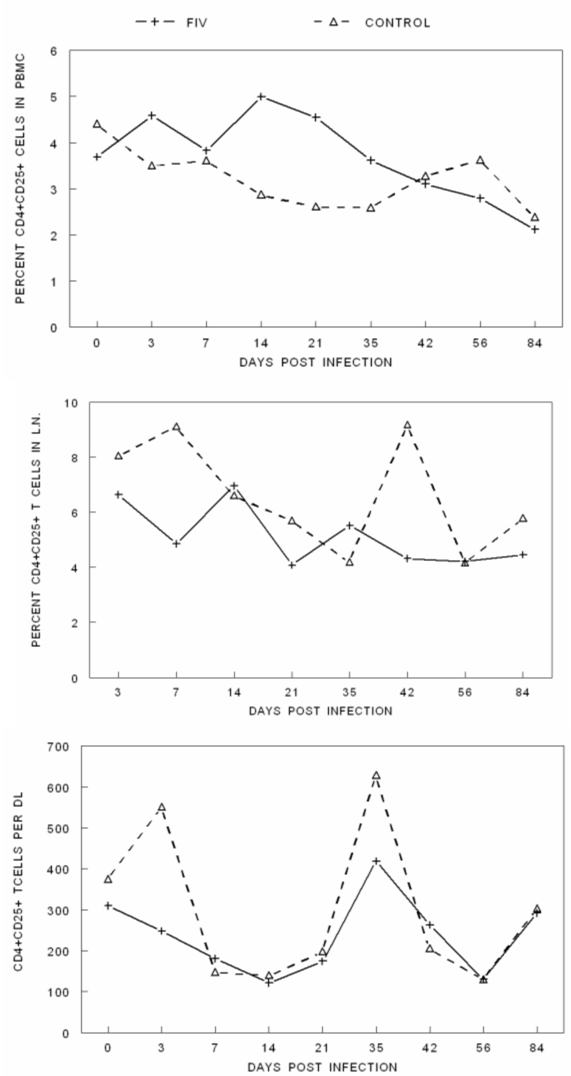
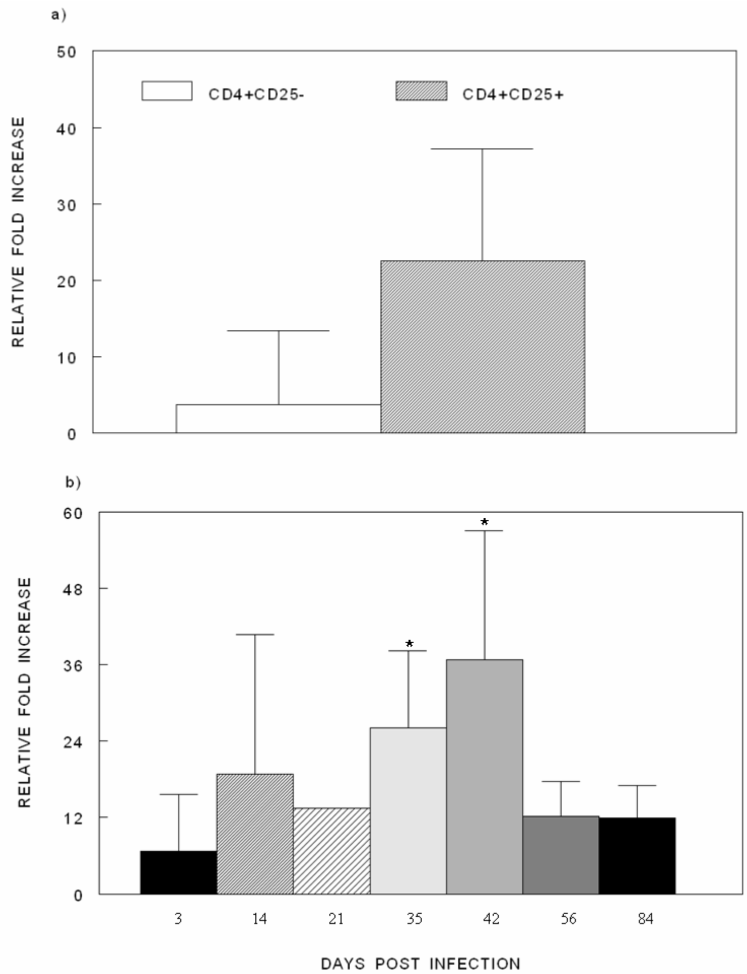
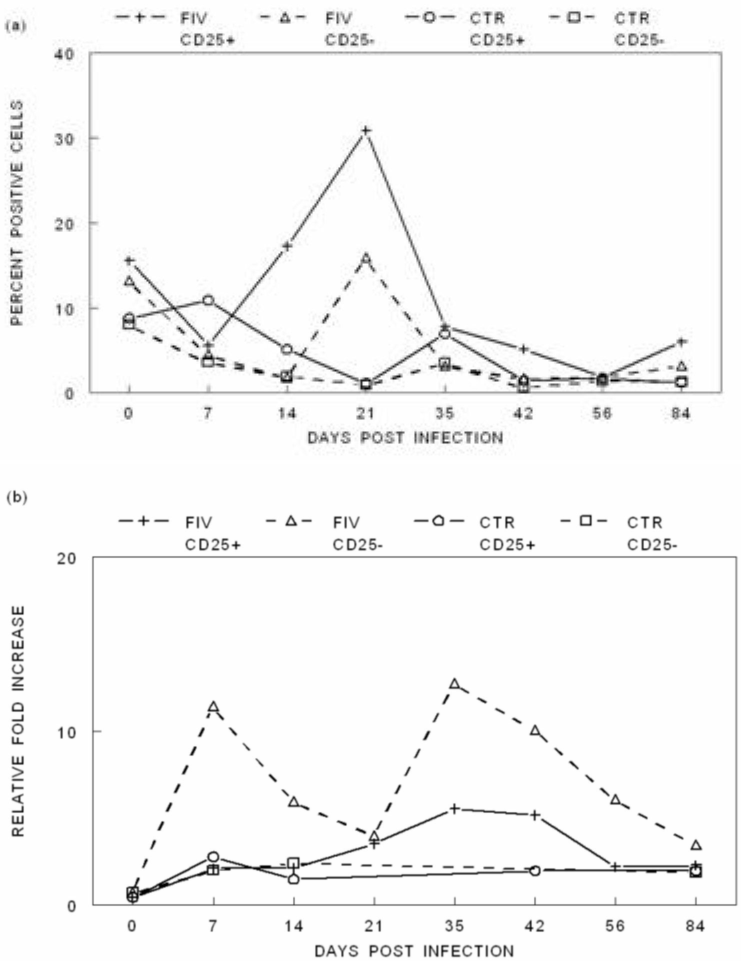


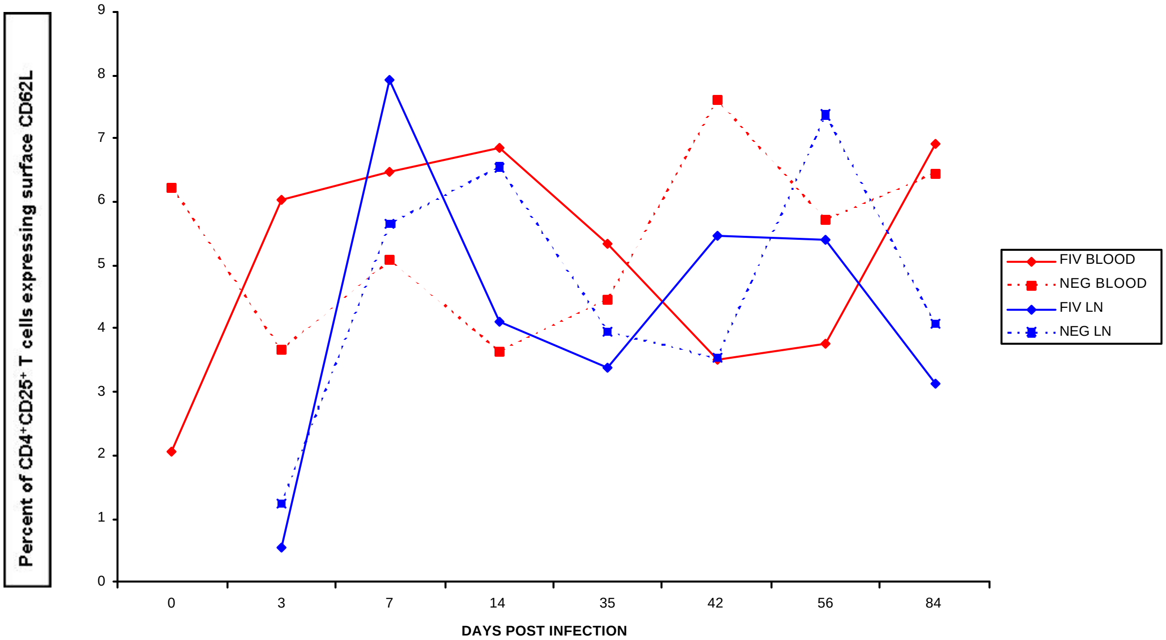
Figure 11. **Changes in $CD4^{+}CD25^{+}$ T cell population with FIV.**
The average percent of $CD4^{+}CD25^{+}$ T cells from total lymphocytes was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group. Values from the blood (a) and lymph nodes (b) are shown separately. The absolute number of $CD25^{+}$ cells in $CD4^{+}$ populations from the blood was calculated by multiplying the total number of lymphocytes counted in a CBC by the mean percent of $CD25^{+}$ T cells from the $CD4^{+}$ T cell population (c).



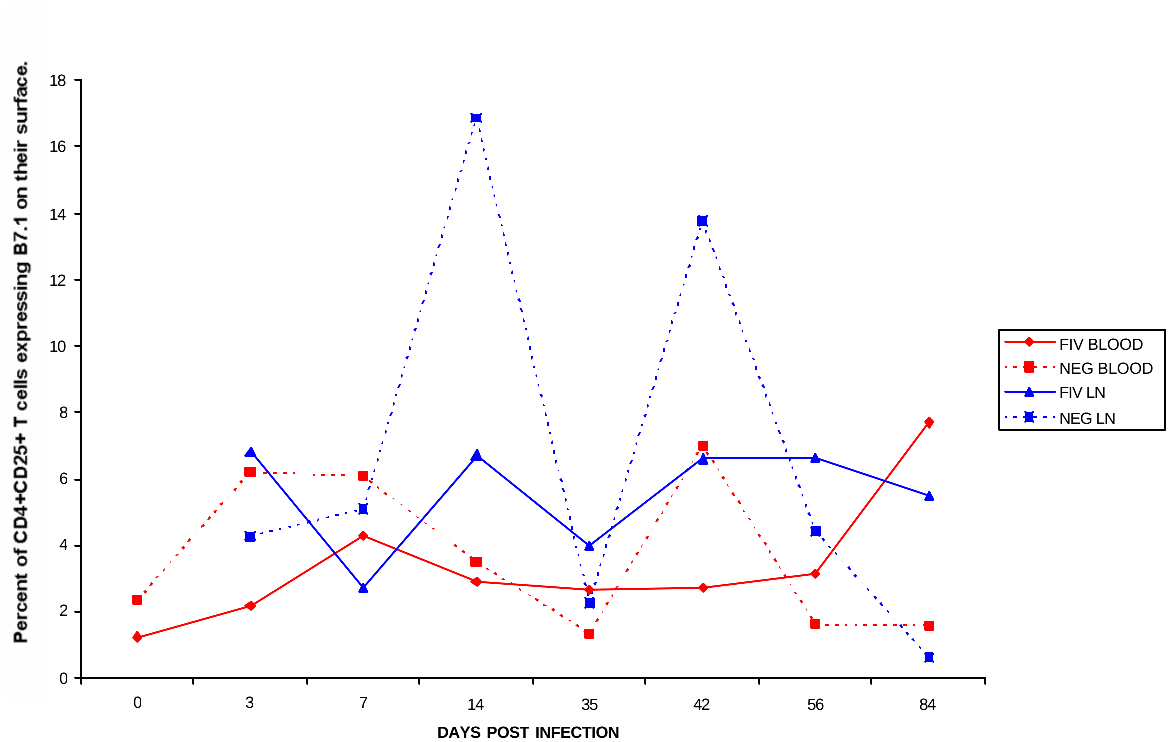
<u>Figure 12.</u> Relative quantification of FoxP3 mRNA in feline lymphocyte subsets.
Sorted subsets of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells isolated from FIV-infected and control cats were maintained in RNA later for analysis of FoxP3 mRNA levels by reverse transcription and quantitative real-time PCR. Equal amounts of RNA were used in each test. The mean and standard deviation of relative values for each group are represented in the bar graph. $CD4^+CD25^+$ T cells from control cats have approximately 20 times more FoxP3 mRNA than $CD4^+CD25^-$ T cells from either cat group at any time point (a). $CD4^+CD25^-$ Tcells contained very small amounts of FoxP3 mRNA at all time-points in infected cats (data not shown). The relative levels of FoxP3 mRNA in the $CD4^+CD25^+$ T cells from FIV-infected cats changed significantly over time and the groups of cells evaluated for each time point are represented individually (b). In this subset, there was a significant increase in FoxP3 transcription on days 35 and 42 post infection p<0.1).



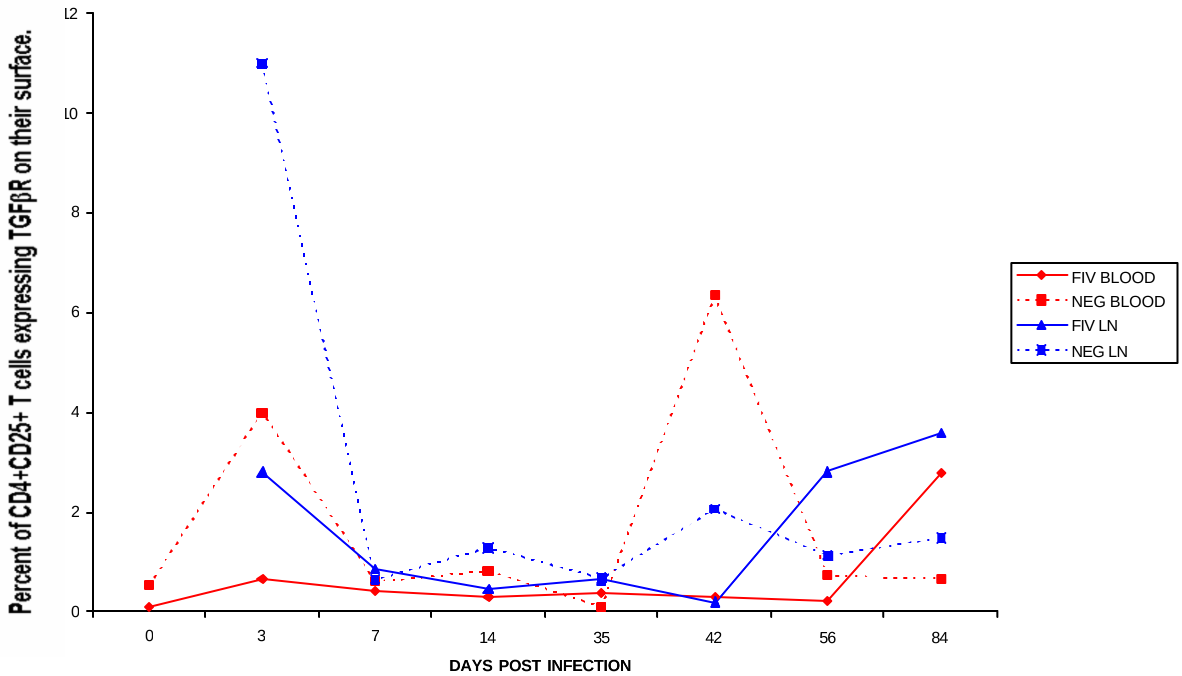
<u>Figure 13.</u> Surface expression and mRNA levels of TGF-b in feline lymphocytes.
The surface expression of TGF-β in $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes from the lymph nodes (a) of FIV-infected and control cats was evaluated by flow cytometry. Each data point represents the mean of means (two measurements for each cat) obtained in each group of cats (4 cats in FIV-groups and 1-2 cats in control groups) at each time-point. The relative levels of TGF-β mRNA were measured in sorted cells from each cat as described by reverse transcription and RT-PCR (b). $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from FIV-infected and control cats are depicted separately in each graph. Each value represents the mean relative fold increase using the delta delta CT method of quantification for 1-4 samples obtained.



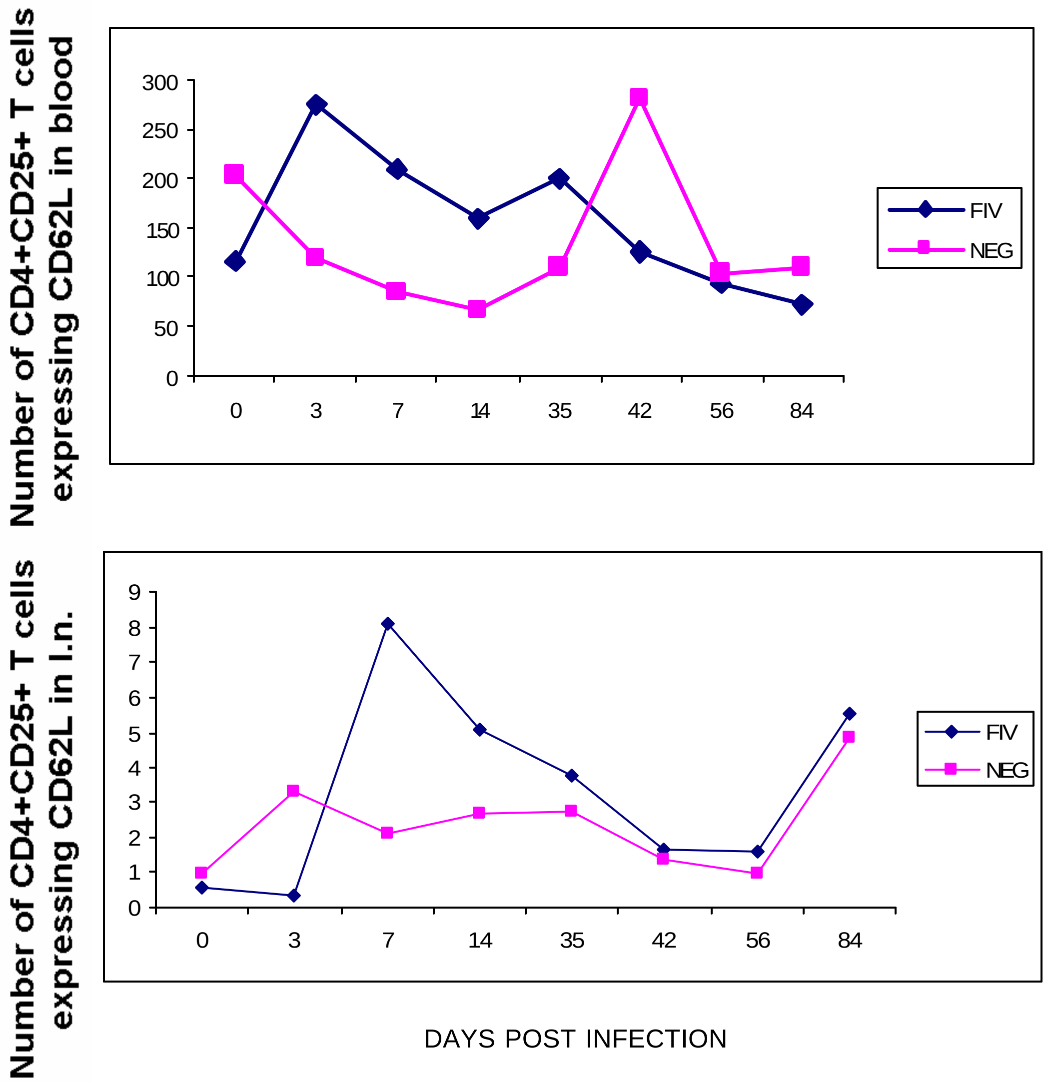
<u>Figure 14.</u> Percent of $CD4^+CD25^+$ T cells expressing surface CD62L.
The average percent of $CD4^+$ lymphocytes expressing CD25 and CD62L on their surface was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group. Values from the blood and lymph nodes are shown.



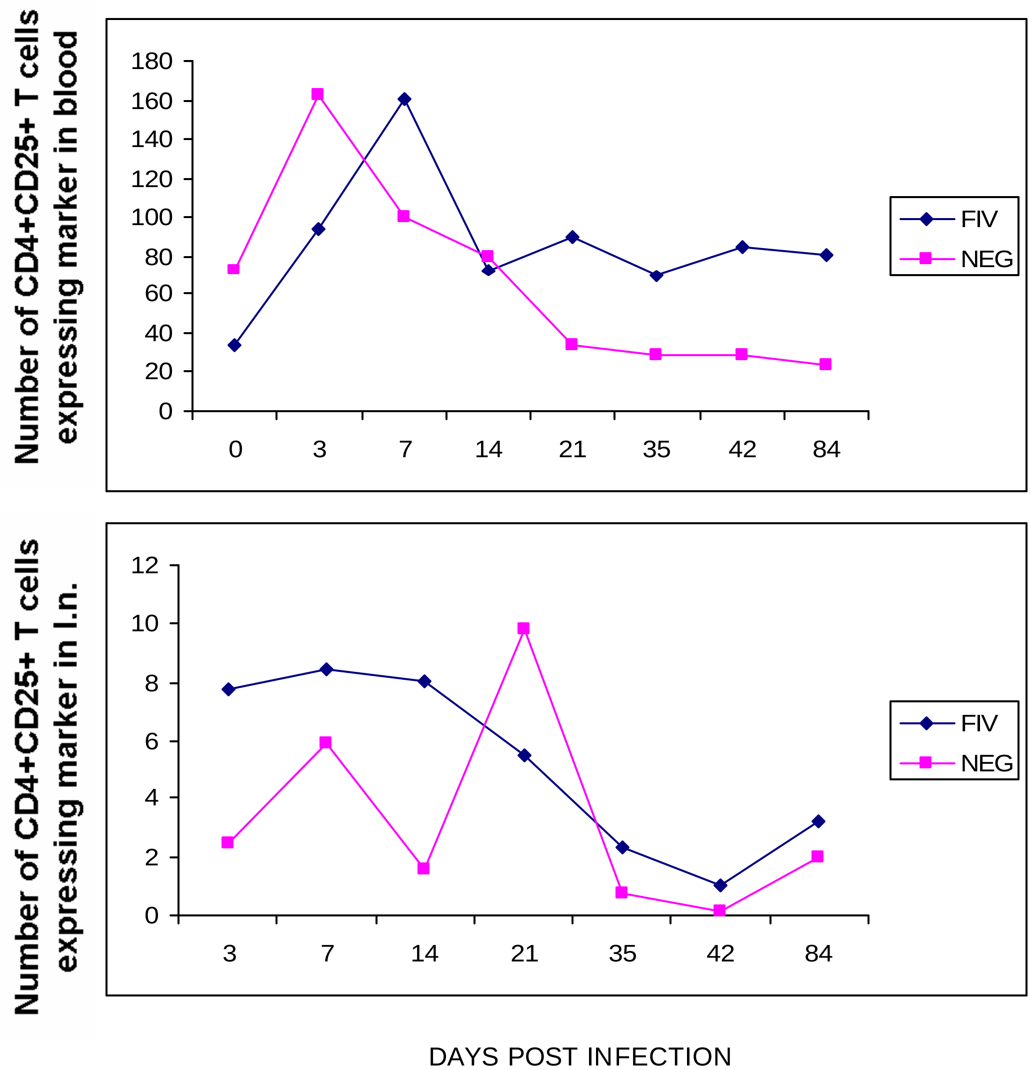
<u>Figure 15.</u> Percent of $CD4^+CD25^+$ T cells expressing B7.1 on their surface.
The average percent of $CD4^+$ lymphocytes expressing CD25 and B7.1 on their surface was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group. Values from the blood and lymph nodes are shown.



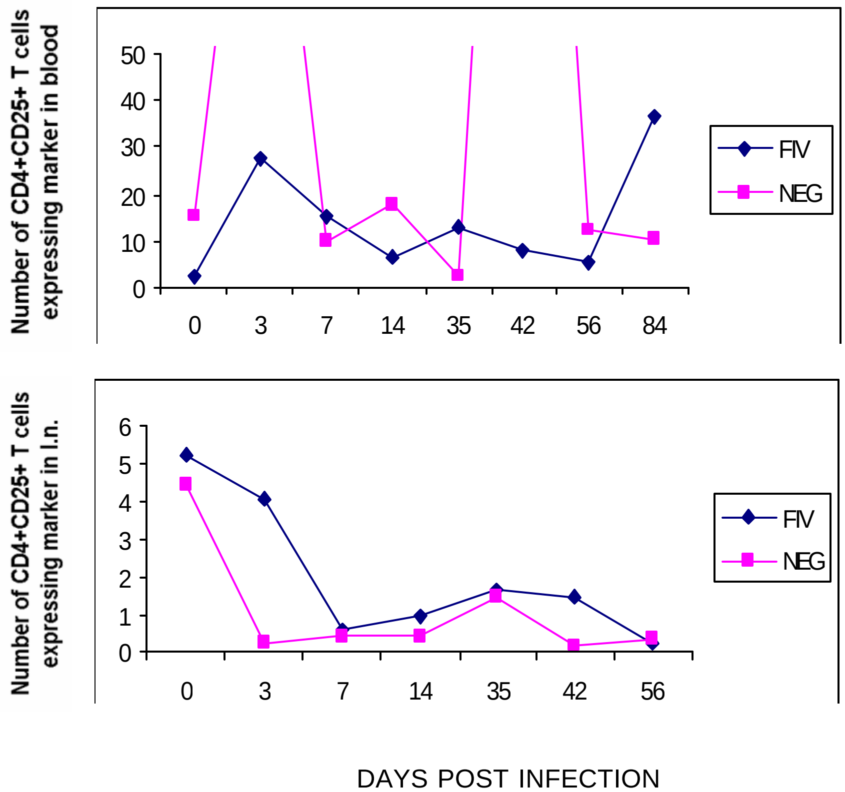
<u>Figure 16.</u> Percent of $CD4^+CD25^+$ T cells expressing TGFbR on their surface.
The average percent of $CD4^+$ lymphocytes expressing CD25 and the receptor for TGFβ on their surface was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group. Values from the blood and lymph nodes are shown.



<u>Figure 17.</u> Absolute number of CD4$^+$CD25$^+$ T cells expressing CD62L in blood and lymph nodes.
The average percent of CD4$^+$ Tcells expressing CD25 and CD62L on their surface was tabulated from duplicate measures for each cat after analysis by flow cytometry. The absolute number of CD25$^+$ CD62L$^+$ cells in CD4$^+$ populations from the blood and lymph nodes was calculated by multiplying the total number of lymphocytes counted in a CBC or lymph node by the percent of CD25$^+$ CD62L$^+$ T cells from the CD4$^+$ T cell population. Mean values for each group are represented for the blood samples per dl (a) or lymph nodes x10^6 (b) for each group of cats. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group.

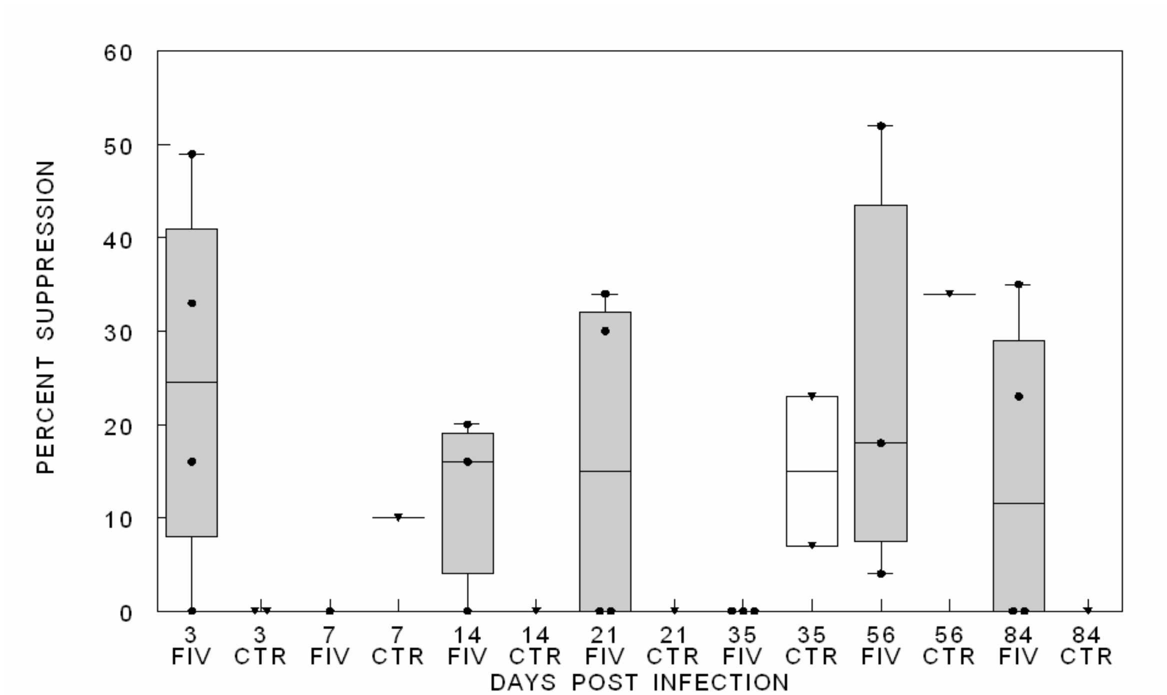


<u>Figure 18.</u> Absolute number of $CD4^{+}CD25^{+}$ T cells expressing B7.1 in blood and lymph nodes
The average percent of $CD4^{+}$Tcells expressing CD25 and B7.1 on their surface was tabulated from duplicate measures for each cat after analysis by flow cytometry. The absolute number of $CD25^{+}$ B7.1 cells in $CD4^{+}$ populations from the blood and lymph nodes was calculated by multiplying the total number of lymphocytes counted in a CBC or lymph node by the percent of $CD25^{+}$ B7.1 T cells from the $CD4^{+}$ T cell population. Mean values for each group are represented for the blood samples per dl (a) or lymph nodes x10$^{\wedge 6}$ (b) for each group of cats. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group.

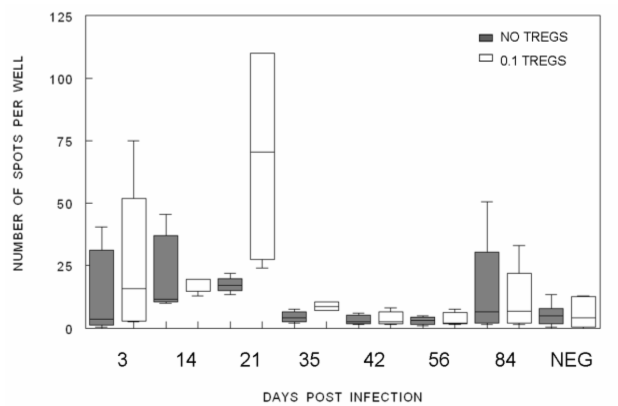


<u>Figure 19.</u> Absolute number of CD4$^+$CD25$^+$ T cells expressing the receptor for TGFb in blood and lymph nodes.

The average percent of CD4$^+$Tcells expressing CD25 and the receptor for TGFβ on their surface was tabulated from duplicate measures for each cat after analysis by flow cytometry. The absolute number of CD25$^+$ TGFβR$^+$ cells in CD4$^+$ populations from the blood and lymph nodes was calculated by multiplying the total number of lymphocytes counted in a CBC or lymph node by the percent of CD25$^+$ TGFβR$^+$ T cells from the CD4$^+$ T cell population. Mean values for each group are represented for the blood samples per dl (a) or lymph nodes x10$^{\wedge 6}$ (b) for each group of cats. Each point represents the mean values for 4 cats in each FIV-infected group and 2 cats in each control group except data from day 21 and 84 which represents only one cat in the control group.



<u>Figure 20.</u> $CD4^+CD25^+$ Mediated Suppression of IL-2 by ELISPOT assay.
$2x10^{5}$ sorted $CD4^+CD25^-$ (target cells) and $2x10^{5}$ sorted $CD4^-CD8^-$ (APC) were stimulated with Con A ($5ug/10^{6}$ 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). $2x10^{4}$ 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 T regulatory function from an individual cat, at each time-point. Circles correspond to FIV-infected cats and triangles to the control cats. The box and whisker plots show the median, 25 and 75 percentile values for a group of cats at each time-point.



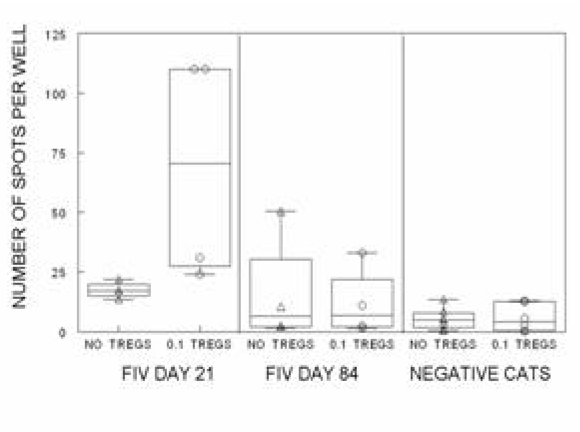


Figure 21. FIV-gag (anti-CD3) stimulated IL-2 ELISPOT assays.
2x10^5 sorted $CD4^{+}CD25^{-}$ (target cells) and 2x10^5 sorted $CD4^{-}CD8^{-}$ (APC) were stimulated with recombinant FIV-gag protein (50mg/10^6 cells) overnight at 37°C and 5% CO2, washed and plated in each well of the 96-well feline IL-2 ELISPOT plate provided by the manufacturer (R&D Systems). 2x10^4 un-stimulated $CD4^{+}CD25^{+}$ (Tregs) 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 is shown. Each marker represents the average number of SFC/well in 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. At early time-points (day 21) following FIV infection, the number of IL-2 spots increases when $CD4^{+}CD25^{+}$ T cells are noted, but this effect is not present at later time points (day 84), or in FIV-negative cats.

CHAPTER 2

2007

ACUTE PHASE OF FIV INFECTION STUDY

(STUDY #2)

ABSTRACT

HIV-induced AIDS may be mediated by the activation of immunosuppressive $CD4^+CD25^+$ T regulatory cells (Treg cells). The results of the first acute infection study showed peak viremia levels correlate with maximal infectivity in lymph node T cell populations and FIV-gag-mRNA levels were higher in $CD4^+CD25^+$ T cells than $CD4^+CD25^-$ lymph node T cells. Activation of FoxP3 and increased expression of TGF-ß in $CD4^+CD25^+$ cells correlated with peak plasma viremia and FIV-gag-mRNA levels in $CD4^+CD25^+$ T cells. However, due to a small sample size and variable results within each group, statistical significance was not reached for some of our findings. Therefore, a second study was designed to expand our findings using similar and additional methods. Cats were experimentally infected with FIV-NCSU^{-1} and blood and lymph node biopsies were collected at 1 week intervals following inoculation. In addition to the methods described in Chapter 1, intracellular staining for FoxP3 was analyzed and Treg suppression of $CD4^+$ T helper cell proliferation was assessed by $^{3-}$H-thymidine incorporation and CFSE flow cytometry assays. Our findings corroborate earlier results and help support the hypothesis that early activation of immune suppressor function in Treg cells may limit an effective anti-FIV response contributing to the establishment of chronic infection and immunodeficiency caused by this virus.

INTRODUCTION

The feline immunodeficiency virus (FIV) of domestic cats is a well established model for the study of infections with human immunodeficiency virus (HIV) and the related immunodeficiency syndrome (AIDS) because HIV and FIV share morphologic, physiologic and pathologic characteristics (21, 22). Both HIV and FIV infections are characterized by progression from a short acute phase with high but self-limiting viremia to a long latent, subclinical phase with low viremia, followed by severe immunodeficiency, a subsequent rise in virus levels and eventually, death from opportunistic infections or lympho-proliferative diseases (3). Interestingly, during the acute phase there is evidence of innate, humoral, and cell-mediated anti-viral immune responses. In fact, there is a rise in virus specific $CD8^+$ T cell numbers that corresponds with the sharp decrease in plasma viremia, which suggests a potential mechanism to clear the virus early after infection (4). However, this process is interrupted early on, and in most cases the virus is able to persist at low levels in quiescent cell populations (5).

The ability to establish a persistent infection requires early evasion and alteration of the immune response. Indeed, evidence of immune dysregulation is present before a significant decline in the number of $CD4^+$ T cells appears. Aspects of this immune dysregulation include both a global T cell immune hyper-activation, and a paradoxical hypo-responsiveness to virus antigens (12, 13). The activation of $CD4^+CD25^+$ regulatory T cells has recently been proposed as a mechanism for the immune suppression associated with retroviral infections. The idea that Treg cells might influence functional anti-viral immunity may have serious implications for the prevention of chronic infections with HIV and other viruses (13). In the previous chapter, we show that $CD4^+CD25^+$ T cells harbor greater amounts of FIV-gag-mRNA than $CD4^+CD25^-$ cells, are activated, and functionally suppressing CD4 Th cell responses during the acute phase of FIV infection. Peak plasma viremia levels correlated with maximal infectivity in lymph node cell populations and were highest at day 14 post infection. Higher viral mRNA levels in $CD4^+CD25^+$ T cells than their $CD4^+CD25^-$ counterparts, suggested either preferential infectivity or increased viral replication in Treg cells early after infection with FIV. The induction of FoxP3 mRNA transcription and an increase in surface expression of TGF-β was evidence of Treg cell activation occurring during the first 12 weeks post infection. High levels of suppressor function could be measured in some cats as early as day 3 post FIV infection. These results are consistent with previous observations of Treg cell function in long-term, chronically infected cats (14). However, due to the small sample sizes and variability within each group, statistical significance was not reached for most of our findings. Therefore, a second study was designed to expand our results using a second group of newly infected cats and focusing on the first four weeks post infection, the period before $CD4^+$ T cell numbers decline, in order to corroborate the results of the first study and attempt to reach statistical significance for our conclusions. In addition, we utilized alternative methods to further confirm our findings. These additional experiments included the use of intracellular staining for

FoxP3 and the measurement of suppression of target cell proliferation by $CD4^+CD25^+$ Treg cells. The results of this study confirmed the previous findings and expanded the evidence that Tregs are activated and functional following inoculation of a cat with FIV, supporting the hypothesis that these cells play a role in the early immune deficiency associated with retroviral infections in general.

The intracellular fork-head family transcription factor (FoxP3) is the most specific marker of regulatory cells that has been identified to date (104, 105). Studies suggest this protein is important in the regulation of immune responses to self antigens, and it is now well recognized that FoxP3 is important in the development, maintenance, and function of $CD4^+CD25^+$ T cells with regulatory functions (107, 108). Signals in the thymus (from stromal or dendritic cells) determine the regulatory lineage of a subset of $CD4^+$ T cells during development (109, 110). At later stages of differentiation, FoxP3 gets induced by a signal that includes IL2 and TGF-β participation (111). The expression of FoxP3 in these cells then results in the down-regulation of the IL2 promoter and the up-regulation of other Treg-associated surface proteins such as CTLA4 and GITR (112). However, none of the proteins expressed on the cell surface are selectively expressed on regulatory cells. Activation through the T cell receptor in non-regulatory T cells also results in the up-regulation of CD25, CTLA4, and GITR. Contact between these molecules on FoxP3 expressing Treg cells and their receptors on target $CD4^+$ effector cells signals them to reduce IL2 production inhibiting their proliferation (113), while in activated T cells that do not express FoxP3, this signaling leads to increased IL2 production and cell proliferation

(110, 112-114). In the periphery, certain feline and human $CD4^{+}CD25^{-}$ T cells can be converted into Treg cells in the presence of IL2 and TGFβ (115, 116). These induced Treg cells also up-regulate FoxP3 expression and develop suppressor functions. Induced Treg cells expressing FoxP3 also have decreased IL-2 production and mitogenesis in response to TCR stimulation. While expression of FoxP3 is not confined to $CD4^{+}CD25^{+}$ T cells in humans (117), it is believed that FoxP3 expressing $CD4^{+}CD25^{-}$ T cells constitute a reservoir of committed regulatory cells in the periphery (118). In addition, it has been shown that genetically reprogrammed FoxP3 expressing lymphocytes become increasingly susceptible to HIV infection via an unpregulation of CCR5 which follows FoxP3 induction (119). CCR5 is one of the primary virus binding proteins on the surface of T cells. Therefore, an increase in FoxP3 expression has been associated with increased regulatory function and is used in our work to measure the activation of Treg cells by FIV in vivo. During the previous experiment we were only able to measure the expression of FoxP3 in groups of sorted cells by RT-PCR. In this study, we also observed changes in the expression of FoxP3 protein by intracellular staining using fluorescent anti-FoxP3 antibodies to detect the protein in $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+}$ T cell subsets in the blood and lymph nodes of FIV infected cats. FoxP3 is primarily intra-nuclear, which means cells have to be fixed and permeabilized prior to the detection of this protein. Therefore, we have been unable to use the expression of this protein as a marker in live cells, making it impossible to selectively sort Treg cells for ex-vivo analysis of cytokine production or proliferation inhibition based on their expression of FoxP3.

However, using this technique, we correlate changes in surface phenotype and function of $CD4^+CD25^+$ T cells to increased expression of FoxP3 as a marker of regulatory capacity. We show that while the number of cells expressing FoxP3 increases in all compartments, the mean fluorescent intensity of FoxP3 staining increases significantly only in $CD4^+CD25^+$ T cells, suggesting up-regulation of this protein is associated with FIV infection and results in the induction of regulatory function early after infection with the virus.

In the previous chapter we described Treg cell function associated with the inhibition of IL-2 production in response to Con A stimulation following infection with FIV. Here, we propose to measure suppressor function by detecting changes in $CD4^+$ Th proliferation assays. Traditional suppressor assays measure incorporation of tritiated thymidine during mitogenesis as a measure of proliferation within whole cell populations. Here we attempted to corroborate the results of these methods with the additional use of a measure of proliferation at the single cell level using the membrane dye CFSE. For these experiments, target cell populations consisting of $CD4^+CD25^-$ Th cells, were treated with the membrane staining dye (CFSE) prior to TCR stimulation with Con A and cultured with or without different concentrations of $CD4^+CD25^+$ Treg cells for 3-6 days. Tritiated thymidine was then added to half of the wells during the last 18 hours of culture in order to assess proliferation of T cells using both methods simultaneously. The results of these experiments were also compared to those from the IL-2 production assays to determine if Treg cells are functionally suppressing CD4 T cell responses during the acute phase of FIV infection.

RESULTS

A. Plasma and Cell Associated Viremia

To confirm infection of the cats, plasma samples obtained starting at 3 weeks post inoculation were evaluated for the presence of anti-FIV antibodies using a commercially available ELISA snap test (IDEXX). All of the cats in this study tested positive for anti-FIV antibodies starting at 4 weeks post inoculation. Stored plasma samples obtained from negative controls were used to assess the validity of these results and tested negative for anti-FIV antibodies, as expected. Of the two plasma samples collected at three weeks post infection that were tested, one had a weak positive result and the other was negative. Quantitative RT-PCR, used to evaluate plasma viremia revealed the peak mean number of FIV-gag mRNA copies per ml of plasma occurred at day 14 post infection as was seen in the first study. This was followed by a decline to low but positive levels thereafter as seen before (Figure 1). The calculated number of copies of FIV-gag mRNA/ml of plasma from individual cats at each time point is also shown in Table 1. There was significant variability between cats in each group as was noted in the previous experiment. In general, however, the number of FIV-gag-mRNA copies detected per ml of plasma was lower in this study than in the previous one. Also in comparison with the previous study, more cats here had levels of virus that were below the detectable measure (5 copies) in this reaction. In fact, at least one cat at each time point had less than 5 copies of FIV-gag-mRNA per ml of plasma. All of these cats tested positive for infection by FIV ELISA and were effectively infected with the virus as established by cell-based PCR analysis. Table 2 shows the individual cats that had less than 5 copies of FIV mRNA per ml of plasma at each time point. Of the 8 cats in this list, only two (RGM1 and JIX3) had undetectable FIV-mRNA levels at both time points examined, but both had detectable FIV mRNA in cell samples. Only one of the cats (BHE3) did not have FIV mRNA detected in cell samples but this cat had a positive plasma viremia test at the second time point (Wk 4). In addition, two of the cats tested had extremely high plasma mRNA levels indicating the low levels in other cats were not assay-dependent (RGS2, RGS3).

To determine if $CD4^+CD25^+$ Treg cells become infected with FIV during the acute stage of infection, relative quantities of viral mRNA were compared in FACS sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes from FIV-infected cats using reverse transcription and real time PCR. Peak virus level means were found in $CD4^+CD25^+$ Treg cells from the lymph nodes at 2 weeks (14 days) post infection, which closely paralleled the plasma viremia pattern described above. Average virus levels in PBMC's, however, were slightly higher at week 3 than week 2 in $CD4^+CD25^+$ cells. The relative quantities of virus mRNA were greater in $CD4^+CD25^+$ than in $CD4^+CD25^-$ cells in both lymph node and blood compartments at all times, for each cat tested, except at week 1 post infection in the blood (Figure 2). Again these results closely mimic those of the previous acute phase infection study.

B. Lymphocyte Phenotype Changes in Acutely FIV-Infected Cats

As expected a decrease in the CD4:CD8 ratio was again detected in the blood and lymph nodes of FIV-infected cats starting at week 5 post inoculation (Figure 3). To determine if acute FIV infection altered Treg cells prior to a drop in CD4:CD8 ratios, we focused our analysis on results from the first four weeks post infection. At weeks 5 and 8, significant variability between cats in the same group was observed in terms of cell numbers, making the results of our other tests difficult to interpret. Thus, we compared the percent and absolute numbers of $CD4^+CD25^+$ T cells in FIV-infected cats during the first four weeks post inoculation. First we tabulated the numbers of cells obtained from a whole popliteal lymph node (Figure 4). The percent of $CD4^+CD25^+$ T cells in the blood and lymph nodes of these cats decreased gradually during the first 4 weeks of infection (Figure 5). The absolute $CD4^+CD25^+$ T cell count in the lymph nodes is represented in Figure 6, showing a decline at 2 weeks post infection followed by the recovery to initial numbers by week 4. Despite the lack of changes in percent and number of $CD4^+CD25^+$ T cells over the first 4 weeks of infection, there was a difference in the phenotype of these cells during this time, suggesting the cells are activated to suppress following FIV infection. During this acute infection study we noted, as before, an increase in the surface expression of TGF-β in $CD4^+CD25^+$ cells at 2 and 3 weeks post infection. This change was specifically noted in the cells of the lymph node as very little surface TGF-ß could be detected on cells from the peripheral blood (Figure 7).

C. Relative quantification of FoxP3 and TGF-b mRNA in T cell Subsets, During the Acute Phase of FIV Infection

As expected, Foxp3 mRNA levels were once again significantly higher in $CD4^+CD25^+$ than CD4+CD25- T cells at all time points (Figure 8). Also, as seen in the previous study, the expression of FoxP3 mRNA in the $CD4^+CD25^+$ T cells of the peripheral blood increased during the first 4 weeks of the study, while it did not change significantly in the lymph nodes. Sufficient peripheral blood $CD4^+CD25^+$ T cells were not available for evaluation from weeks 5 and 8 post infection.

Reverse transcription and RT-PCR was also used to measure differences in the expression of TGF-β mRNA in the blood and lymph node T cell subsets of FIV-infected cats. No significant differences were noted during the first four weeks following FIV infection in these cats, but a slight trend towards increased levels of TGF-β mRNA was noted in all cell subsets over the course of the experiment (Figure 9).

D. Intracellular expression of FoxP3 in T cell subsets following infection with FIV

In addition to the detection of FoxP3 mRNA by RT-PCR, intracellular staining for FoxP3 protein was performed to detect changes in this important marker of regulatory function at the protein level. The results suggested FoxP3 was up-regulated in both $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets of the blood and lymph nodes following FIV infection, as the percent of FoxP3 positive cells increased in all T cell subsets (Figure 10). The change in percent of cells expressing FoxP3 was actually smaller over time in the $CD4^+CD25^+$ subset however, because these cells show a higher baseline of FoxP3 expression prior to infection, and at very early time-points following expression. However, a greater change in the mean fluorescence intensity was noted in the $CD4^+CD25^+$ T cells when compared to the $CD4^+CD25^-$ T cells. Figure 11 shows a representative sample for T cell subsets at weeks 1, 2, and 5 post infection, indicating both the increased $FoxP3^+$ T cell counts noted in all subsets and the increased expression of FoxP3 expression in $CD4^+CD25^+$ T cell subsets. In figure 12 the mean fluorescence intensity (MFI) of all samples analyzed is compared for the first four weeks post infection. As expected, $CD4^+CD25^+$ T cells express higher FoxP3 levels than $CD4^+CD25^-$ T cell subsets at all time points. The MFI for FoxP3 expression was significantly higher in $CD4^+CD25^+$ T cells of the lymph nodes at weeks 3, 4, and 8 post infection, than at weeks 1 and 2. This conclusion was reached by performing individual t tests between sets of values obtained at each time point for each cell subset. Data from week 5 was not included in this analysis due to a probable assay error. The p value obtained was less than 0.05 when statistical significance is mentioned. A similar trend was noted in the $CD4^+CD25^+$ cells from the blood; however, these numbers did not reach statistical significance. There was a relative increase in the FoxP3 MFI on all cell subsets at week 5 post infection. Given the unexpected increases in the $CD4^+CD25^-$ T cell subsets, these results may represent a problem with the assay at that time point.

E. Suppression of Target T cell Proliferation by Treg Cells during Acute Phase FIV Infection

Two methods were employed in this study to measure the inhibition of proliferation of $CD4^+CD25^-$ target cells by $CD4^+CD25^+$ Treg cells, and these results are compared to the IL-2 ELISPOT experiments detailed in Chapter 1. Our aim was to use well established methods to assess cell proliferation in order validate our earlier observations. For this purpose, cells from the lymph nodes of FIV-infected cats were sorted into individual populations of $CD4^+CD25^+$ (Tregs) and $CD4^+CD25^-$ (targets) T cells. Proliferation of CFSE stained target cells was analyzed by flow cytometry and 3-H Thymidine incorporation as described in the methods. The results for the thymidine incorporation assays show suppression of target cell proliferation in some of the cats at weeks 2, 3, and 4 post infection (Figure 13). Using a ratio of one Treg cell to 10 target cells (E:T 0.1) 0 of 3, 1 of 1, 1of 1, and 1 of 2 Treg samples were able to suppress the proliferation of $CD4^+CD25^-$ target cells at 1, 2, 3, and 4 weeks post infection, respectively. Using a ratio of one Treg to 2 target cells (E:T 0.5) 0 of 3, 0 of 1, 1 of 1, and 1 of 2 Treg samples were able to suppress the proliferation of $CD4^+CD25^-$ target cells at 1, 2, 3, and 4 weeks post infection, respectively. These results confirm that Treg cells functionally suppress the proliferation of effector $CD4^+$ T cells during the acute phase of FIV infection and suggest a mechanism involved in early immunosuppression. However, the radiation counts obtained during this analysis were far below those reported in earlier studies suggesting there was relatively little cell proliferation even in the absence of Treg cells.

Proliferation of target cells was also assessed by flow cytometry using the membrane dye CFSE, which divides equally between daughter cells during each cell division event. As described in the methods, cells for these and the previous proliferation experiments were stimulated with Con A prior to the addition of Treg cells. The results of our CFSE studies show very little proliferation of the target cells at day 4 and even 6 of culture following Con A stimulation with and without Treg cells being present in culture making it impossible to accurately detect suppression of proliferation in these experiments. Figure 14 shows the results of flow cytometric analysis of representative samples at one and four weeks post infection. The CFSE (FITC) low cells in some panels indicate $CD4^{+}CD25^{+}$ T cells added to the cultures. M2 encompasses the cells with the highest intensity of CFSE detected in target cells at day 1 (presumably not proliferating cells). M1 encompasses the target cells only at later time points post-stimulation. The percent of inhibition of cell proliferation was examined by comparison of the percent of cells left in M2 out of the percent of cells in M1 at each time point to account for the difference in Treg cells added between samples. Table 3 describes the calculations performed and suppression of proliferation measured in these samples. Due to the lack of proliferation in the target cell population, very little inhibition was noted in most samples and no conclusions can be made from these studies.

F. Comparison of Regulatory Function Markers in Cats with High and Low Plasma Viremia Levels

A wide range of virus mRNA levels was detected in plasma samples from different cats during the first weeks following infection with the same virus doses. This led to a brief analysis of potential differences in T regulatory cell function between cats with high and low viremia levels. 6 out of 28 cats (21%) experimentally infected with FIV in both studies had extremely high plasma viremia levels within the first weeks post infection when compared to the other cats (Figure 15). Sufficient PCR results for comparison of high to low viremia cats from similar sources and time-points were only available for lymph node cells obtained at 8 weeks post infection. The results of this analysis are presented in Figure 16. High plasma viremia correlated with higher FIV viral load and lower FoxP3 and TGF-β levels in $CD4^+CD25^+$ T cells from the lymph nodes at 8 weeks post infection. Data from $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from the peripheral blood was also available from both high and low plasma viremia cats at week 5 post infection, but only from the first study. On average FIV mRNA levels were once again higher and TGF-β mRNA levels were lower in the cats with high plasma viremia levels, but the level of FoxP3 mRNA was higher in the cat with high viremia than in three cats with low viremia levels (Figure 17). In addition small samples for comparison between cats with high and low plasma viremia levels were available for $CD4^+CD25^+$ T cells at week 2 from the first study and $CD4^+CD25^-$ T cells at week 3 from the second study (Figure 18). At this early time points in $CD25^+CD25^+$ T cells FIV mRNA levels were already higher in the cats with high plasma viremia levels, but on average, there were no differences in the amounts of FoxP3 and TGF-β mRNA in these samples. While enough data was not present to confirm these findings at other time points, or from more cats, our results suggest there is a possible relationship between low regulatory function markers and high viremia levels during the acute phase of infection with FIV.

DISCUSSION

Two major aims were completed in this second acute phase infection study. The first was to validate the results of the first experiment and expand the number of cats or samples examined at each time-point using similar methods. The second aim was to apply new techniques in an effort to corroborate and fortify our conclusions. The results validate our hypothesis that $CD4^+CD25^+$ T cells are infected by higher levels of FIV than $CD4^+CD25^-$ T cells, and express activation phenotypes that correlate with regulatory function. The activation of regulatory function in $CD4^+CD25^+$ T cells may negatively impact anti-viral cell-mediated immune responses.

Several findings from the first acute infection study were confirmed with these experiments. Peak viremia levels once again correlated with maximal infectivity in lymph node cell populations, and were highest at two weeks post infection. $CD4^+CD25^+$ T cells had higher levels of viral mRNA than the $CD4^+CD25^-$ counterparts, and FoxP3 mRNA and surface TGF-β were up-regulated during the course of the study. Other aspects such as the high levels of suppressor function prior to week 3 post infection were not duplicated here. The pattern of plasma viremia was similar to that noted in the first study, however, the number of FIV-gag-mRNA copies present in the plasma samples was lower overall than the values noted in the first study. The same dose and isolate of virus was used in both experiments. The virus was maintained in liquid nitrogen and thawed only prior to inoculation. A wide range of plasma viremia levels was noted in both studies, suggesting the variability between studies was due to the individual cats' response to the virus. Nevertheless, this important difference may have secondarily affected other results in this investigation. Within T cell subsets, virus mRNA levels were measured in relation to those of a calibrator sample which differed between experiments. Therefore, it is impossible to compare the actual levels of FIV-gag-mRNA in either subset between studies. The relative difference in FIV mRNA levels between $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets however, was the same in both studies. Again, we show $CD4^+CD25^+$ T cells have higher FIV mRNA levels at most of the time points examined. This result implies $CD4^+CD25^+$ Treg cells are likely infected with FIV, but is not surprising considering activated phenotypes have previously been shown to be the only cell type able to stably carry the infection and replicate the virus.

Another similarity between studies was the inversion of CD4:CD8 ratio which was noted to start at week 5 post infection in both studies. The inversion of this ratio is likely due to both, the expansion of $CD8^+$ T cells, and the reduction of $CD4^+$ T cells associated with the infection. Because our intent is to focus on changes to the immune system that may be present during the period of time preceding a decline in $CD4^+$ T cells, the remainder of our results and conclusions are concentrated around the findings for the first 4 weeks post inoculation. In the previous study, no significant differences between the percent and absolute numbers of $CD4^+CD25^+$ T cells in FIV-infected vs. non-infected controls were observed. In the present investigation, a slight but continuous decline in the mean percent of $CD4^+CD25^+$ T cells was observed in the blood and lymph nodes of FIV-infected cats until week 4 post infection, but once again, these differences were not statistically significant due to variability within each group.

The intracellular transcription factor FoxP3 is recognized as the most specific marker of regulatory function expressed within regulatory T cells. A main focus of this study, was to detect changes in the expression of FoxP3 following FIV infection as a marker for increased regulatory function. In the present study, we utilized two methods to detect changes in FoxP3 expression: RT-PCR and flow cytometry. The results here are compared to previous examination of FoxP3 mRNA levels in T cells subsets from FIV-infected and control cats as described in Chapter 1. Again, we show by RT-PCR that FoxP3 mRNA is produced at much higher levels in $CD4^+CD25^+$ T cells than CD4+CD25- T cells. In addition, we show a statistically significant increase in the expression of FoxP3 mRNA during the first 4 weeks post infection in the blood cells of FIV^+ cats. The reason for a difference in FoxP3 expression in the blood and not the lymph node in both studies was not clearly defined. However, it is believed that peripheral recruitment of infection activated Treg cells (so called induced Treg cells) happens in the periphery and not the lymph nodes. It is possible that changes in mRNA expression are only noted in this compartment because it is only here that "new" Treg cells are being induced. In contrast to the studies by RT-PCR, changes in expression of FoxP3 protein levels were noted in the cells of FIV-infected cats during the first 4 weeks of infection. Using intracellular staining techniques on fixed and permeabilized cells, we show here there is a significant increase in the expression of FoxP3 protein in $CD4^{+}CD25^{+}$ cells of the lymph node at weeks 3 and 4 post infection, while a similar change was smaller and not statistically significant in the blood cells. Taken together, the results of both studies suggest important changes in FoxP3 expression occur following FIV infection in cats, but further studies are needed to conclusively define the extent and significance of these changes.

TGF-β is another factor associated with regulatory function that had been shown in previous studies to be expressed at higher levels on the surface of activated $CD4^{+}CD25^{+}$ Treg cells, suggesting TGF-β expressing $CD4^{+}CD25^{+}$ T cells are functional regulatory T cells. Furthermore, it was shown that Tregs from chronically FIV infected cats but not control cats expressed TGF-β on their surface, suggesting FIV infection induces regulatory function in T cells. A transient increase in membrane TGF-β expression was also noted in the previous acute phase infection study. Here an increase in surface TGF-β expression was again noted in cells from the lymph nodes at weeks 2 and 3 post infection. No such changes were noted in the 12 cats reported here either by flow cytometry, or RT-PCR during the first four weeks following FIV infection in the peripheral blood T cell subsets. It is possible that the low viremia levels recorded in this group of cats had a significant effect on the up-regulation of TGF-β, eliminating our ability to detect any such changes. The function of TGF-β is closely regulated by several pre and post-transcriptional effects making any changes in mRNA and protein, or lack thereof, difficult to interpret. On the other hand, the changes observed in TGF-β expression on $CD4^{+}CD25^{+}$ cells from the lymph nodes may suggest the activation of Treg cells to suppress effector T cell responses following FIV infection.

Lastly, attempts were made to detect the suppression effects of Treg cells on the proliferation of target $CD4^{+}CD25^{-}$ T cells using well established methods. The primary aim of these experiments was to compare the results to the levels of IL-2 inhibition reported in the first acute infection study. For this reason, we attempted to keep the methods used in these suppression assays similar to those used in the IL-2 suppression assays. Nevertheless, important factors may have affected the results of these studies. Both methods employed in this study suffered from poor proliferative responses in the target cell populations. This is illustrated best in the CFSE experiments, but low radioactivity levels measured in the thymidine assays corroborate this finding. One important difference between our study and the previously reported studies is the lack of IL2 used in the culture of cells during the suppression assays. It has been shown that IL2 is required not only for the maintenance and proliferation of $CD4^{+}CD25^{+}$ T cells, but also for the proliferation of effector $CD4^{+}CD25^{-}$ T cells following TCR stimulation. This is evidenced best by the proposed mechanism of regulation suggested here and in other studies, which is the inhibition of IL2 production in target cells. Our desire to compare the results of these proliferation studies with those on IL-2 production precluded our ability to use IL2 in the cultures, but likely resulted in inhibited levels of T cell proliferation in the target population. In spite of this effect, however, the tritiated thymidine studies showed some degree of inhibition by $CD4^{+}CD25^{+}$ T cells on the proliferation of $CD4^{+}CD25^{-}$ T cells. The higher levels of suppression noted at an E:T of 0.5 vs. 0.1 also give credence to our methods and results in these experiments.

Perhaps the most striking difference among different cats in this and the previous study was the wide range of virus mRNA levels detected in plasma samples during the first weeks following infection with the same virus doses. Even when the overall number of copies of FIV-gag mRNA seemed significantly lower in the second study, two cat plasma samples registered such high plasma FIV levels that the average plasma viremia level was higher for high viremia level cats in the 2007 than the 2006 study. Sufficient PCR results for comparison of high to low viremia cats from similar sources and time-points were only available for limited samples in this retrospective analysis. In general, high plasma viremia correlated with higher FIV viral load and lower FoxP3 and TGF-β levels in $CD4^{+}CD25^{+}$ T cells from the blood and lymph nodes at 5 and 8 weeks post infection, respectively, suggesting a possible relationship between low regulatory function markers and high viremia levels during the acute phase of infection with FIV. It is impossible to infer causation from this brief and limited analysis, however, these results warrant further exploration of the effects of viral containment on activation of regulatory function (and vice versa) in the early stages of FIV infection in cats. On the other hand, the high viremia levels in some cats may be associated with increased proliferation of activated T helper cells and not Treg cells, resulting in relatively lower levels of Treg function in the $CD4^+CD25^+$ T cell compartment overall.

In conclusion, this experiment helped to clarify the results of the previous acute phase infection study. Further studies on the functional suppression of T helper cell proliferation by Treg cells in FIV infected cats during this phase of infection are needed, but IL2 appears to be required for the proliferation of target cells in such studies. Furthermore, there appears to be a correlation between decreased FoxP3 and TGF expression and a high viremia level, but additional studies are needed in this regard. Nonetheless, the results here suggest there is evidence of regulatory T cell function suppressing $CD4^+$ T cell responses early in the course of FIV infection.

TABLE 1. Real-time RT-PCR analysis of plasma from FIV-infected cats

(copies FIV-gag-mRNA/ml of plasma).

WEEKS PI	1	2	3	4	5	8
CAT #1	<5	12.35	<5	5.372	10.92	18.88
CAT #2	8.652	<5	16	141.6	<5	0
CAT #3	5.198	33.24	1974	0	<5	31.4
CAT #4	0	6378	<5	21.48	21.84	0

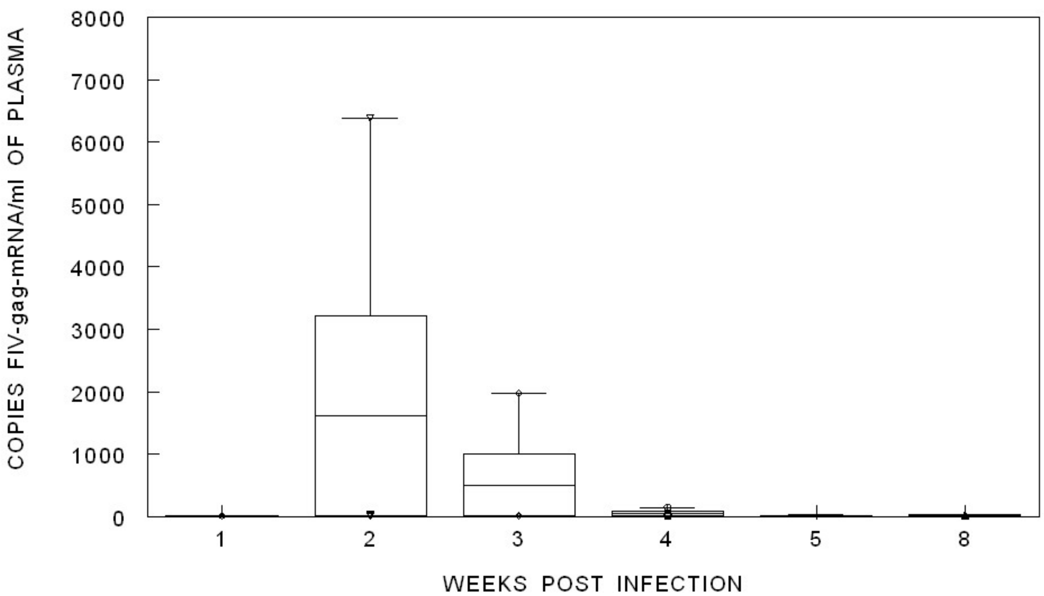
TABLE 2. Cats with less than 5 copies of FIV-gag-mRNA per ml of plasma.

Week Sample was taken:	Cat number:
Week 1	BHE3 RGL3
Week 2	RGM1*
Week 3	RGL5 JIX3*
Week 4	RGK4
Week 5	RGM1* RGS1
Week 8	RGL6 JIX3*

*Cats with <5 copies/ml of plasma on more than one sample.

TABLE 3. Inhibition of cell proliferation by CFSE assays.

WEEKS POST INFECTION	DAY OF CELL CULTURE	E:T		% CELLS GATED		% INHIBITION OF CELL PROLIFERATION
			M1	M2	M2/M1	
1	4	0	99.70	32.04	0.321364	
		0.5	54.90	18.17	0.330965	3.0%
4	4	0	99.55	12.32	0.123757	
		0.1	93.99	13.31	0.141611	14.4%
		0.5	72.99	9.29	0.127278	2.8%
4	6	0	99.87	2.32	0.02323	
		0.1	71.42	1.27	0.017782	-23.5 (no supp)
		0.5	44.00	0.63	0.014318	-38.4 (no supp)



<u>Figure 1.</u> Quantification of FIV-gag mRNA in plasma samples by real-time PCR. Absolute quantification of viral mRNA was evaluated in 1 ml of plasma obtained from FIV-infected cats at different time-points in the study. Serial dilutions of a previously quantified sample were run in parallel in each assay to create a standard curve. The assay can detect as little as five copies of FIV-gag mRNA in a sample. Each of the unknown and standard curve samples were evaluated in duplicate wells and the assay was repeated twice. All of the results for each sample were averaged individually and are tabulated in table 4. Each marker represents the value obtained from one specific cat. The mean, 25 and 75 percentiles are represented in the box and whisker plots.

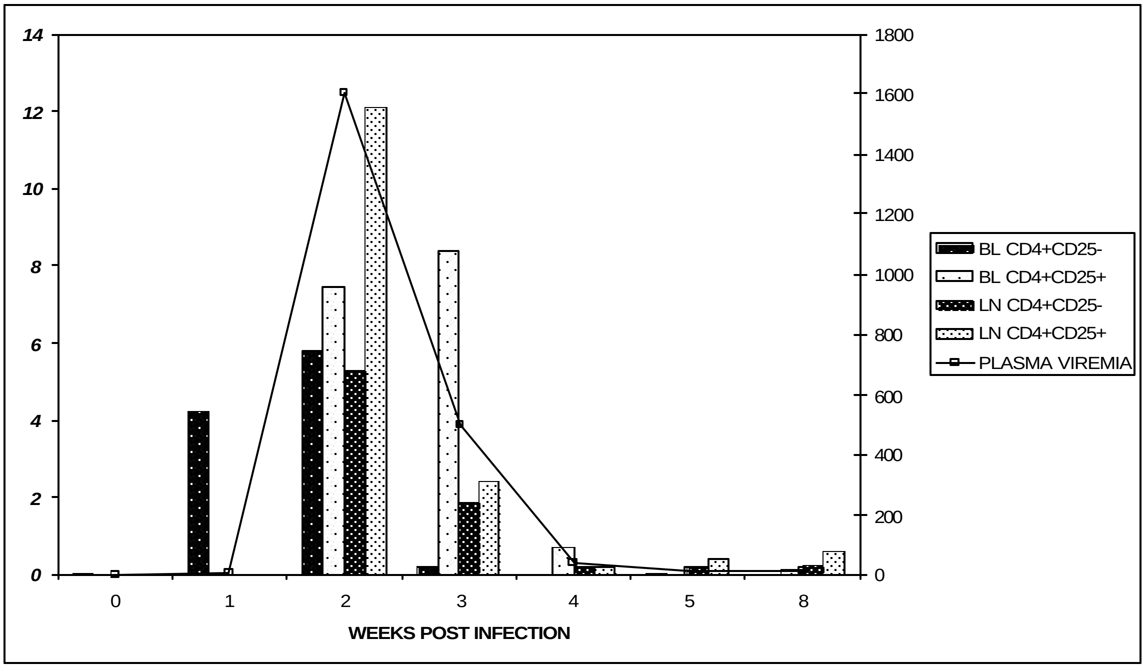


Figure 2. **Relative quantification of viral load in subsets of feline lymphocytes by Real-time PCR.**

Sorted populations of 0.5X10^6 $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from blood and lymph nodes were maintained in RNA later 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 1-4 cats were evaluated individually. 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 mean plasma viremia levels at each time as described in figure 1.

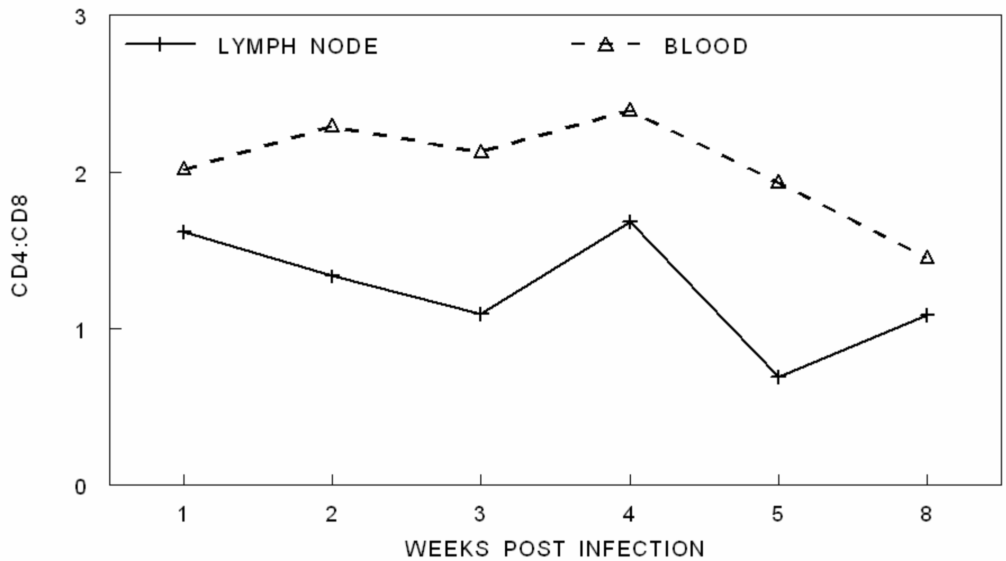
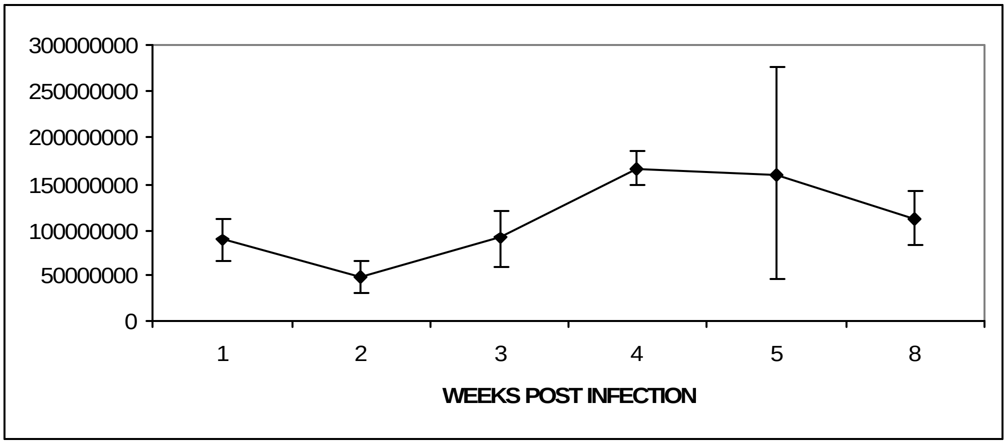


Figure 3. CD4:CD8 ratios decrease in FIV infected cats.
The numbers and ratios of $CD4^+$ to $CD8^+$ T lymphocytes were calculated for each cat evaluated at each time-point during the first 8 weeks following FIV inoculation by flow cytometry. Each measurement is the average of values obtained from 4 FIV-infected cats. The average CD4:CD8 ratios at each time-point in the blood and lymph node begin to drop at week 5 post infection.



<u>Figure 4.</u> Number of cells obtained from each lymph node.
Popliteal lymph nodes were removed as described in the material and methods and maintained on ice in CTL culture media. Connective tissue surrounding each node was gently removed and the node was perforated several times with a 20g hypodermic needle. Media was then injected through the node several times to release the cells contained. The cells were then washed and counted as described in the appendix and the total number of cells was recorded for each lymph node obtained. The graph above represents the mean number and standard deviation of total cells obtained from the popliteal lymph node of 4 cats at each time-point.

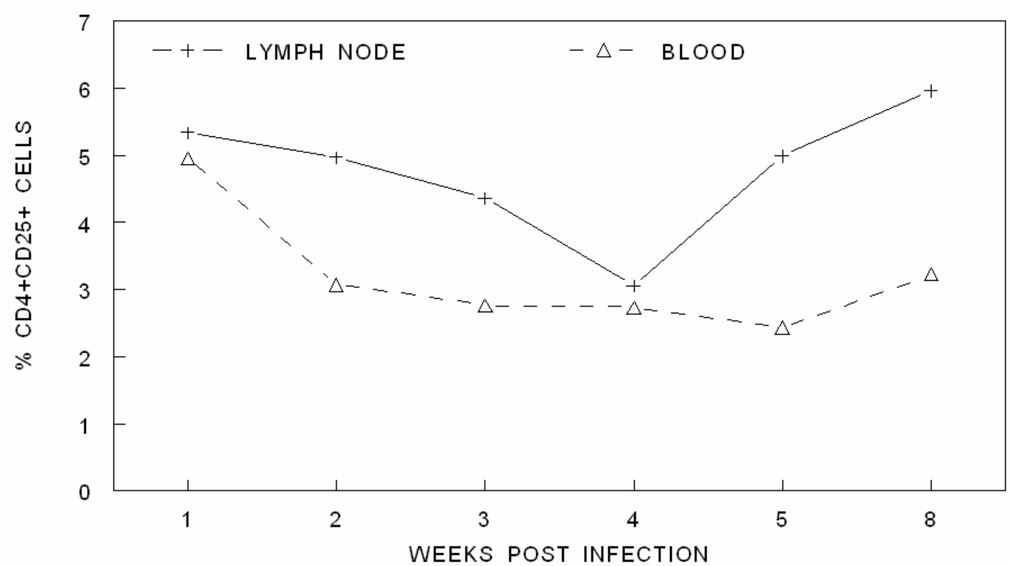
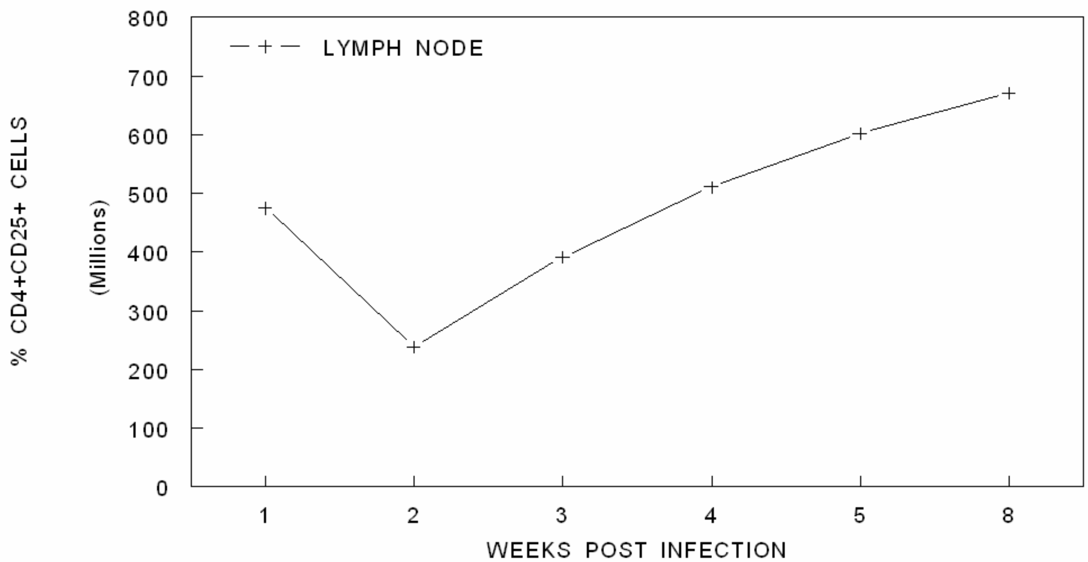
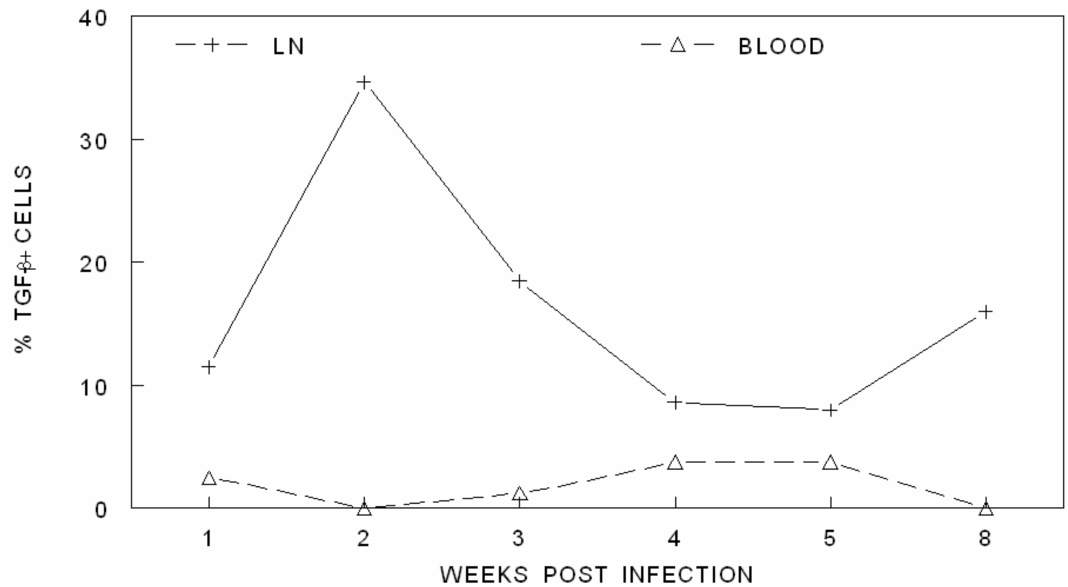


Figure 5. **Changes in CD4$^+$CD25$^+$ T cell population in cats with FIV.**
The average percent of CD4$^+$CD25$^+$ T cells from total lymphocytes was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each group. Values from the blood and lymph nodes are represented.



<u>Figure 6.</u> Changes in absolute number of $CD4^+CD25^+$ T cells in cats with FIV.
The absolute number of $CD4^+CD25^+$ T cells from total lymphocytes was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each group. Values from the lymph nodes are represented.



<u>**Figure 7.**</u> **Surface expression of TGF-b on $CD4^+CD25^+$ Lymphocytes.**
The percent of $CD4^+CD25^+$ T cells expressing surface TGF-β was tabulated from duplicate measures for each cat after analysis by flow cytometry. Each point represents the mean values for 4 cats in each group. Values from the blood and lymph node samples are represented.

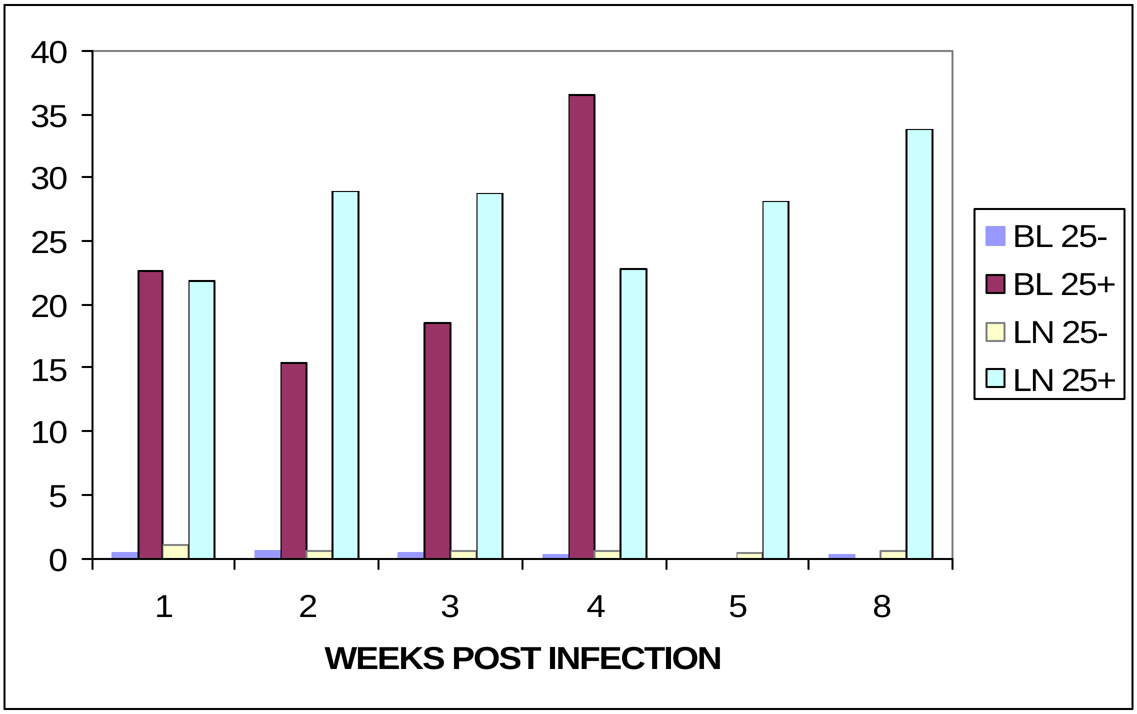
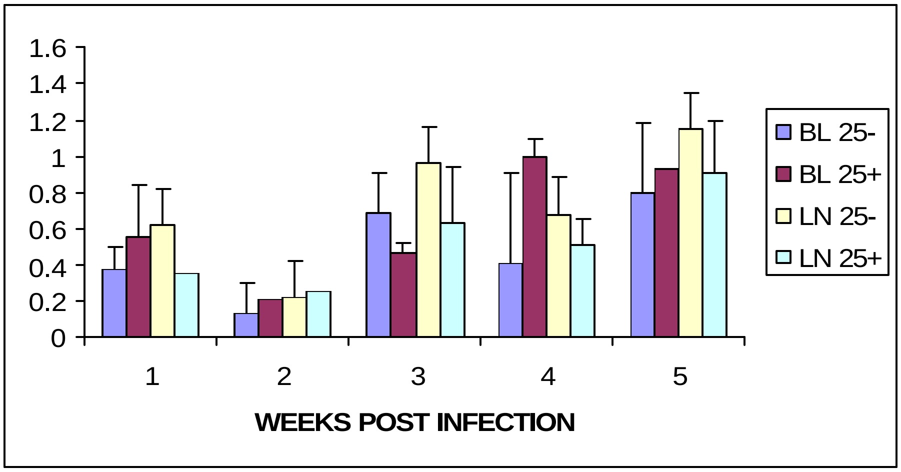


Figure 8. Relative quantification of FoxP3 mRNA in feline lymphocyte subsets.
Sorted subsets of $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ T cells isolated from FIV-infected cats were maintained in RNA later for analysis of FoxP3 mRNA levels by reverse transcription and quantitative real-time PCR. Equal amounts of RNA were used in each test. The mean of relative values for each group are represented in the bar graph. $CD4^{+}CD25^{-}$ T cells contained relatively small amounts of FoxP3 mRNA at all time-points in infected cats. However, increases were noted at weeks 1 and 3 in PBMC $CD4^{+}CD25^{-}$ T cells. The relative levels of FoxP3 mRNA in the $CD4^{+}CD25^{+}$ T cells from FIV-infected cats increased slightly over time and the groups of cells evaluated for each time point are represented individually. In this subset, there was a significant increase in FoxP3 transcription at week 4 post infection in the blood of FIV-infected cats ($p<0.1$).



<u>Figure 9.</u> Relative quantification of TGF-b mRNA in feline lymphocyte subsets.
The expression of TGF-β mRNA in $CD4^+CD25^+$ and $CD4^+CD25^-$ lymphocytes from the lymph nodes and blood of FIV-infected cats was evaluated by reverse transcription and RT-PCR. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from FIV-infected cats are depicted separately. Each bar represents the mean relative fold increase using the delta delta CT method of quantification for 1-4 samples obtained in duplicate assays.

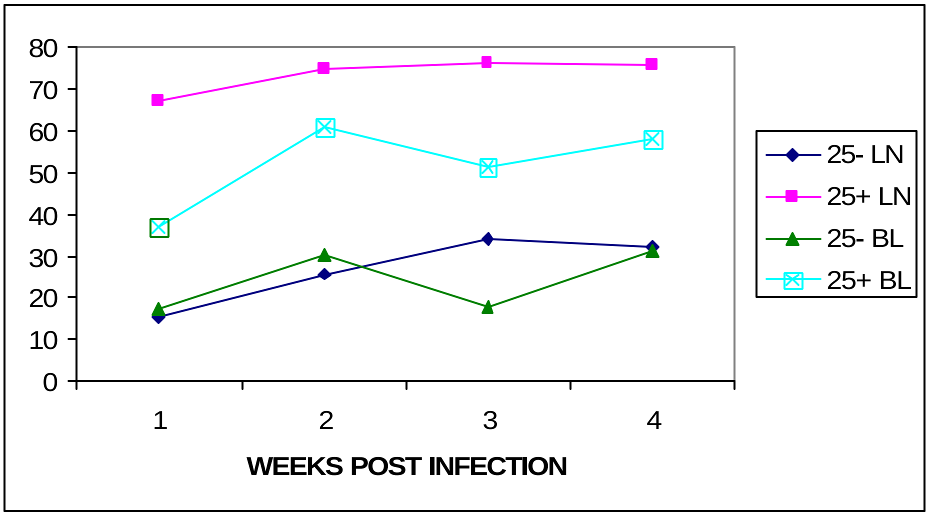
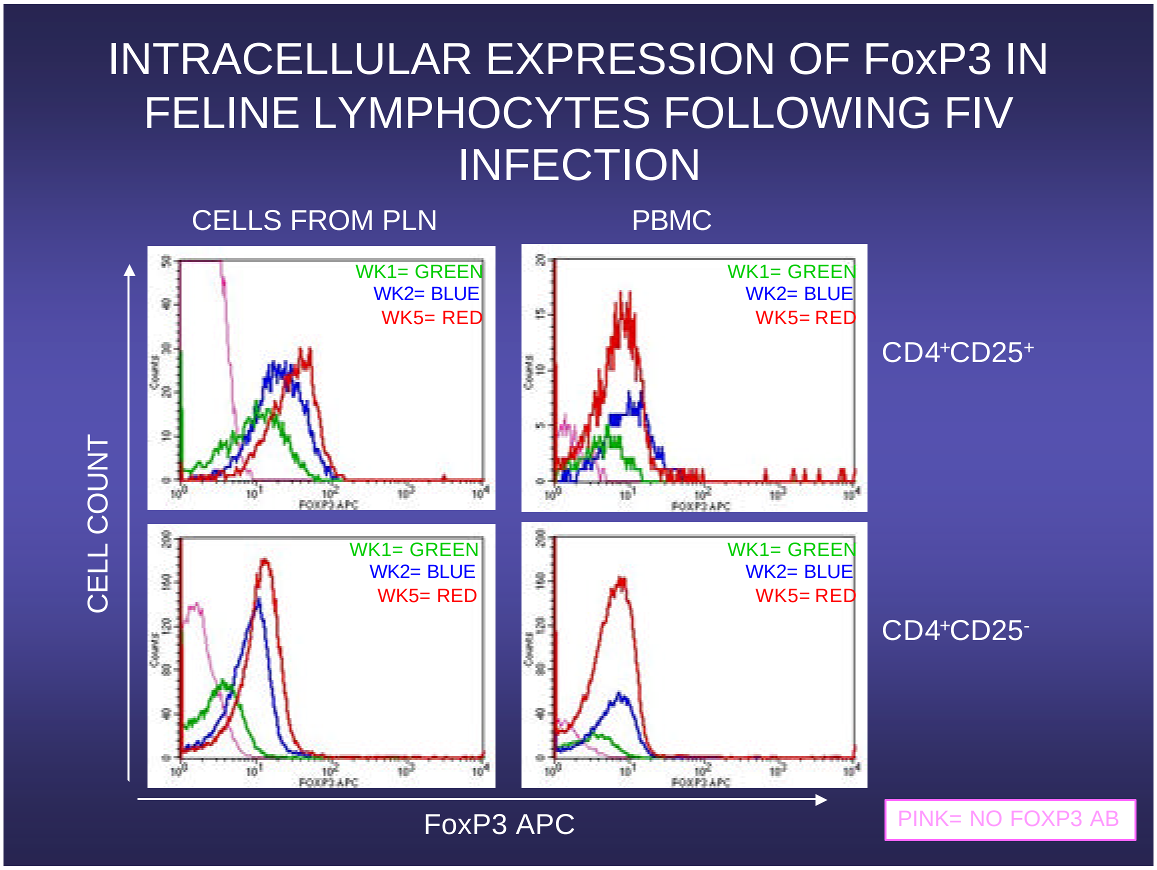


Figure 10. **Intracellular staining for FoxP3 in feline T cell subsets.**
Total cells samples from blood (BL) or lymph nodes (LN) were routinely stained for surface CD4 and CD25, fixed and permeabilized according to the manufacturers instructions, and incubated with anti-FoxP3-APC (1µl.tube). The cells were washed in staining buffer over-night and analyzed by flow cytometry. The mean percent of FoxP3 positive cells from 4 cat samples is represented for each subset at each time point.



<u>Figure 11.</u> Changes in FoxP3 Expression in Lymphocyte Subsets over time.
The expression of intracellular FoxP3 was detected in lymphocyte subsets by flow cytometry. Histogram overlays of representative samples for weeks 1, 2 and 5 are presented. Samples containing no FoxP3 antibody served as negative controls.

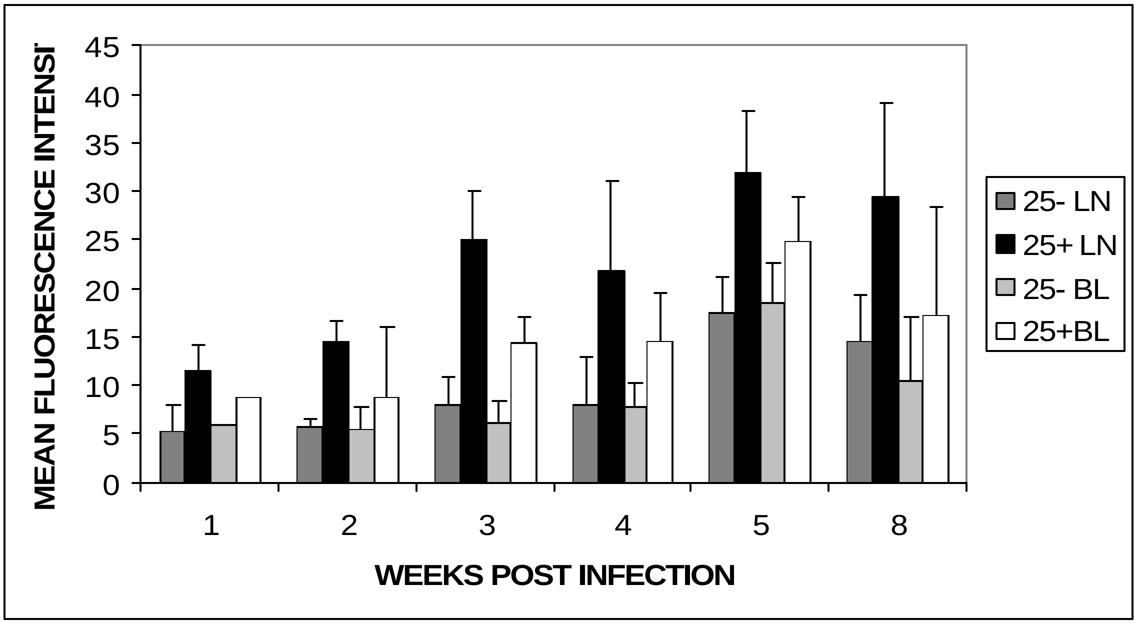
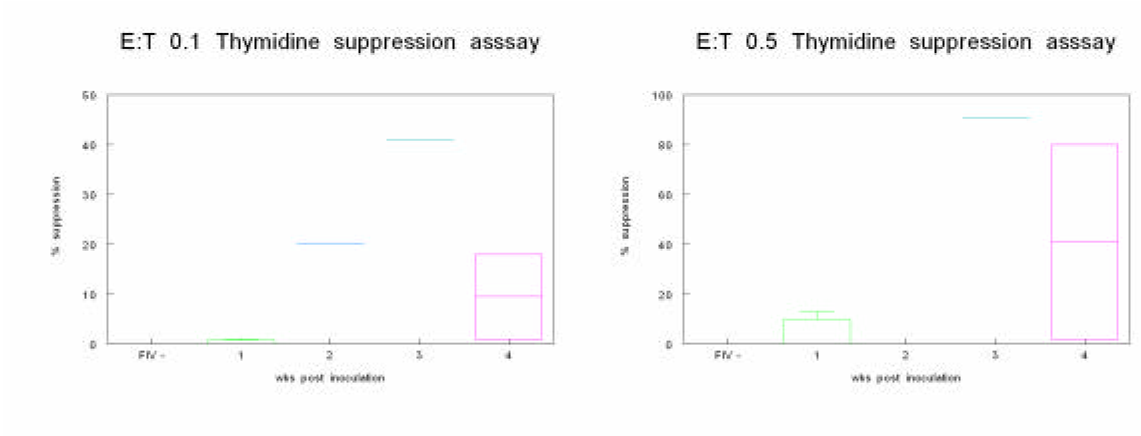
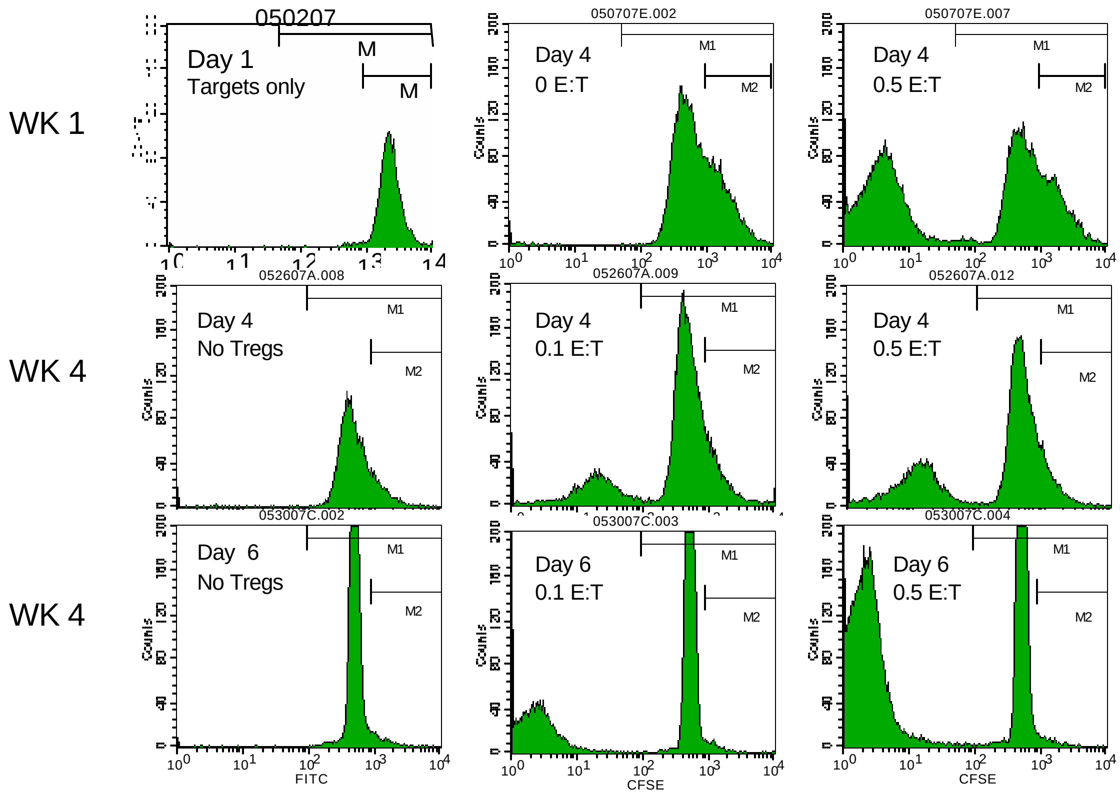


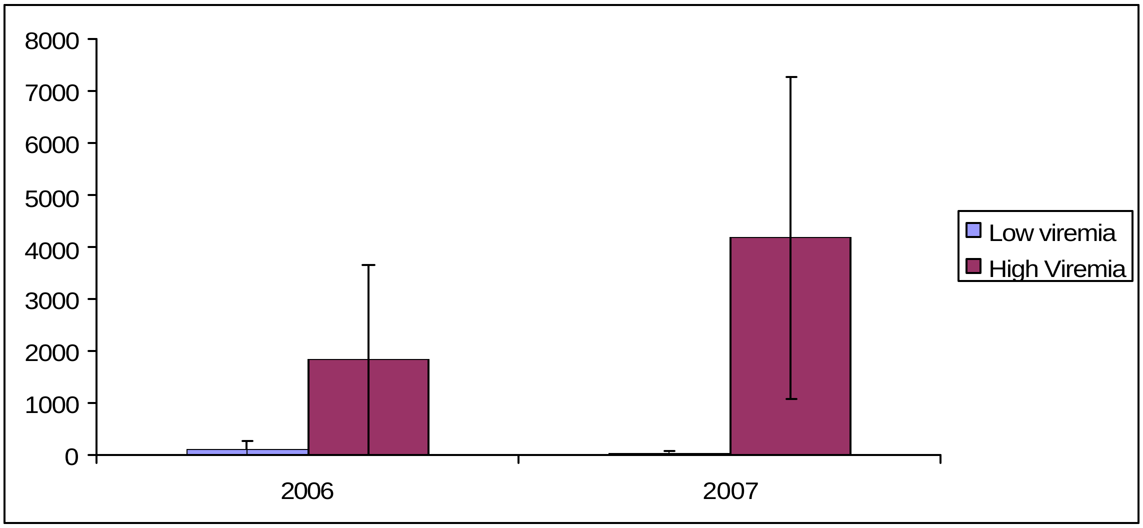
Figure 12. Mean Fluorescence Intensity of FoxP3 Staining in Lymphocyte Subsets. Staining for FoxP3 was analyzed in $CD4^+$ lymphocytes from the blood (BL) and lymph nodes (LN) of acutely FIV infected cats at 1 to 4 weeks post inoculation. Four samples were analyzed at each time point. The expression of FoxP3 in $CD4^+CD25^+$ lymphocytes from the lymph nodes was significantly higher at weeks 3 and 4 compared to weeks 1 and 2 post infection. There was significantly more FoxP3 expression in this T cell subset than all the others at all time points examined.



<u>Figure 13.</u> CD4$^+$CD25$^+$ Mediated Suppression of Proliferation by 3-H-Thymidine Incorporation. 4x10$^{\wedge 5}$ sorted CD4$^+$CD25$^-$ (target cells) were stained with CFSE and stimulated with Con A (5ug/10$^{\wedge 6}$ cells) overnight at 37 C and 5% CO2, washed and plated in each well of a 96-well plate. CD4$^+$CD25$^+$ (Treg cells) were added to some wells to obtain a E:T cell ratio of 0.1 or 0.5 accordingly. The plate was incubated at 37 C and 5% CO2 for 72 hours. Tritiated thymidine (1µCi/well) was added to each well and the cells were left to incubate for another 18 hrs. The plates were frozen at -20° C and analyzed at the end of the study. Samples were tested in duplicate wells and the radiation counts were measured. Percent suppression was calculated as the average radiation count in target only wells minus the average radiation count at the E:T=0.1 or 0.5 divided by the average radiation count in the target only wells for each cat. Each marker represents the percent of suppression from T regulatory function from an individual cat sample 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 when more than one sample was available.



<u>Figure 14.</u> CD4^{+}CD25^{+} Mediated Suppression of Proliferation by Flow Cytometry.
4x10$^{\wedge 5}$ sorted CD4^{+}CD25^{-} (target cells) were stained with CFSE and stimulated with Con A (5ug/10$^{\wedge 6}$ cells) overnight at 37 C and 5% CO2, washed and plated in each well of a 96-well plate. CD4^{+}CD25^{+} (Treg cells) were added to some wells to obtain a E:T cell ratio of 0.1 or 0.5 accordingly. The plate was incubated at 37 C and 5% CO2 for 4-6 days. Cells were transferred to tubes for flow cytometry analysis. Percent suppression was calculated as the % gated cells in M2/M1 (cells that did not proliferate out of total target cells) at either E:T=0.1 or 0.5 divided by the % target cells for each sample. Table 6 shows the numbers and calculation for these representative samples.



<u>Figure 15.</u> High and low plasma viremia levels in cats from the two studies.
Cat samples used in the 2006 and 2007 acute infection studies were separated into two groups based on high or low plasma viremia levels measured by RT-PCR. Four out of 16 cats in the 2006 study and 2 out of twelve cats in the 2007 study had extremely high plasma viremia levels. In the high viremia groups the average viremia level was higher in the 2007 than the 2006 study, while the reverse was true in the low viremia cats, which constituted the majority in both studies.

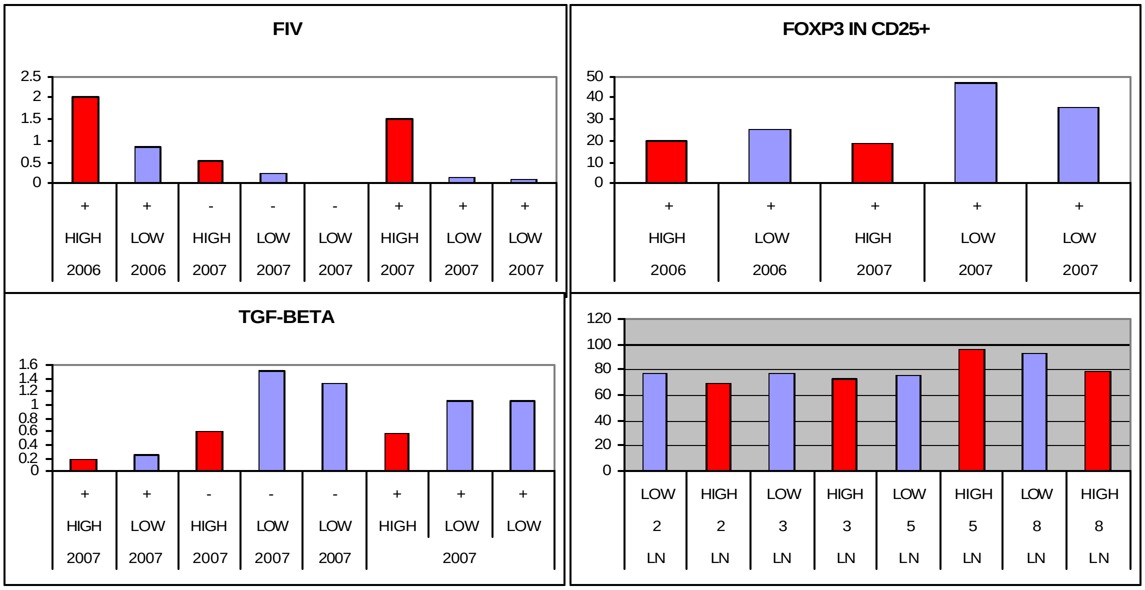
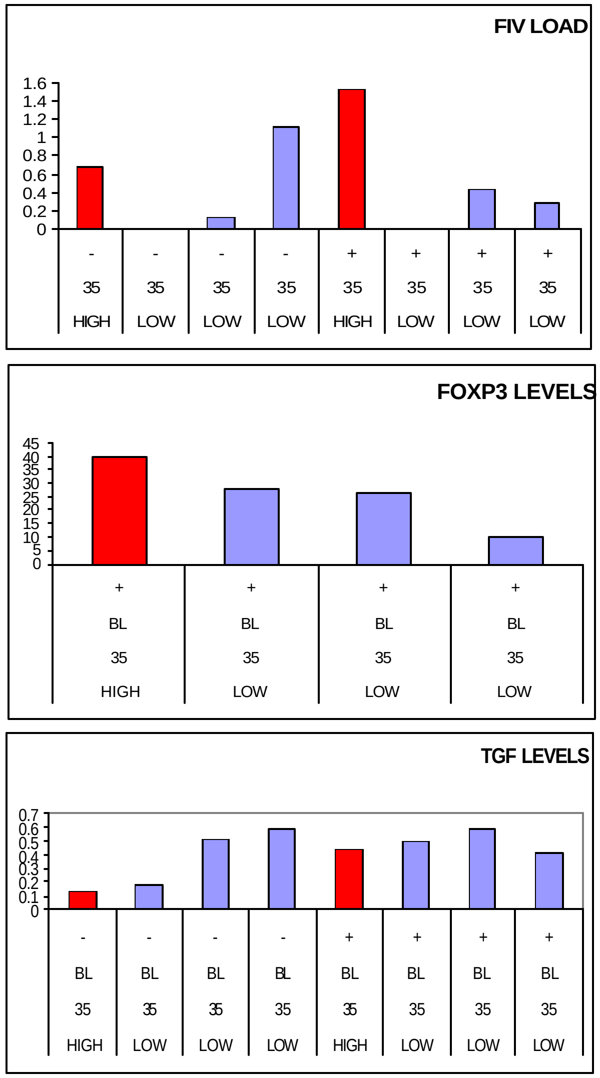


Figure 16. Comparison of regulatory T cell markers and viral load in two studies at 8 w.p.i in lymph node cells.
RT-PCR and Intracellular FoxP3 staining data from 2006 and 2007 acute phase infection studies was retrospectively analyzed for time points containing data from high and low plasma viremia cats. Both studies had data from $CD4^+CD25^+$ and $CD25^+CD25^-$ T cells from the lymph nodes at week 8 post infection. Relative quantification of FIV (a), FoxP3 (b), and TGF-β (c) mRNA and the mean of percent $FoxP3^+$, $CD4^+CD25^+$ T cells at several time points (d) are demonstrated above.



<u>Figure 17.</u> Comparison of regulatory T cell markers and viral load in 2006 study at 5 w.p.i in peripheral blood cells.
RT-PCR from 2006 acute phase infection studies was retrospectively analyzed for time points containing data from high and low plasma viremia cats. $CD4^{+}CD25^{+}$ and $CD25^{+}CD25^{-}$ T cells from the peripheral blood of cats with high and low viremia levels were analyzed at 5 weeks post infection. Relative quantification of FIV (a), FoxP3 (b), and TGF-β (c) mRNA from individual cat samples are demonstrated above for one high viremia cat and three low viremia cats in each case.

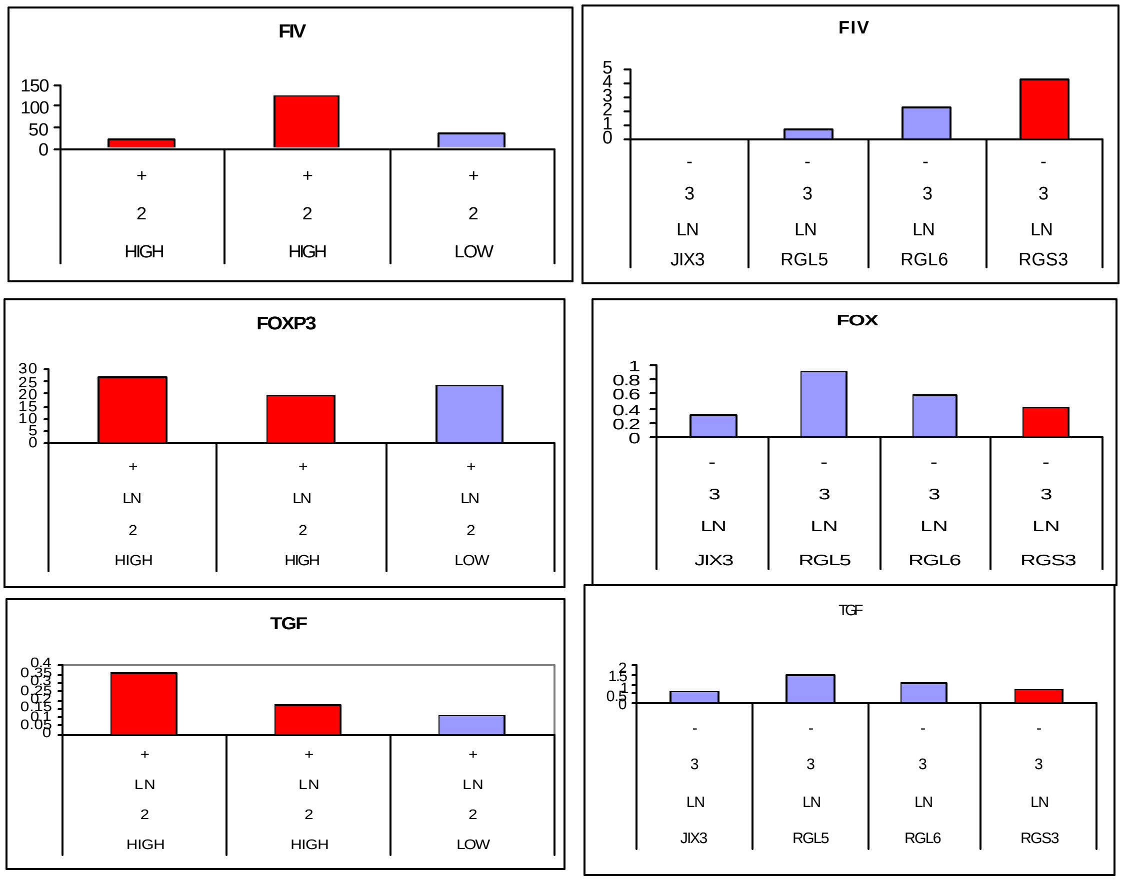


Figure 18. Comparison of regulatory T cell markers and viral load at 2 and 3 w.p.i in lymph node cells.

RT-PCR from 2006 and 2007 acute phase infection studies was retrospectively analyzed for time points containing data from high and low plasma viremia cats. $CD4^+CD25^+$ and $CD25^+CD25^-$ T cells from the lymph nodes of cats with high and low viremia levels were analyzed at week 2 for $CD25^+$ cells in the first study and at week 3 for CD25- cells in the second study. Relative quantification of FIV, FoxP3, and TGF-β mRNA from individual cat samples are depicted above for one or two high viremia cats and one or three low viremia cats in each case.

SUMMARY

During the course of three years, two studies were conducted to test the hypothesis that $CD4^{+}CD25^{+}$ Treg cells become infected and activated during the acute stage of FIV infection, and subsequently inhibit $CD4^{+}$ T helper cell immune responses. We believe these activated immunosuppressive cells contribute to the early immune dysfunction that is associated with FIV infection prior to the decrease of $CD4^{+}$ T cell numbers, and evidence of their infection and activation would support the role of Treg cells in lentivirus related immune suppression. The results indicate there is evidence of FIV infection in $CD4^{+}CD25^{+}$ T cells, activation markers are up-regulated at very early time points after infection, and these cells are capable of suppressing the proliferation and cytokine production of target $CD4^{+}CD25^{-}$ T cells in some cats. The studies comprised a total of 28 cats experimentally infected with the $NCSU^{-1}$ FIV isolate and 7 control cats that received a sham inoculation. Samples collected and analyzed from infected cats included lymph nodes and blood collected from 4 cats at 3 days, 6 weeks and 12 weeks post infection and 8 cats at 1,2,3,5 and 8 weeks post infection. Samples were collected from sham inoculated cats only during the first study and these included lymph nodes and blood collected from 2 cats at day 3, and 1,2,5,6, and 8 weeks post inoculation and one cat at 3 and 12 weeks post inoculation. In addition to the studies described herein, the samples obtained at each time point were shared with other members of the laboratory for additional experiments, resulting in limited numbers of $CD4^{+}CD25^{+}$ T cells for use in each

experiment. This resulted in less than 4 or 8 results obtained in some analyses for some experiments at some time points. There was very good correlation between our findings in the first and second studies.

All of the FIV-infected cats showed evidence of a successful infection by seroconversion on a commercially available “snap test” ELISA. Since samples were not collected from all the cats at each time point, it is impossible to determine at what time post infection each individual cat became positive for FIV antibodies by our methods. However, the first positive samples were found in the first study at 5 weeks post infection and in the second study at 4 weeks post infection and all the samples tested thereafter were also positive in each cat tested during both studies. There were no samples collected at week 4 during the first study. Also in support of successful inoculations, the ratio of $CD4^+$ to $CD8^+$ T cells became inverted and began dropping consistently in the blood and lymph node samples from cats in both studies at day 35 (week 5) p.i. This result not only confirmed the successful experimental infection in these cats, but also pointed toward a specific time frame during the acute phase in which $CD4^+$ T cells are not yet reduced, making the first 4 weeks of infection the time of interest for our analysis.

Concordant with previously published records, plasma viremia tested by RT-PCR showed a high peak at day 14 (week 2) post infection in both of the acute phase infection studies presented here. There was, however, a very wide range of plasma FIV-gag mRNA levels detected in the cats in both studies. Overall, the numbers of FIV-gag mRNA copies in the plasma of cats from the second study were lower than those found in the plasma of cats from the first study, but some cat samples carried very large numbers of FIV-gag mRNA copies per ml. Due to this discrepancy, six of the 28 cats infected with FIV in these experiments were later classified as having high plasma viremia levels and were analyzed separately for markers of regulatory function in the blood and lymph nodes.

The relative amounts of cellular FIV-gag mRNA were also measured in $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ T cell subsets from the blood and lymph nodes in each study. Cellular FIV-gag mRNA levels peaked in $CD4^{+}CD25^{+}$ T cells from the lymph nodes at day 14 (week 2) post infection in cats from both studies, which correlated to peak plasma viremia levels. In the peripheral blood, however, the level of FIV-gag mRNA peaked later in $CD4^{+}CD25^{+}$ T cells, at day 35 (week 5) p.i. in the first study, and day 21 (week 3) p.i. in the second study. It is interesting that FIV mRNA levels would correlate better between plasma and lymph node cells than plasma and PBMC levels, but the significance of this observation is unknown. Levels of FIV-gag mRNA were consistently higher in $CD4^{+}CD25^{+}$ than $CD4^{+}CD25^{-}$ T cells in both studies. This finding supports the hypothesis that $CD4^{+}CD25^{+}$ T cells are infected with FIV early after inoculation. However, because the $CD4^{+}CD25^{+}$ T cell compartment also contains proliferating $CD4^{+}$ T helper cells that up-regulate CD25 upon activation, it is impossible to make this a definitive conclusion. It has long been recognized that activated T cells preferentially replicate FIV and HIV when compared to resting T cell subsets. As such it is not surprising to have found higher FIV mRNA levels in this cell subset overall. The fact that viral levels rise concurrently to the expression of regulatory function markers in these cells, such as FoxP3 and TGF-β, however, make it possible to conclude that infection of Treg cells has an effect on the activation of their regulatory capacity. Both of the studies presented here failed to show significant changes in the number or percent of $CD4^{+}CD25^{+}$ T cells following inoculation of the cats with FIV. On one hand, this suggests the possibility that more of the cells expressing CD25 are actually regulatory instead of activated T helper cells, as this latter category would definitely be expected to proliferate under the effect of TCR engagement. There is therefore, a relative degree of anergy evident in these experiments which may correspond with a high degree of regulatory function in this T cell subset. To that we add the evidence of a phenotype in this cell subset previously recognized in activated Treg cells, the induction of FoxP3 expression and increases in surface TGF-β levels; making it more likely that we are seeing the activation and infection of Treg cells as a result of FIV infection.

We analyzed changes in FoxP3 expression using two methods. In the first and second study, sorted $CD4^{+}CD25^{+}$ and $CD4^{+}CD25^{-}$ T cells from the blood and lymph nodes of FIV-infected cats were tested by RT-PCR for their relative FoxP3 mRNA content. FoxP3 mRNA levels were significantly increased in the $CD4^{+}CD25^{+}$ T cells of the peripheral blood on weeks 5 and 6 post infection in the first study and by week 4 post infection in the second study. We also analyzed the expression of FoxP3 protein in cells by intracellular staining and flow cytometry in the second study. The number of $FoxP3^{+}$ $CD4^{+}CD25^{+}$ T cells in the blood doubled from 30% at week 1 to 60% at week 4. We then analyzed changes in the mean fluorescence intensity of

FoxP3 staining in T cell subsets and noted a significant increase in FoxP3 expression by this method in $CD4^{+}CD25^{+}$ T cells of the lymph nodes starting at week 3 post infection and continuing to rise through week 8. Using this method it appeared all of the T cell subsets had unusually high FoxP3 levels at 5 weeks post infection, which may denote an error in the assay. Using this method, only a trend towards increasing FoxP3 expression was noted in the $CD4^{+}CD25^{+}$ T cells of the peripheral blood. Overall, there were changes in FoxP3 expression following the infection of the cats with FIV, which may correlate with the induction of regulatory function in T cell subsets. It is possible that FIV infection is inducing the recruitment of new regulatory cells in the blood and lymph nodes during the acute phase of infection.

Changes in the expression of TGF-β, another marker of regulatory function in $CD4^{+}CD25^{+}$ T cells were also analyzed by flow cytometry and RT-PCR in T cell subsets from the blood and lymph nodes of cats experimentally infected with FIV. During both studies analysis by flow cytometry for membrane bound TGF-β showed higher levels of the protein expressed in $CD4^{+}CD25^{+}$ T cells from the lymph nodes at weeks 2 and 3 post infection with FIV. This transient increase in TGF-β expression may have an effect on the regulatory capacity of this T cell subset during the acute phase of FIV infection. Also in both studies, there was a trend towards increasing levels of TGF-β mRNA in $CD4^{+}CD25^{+}$ T cells measured by RT-PCR during the first weeks of FIV infection, but this difference failed to reach statistical significance. In the first study, there were also significantly higher levels of TGF-β mRNA in

$CD4^+CD25^-$ T cells of FIV infected cats compared to other T cell subsets, but this finding was not repeated in the second study.

In order to determine if activated regulatory T cells contained in the $CD4^+CD25^+$ compartment are functionally able to suppress the cytokine production and proliferation of $CD4^+$ target helper cells during the acute phase of FIV infection, suppression assays were performed in both studies. During the first acute infection study, we analyzed the suppressor function of Treg cells using feline specific IL-2 ELISPOT assays. Using this technique we could detect the ability of $CD4^+CD25^+$ Treg cells to suppress the IL-2 production of Con A stimulated $CD4^+CD25^-$ target cells in the lymph node cell samples of some FIV-infected cats. 11 of 22 (50%) of the cell samples tested from FIV-infected cats showed the ability of Treg cells to suppress IL-2 production from their corresponding target cells. However, in these assays, 4 out of 9 (44%) of the cell samples obtained from sham-inoculated, control cats also demonstrated the ability to suppress IL-2 responses. While we do not suggest the activation of Treg cells is necessarily an FIV-specific response, we could not identify the factors that may have resulted in the activation of Treg cells in the control cats. It is possible to suggest, however, that if antigenic stimulation by FIV resulted in the activation of only T helper $CD4^+$ T cells (and not Treg cells), we would have expected less FIV-infected cats to suppress IL-2 production in the ELISPOT assays. The fact that regulatory function could still be demonstrated in the acute phase of an infection in the $CD4^+CD25^+$ T cell compartment of FIV-infected cats suggests Treg cells are activated to suppress $CD4^+$ T helper responses. We also attempted to detect anti-FIV specific suppression of IL-2 production by stimulating $CD4^+CD25^-$ target cells with a recombinant FIV-gag peptide in vitro instead of Con A. For the first 5 weeks following infection of the cats with FIV, there were more IL-2 spots in the wells containing $CD4^+CD25^+$ (Treg) cells than in those that did not. These assays failed to demonstrate any suppressive function of IL-2 production, suggesting the presence of activated FIV-specific T helper cells in these wells. Again, we are not surprised to see the $CD4^+CD25^+$ compartment contained activated $CD4^+$ T helper cells, but this finding made it impossible to detect anti-FIV specific suppression by our methods.

We then attempted to corroborate the results of these IL-2 ELISPOT assays in the second study, by measuring the suppression of proliferation of target $CD4^+CD25^-$ T cell following Con A stimulation in the presence and absence of $CD4^+CD25^+$ T cells. Our findings here were significantly limited due to the lack of exogenous IL-2 in the culture wells, which blunted the proliferative capacity of target cells in these assays. Nevertheless, we were able to detect the functional suppression of target cell proliferation in samples from FIV-infected cats at 2, 3, and 4 weeks post infection by tritiated thymidine incorporation assays and at week 4 post infection by flow cytometry analysis of CFSE staining. Altogether, the results of these analyses support the hypothesis that activated Treg cells suppress $CD4^+$ responses such as cytokine production and cell proliferation during the acute phase of FIV infection.

Lastly, we analyzed cellular markers of regulatory function including FoxP3 and TGF-β expression in the $CD4^+CD25^+$ cells of cats that exhibited high levels of plasma viremia compared to cats with low plasma viremia levels. In general, the plasma samples that contained high levels of FIV-gag mRNA were obtained from cats whose cells also contained higher levels of FIV-gag mRNA and lower levels of FoxP3 and TGF-β. While we are unable to establish causation in this analysis, the results suggest a possible relationship between the limited control of plasma viremia and decreased regulatory function. It is also possible to suggest, however, that cats having high plasma and cell viremia levels simply have a greater proportion of FIV-activated T helper $CD4^+CD25^+$ T cells to $CD4^+CD25^+$ Treg cells than cats with lower virus levels.

All of our analyses were limited by our inability to separate $CD4^+CD25^+$ Treg cells from activated T helper cells in the face of acute FIV exposure. Further studies are needed to confirm our hypothesis that FIV infection in cats, results in the infection, activation, and functional conversion of Treg cells to suppress anti-viral immune responses. Nevertheless, the results of these experiments lend support to this hypothesis and show that $CD4^+CD25^+$ T cells contain high levels of FIV-gag mRNA, exhibit activation and regulatory phenotypes, and are able to suppress the cytokine production and proliferation of target $CD4^+CD25^-$ T cells during the acute phase of FIV infection in cats.

CONCLUSIONS

Lentivirus infections with human and animal retroviruses, including HIV and FIV, are characterized by the induction of slowly progressive, severe immune suppression. Transmission through direct contact is followed by a short acute phase characterized by fever, malaise and transient lymphadenopathy. During the acute phase of infection there is evidence of innate, humoral, and cell-mediated anti-viral immune responses. However, this process is interrupted early on, and in most cases the virus is able to persist at low levels despite the initial immune response. The ability of lentiviruses to establish a persistent infection requires an early evasion and alteration of the T cell immune system. Aspects of this immune dysregulation include both a global T cell immune hyper-activation, and a paradoxical hypo-responsiveness to virus antigens. The exact mechanisms mediating this T cell immune hypo-responsiveness have not been fully explained. One cell population recently implicated in mediating virus specific T cell anergy is the immunosuppressive $CD4^+CD25^+$ Treg cell. The hallmark characteristic of $CD4^+CD25^+$ Treg cells is their ability, once activated via TCR stimulation, to interact with other antigen-activated $CD4^+$ Th and $CD8^+$ T cells and prematurely abort their expansion by inducing anergy, thus preventing an effective immune response. Treg cells are now recognized to also be peripherally activated (adaptive Treg cells or pathogen-induced Treg cells), and modulate $CD4^+$ and $CD8^+$ immune responses to microbial pathogens, including viruses. The potential role of Treg cells in HIV and FIV infection has recently been explored by several independent groups. It is currently not known however, what effect the lentivirus infection of Treg cells has on their function and/or survival. Also unknown are the consequences that impairment or, in contrast, activation/expansion of these cells in number or function would have on the establishment of asymptomatic or clinical disease. We hypothesized that Treg cells become infected with FIV during the acute phase of infection and subsequently become activated and inhibit anti-FIV $CD4^+$ Th immune responses, thus allowing for the establishment of a long-term productive infection and the eventual development of AIDS. Our experiments were aimed at determining if and when Treg cells become infected with FIV and what effects this infection has on their phenotypic and functional characteristics. We report here that $CD4^+CD25^+$ cells are productively 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 starting as early as day 14 post infection; $CD4^+CD25^+$ T cells have higher viral mRNA levels than $CD4^+CD25^-$ T cells and infectivity in lymph node cells correlates with plasma viremia levels. FoxP3 and TGF-β levels are increased in $CD4^+CD25^+$ T cells during the first 4 weeks of infection, suggesting the activation of cells with a regulatory phenotype in the acute phase of FIV infection. In addition, $CD4^+CD25^+$ Treg suppression of mitogen stimulated $CD4^+$ responses, as measured by IL-2 production and cell proliferation, could be detected early in the acute phase of infection with FIV.

This study utilized the FIV/feline model of acute infection to test the hypothesis that

$CD4^+CD25^+$ Treg cells become infected with FIV and activated during the acute stage of infection, and subsequently inhibit $CD4^+$ T helper cell immune responses. We believe these activated immunosuppressive cells contribute to the early immune dysfunction that is associated with FIV/HIV infections prior to significant decreases in $CD4^+$ T cell numbers. The feline animal model is the only naturally occurring infection with a retrovirus which mimics both, the pathophysiology and course of disease seen in humans with HIV induced AIDS. While the ideal animal model may be considered one that can be infected by the same human virus, working within a species specific context offers the advantage of decreased human risks. If, in addition to decreasing the risk of exposure to a human pathogen, one can mimic the pathogenesis and genetic aspects of the virus in question, the animal model may offer invaluable benefits in the study of the human disease. Such is the case with FIV, a well established yet underutilized animal model of HIV. FIV is highly species-specific. Individuals working with the virus or with infected cats that are known to be a high occupational risk or exposure to feline retroviruses do not seroconvert and the zoonotic potential of FIV is considered minimal. Lastly, working in a well domesticated species offers many advantages to working with primates in a similar setting. For example, the effects of captivity on a primate species are likely very different from those on a domestic cat. And, while the cost of maintaining cats is comparatively more expensive to rodents, it is much less than that of maintaining primates in humane laboratory settings. This allows not only the ability to monitor and study animals throughout the course of disease, but also the advantage of attaining statistical relevance when one can use greater numbers of animals in each study. No single model is ideal for the study of HIV and AIDS. It is through our ability to combine clues from each animal model and the use of in-vitro and in-vivo studies that we are most likely to find the much needed answers to this disease and epidemic. However, it is imperative that we understand the benefits and pitfalls of all of our available resources when we design and fund future investigations. Understanding the basic question we are trying to answer when we design such studies will allow us to choose the best possible scenario in which to answer our specific questions. When we choose appropriate animal models, the responsible and humane use of these animals in research may not only benefit the health of humans in the future, but also that of the species we are studying. The best translational research is therefore, that which mimics the most characteristics of the species we wish to affect and interjects the least number of variables between the model and human disease. In many cases, the cat is such a model for the study of HIV and AIDS.

Here we focus on $CD4^{+}CD25^{+}$ Tregs as potential reservoirs because in addition to maintaining the infection over time, infection of these cells is likely to have early effects on anti-viral immune responses which allows for the establishment of a persistent infection. That is, by infecting $CD4^{+}CD25^{+}$ Treg cells the virus is limiting the effector responses of anti-viral $CD4^{+}$ and $CD8^{+}$ T helper cells. Subsequently, the virus is not eliminated during early phases of infection, perpetuating the established infection in this and other reservoir cell populations. In order to maintain viral reservoirs, however, $CD4^+CD25^+$ Tregs must conserve their initial characteristics of anergy and resistance to apoptosis in the face of FIV infection. This is particularly important because infection with HIV and FIV has been shown to be associated with the increased in vitro apoptosis of activated T cells, and a measure of apoptosis in lymphocytes from FIV infected cats has been shown to correlate with disease progression as measured by CD4 cell counts and CD4:CD8 T cell ratios. Therefore, while increased apoptosis is a characteristic of FIV infection which renders all lymphocytes more susceptible to dying, if $CD4^+CD25^+$ Tregs are to function as effective reservoirs of virus, they must be resistant to this FIV effect compared to other T cell populations. In support of this we did not detect significant changes in the number or percent of $CD4^+CD25^+$ T cells during the acute phase of FIV infection. In other words, we believe the virus is surviving preferentially in $CD4^+CD25^+$ Tregs, which are quiescent, long-lived lymphocytes that resist the death imparted to other $CD4^+$ T cell populations in the face of FIV infection.

The results of our experiments support the hypothesis that Treg cells may play a role in the early alterations to the immune system in retrovirus-associated diseases, which allow for the establishment of a persistent viral infection. In addition, we learned from this experience that the first four weeks following infection likely represent the time at which regulatory function becomes activated in the case of FIV infection. However, important limitations in these studies preclude our ability to make these more definitive conclusions. In particular, we have been unable to differentiate, or eliminate, activated T helper cells, which are present in high numbers during the acute phase of infection, from regulatory T cells based on surface phenotype alone. This has significantly limited our ability to analyze Treg cells specifically and likely contributed to our inability to detect increased regulatory function in the suppression assays described. Previous studies suggested surface TGF-β may serve as a marker of activated Treg cells in FIV infected cats. Here we detected only a transient increase in the expression of this molecule in $CD4^+CD25^+$ T cells during the acute phase of infection suggesting this may only be useful at 2 and 3 weeks post infection. Also based on this analysis, and our established understanding of Treg cell function, it is likely that activated Treg cells actually represent a very small percentage of the $CD4^+CD25^+$ T cells in the blood and lymph nodes. Identifying a different, unique surface marker for regulatory T cells would greatly increase our ability to examine the function of this T cell subset during FIV infection. Nevertheless, we conclude that $CD4^+CD25^+$ T cells include both: activated T helper and activated Treg cells during the acute phase of FIV infection in cats. These cells exhibit a transient up-regulation of FoxP3 and TGF-β, which are markers commonly associated with regulatory function; and they are able to suppress the IL-2 production and cell proliferation of target $CD4^+$ effector cells in some cats during the acute phase of FIV infection.

REFERENCE MATERIALS

1. Clements, J. E., L. Hu, L. Lindstrom, A. Powell, C. Rexroad, and M. C. Zink. 1996. Molecular studies of visna virus gene expression: analysis of envelope gene expression in transgenic sheep. *AIDS Res Hum Retroviruses 12:421*.

2. Anderson, E. R. e. a. 2006. Animal Model Systems of HIV-Diseases. In *Infectious agents and Pathogenesis*. S. S. Friedman H., Bendinelli M., ed. Springer Science and Business Media, Inc., New York, New York, p. 21.

3. Lehmann, R., B. von Beust, E. Niederer, M. A. Condrau, W. Fierz, A. Aubert, C. D. Ackley, M. D. Cooper, M. B. Tompkins, and H. Lutz. 1992. Immunization-induced decrease of the CD4+:CD8+ ratio in cats experimentally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol 35:199*.

4. Tompkins, M. B., M. E. Bull, J. L. Dow, J. M. Ball, E. W. Collisson, B. J. Winslow, A. P. Phadke, T. W. Vahlenkamp, and W. A. Tompkins. 2002. Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis. *J Infect Dis 185:1077*.

5. Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins. 2005. Different thresholds of T cell activation regulate FIV infection of CD4+CD25+ and CD4+CD25- cells. *Virology 335:212*.

6. Liaison, O. o. C. a. P., N. I. o. A. a. I. Diseases, and N. I. o. Health. 2004. How HIV causes AIDS.

7. Zhang, L., B. Ramratnam, K. Tenner-Racz, Y. He, M. Vesanen, S. Lewin, A. Talal, P. Racz, A. S. Perelson, B. T. Korber, M. Markowitz, and D. D. Ho. 1999. Quantifying residual HIV-1 replication in patients receiving combination antiretroviral therapy. *N Engl J Med 340:1605*.

8. Nielsen, M. H., F. S. Pedersen, and J. Kjems. 2005. Molecular strategies to inhibit HIV-1 replication. *Retrovirology 2:10*.

9. Arrode, G., J. S. Finke, H. Zebroski, F. P. Siegal, and R. M. Steinman. 2005. CD8+ T cells from most HIV-1-infected patients, even when challenged with mature dendritic cells, lack functional recall memory to HIV gag but not other viruses. *Eur J Immunol 35:159*.

10. Levy, J. K., J. W. Ritchey, J. B. Rottman, M. G. Davidson, Y. H. Liang, H. L. Jordan, W. A. Tompkins, and M. B. Tompkins. 1998. Elevated interleukin-10-to-interleukin-

12 ratio in feline immunodeficiency virus-infected cats predicts loss of type 1 immunity to Toxoplasma gondii. *J Infect Dis 178:503*.

11. Miedema, F., A. J. Petit, F. G. Terpstra, J. K. Schattenkerk, F. de Wolf, B. J. Al, M. Roos, J. M. Lange, S. A. Danner, J. Goudsmit, and et al. 1988. Immunological abnormalities in human immunodeficiency virus (HIV)-infected asymptomatic homosexual men. HIV affects the immune system before CD4+ T helper cell depletion occurs. *J Clin Invest 82:1908*.

12. Eggena, M. P., B. Barugahare, N. Jones, M. Okello, S. Mutalya, C. Kityo, P. Mugyenyi, and H. Cao. 2005. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol 174:4407*.

13. Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins. 2005. The role of CD4+CD25+ regulatory T cells in viral infections. *Vet Immunol Immunopathol 108:219*.

14. Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins. 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol 172:4752*.

15. Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci. 2004. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med 200:331*.

16. Aandahl, E. M., J. Michaelsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon. 2004. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol 78:2454*.

17. Thornton, C. A., J. W. Upham, M. E. Wikstrom, B. J. Holt, G. P. White, M. J. Sharp, P. D. Sly, and P. G. Holt. 2004. Functional maturation of CD4+CD25+CTLA4+CD45RA+ T regulatory cells in human neonatal T cell responses to environmental antigens/allergens. *J Immunol 173:3084*.

18. Ramilo, O., K. D. Bell, J. W. Uhr, and E. S. Vitetta. 1993. Role of CD25+ and CD25- T cells in acute HIV infection in vitro. *J Immunol 150:5202*.

19. Bell, K. D., O. Ramilo, and E. S. Vitetta. 1993. Combined use of an immunotoxin and cyclosporine to prevent both activated and quiescent peripheral blood T cells from producing type 1 human immunodeficiency virus. *Proc Natl Acad Sci U S A 90:1411*.

20. Borvak, J., C. S. Chou, K. Bell, G. Van Dyke, H. Zola, O. Ramilo, and E. S. Vitetta. 1995. Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection. *J Immunol 155:3196*.

21. Levy, J. A. 2006. HIV pathogenesis: knowledge gained after two decades of research. *Adv Dent Res 19:10*.

22. Burkhard, M. J., and G. A. Dean. 2003. Transmission and immunopathogenesis of FIV in cats as a model for HIV. *Curr HIV Res 1:15*.

23. Pedersen, N. C., J. K. Yamamoto, T. Ishida, and H. Hansen. 1989. Feline immunodeficiency virus infection. *Vet Immunol Immunopathol 21:111*.

24. Dias, A. S., M. J. Bester, R. F. Britz, and Z. Apostolides. 2006. Animal models used for the evaluation of antiretroviral therapies. *Curr HIV Res 4:431*.

25. Yang, J. S., R. V. English, J. W. Ritchey, M. G. Davidson, T. Wasmoen, J. K. Levy, D. H. Gebhard, M. B. Tompkins, and W. A. Tompkins. 1996. Molecularly cloned feline immunodeficiency virus NCSU1 JSY3 induces immunodeficiency in specific-pathogen-free cats. *J Virol 70:3011*.

26. English, R. V., C. M. Johnson, D. H. Gebhard, and M. B. Tompkins. 1993. In vivo lymphocyte tropism of feline immunodeficiency virus. *J Virol 67:5175*.

27. Yamamoto, J. K., E. Sparger, E. W. Ho, P. R. Andersen, T. P. O'Connor, C. P. Mandell, L. Lowenstine, R. Munn, and N. C. Pedersen. 1988. Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. *Am J Vet Res 49:1246*.

28. English, R. V., P. Nelson, C. M. Johnson, M. Nasisse, W. A. Tompkins, and M. B. Tompkins. 1994. Development of clinical disease in cats experimentally infected with feline immunodeficiency virus. *J Infect Dis 170:543*.

29. Lombardi, S., C. Garzelli, C. La Rosa, L. Zaccaro, S. Specter, G. Malvaldi, F. Tozzini, F. Esposito, and M. Bendinelli. 1993. Identification of a linear neutralization site within the third variable region of the feline immunodeficiency virus envelope. *J Virol 67:4742*.

30. Tozzini, F., D. Matteucci, P. Bandecchi, F. Baldinotti, K. Siebelink, A. Osterhaus, and M. Bendinelli. 1993. Neutralizing antibodies in cats infected with feline immunodeficiency virus. *J Clin Microbiol 31:1626*.

31. Matteucci, D., F. Baldinotti, P. Mazzetti, M. Pistello, P. Bandecchi, R. Ghilarducci, A. Poli, F. Tozzini, and M. Bendinelli. 1993. Detection of feline immunodeficiency virus in saliva and plasma by cultivation and polymerase chain reaction. *J Clin Microbiol 31:494*.

32. Hohdatsu, T., R. Pu, B. A. Torres, S. Trujillo, M. B. Gardner, and J. K. Yamamoto. 1993. Passive antibody protection of cats against feline immunodeficiency virus infection. *J Virol 67:2344*.

33. Yamamoto, J. K., T. Hohdatsu, R. A. Olmsted, R. Pu, H. Louie, H. A. Zochlinski, V. Acevedo, H. M. Johnson, G. A. Soulds, and M. B. Gardner. 1993. Experimental vaccine protection against homologous and heterologous strains of feline immunodeficiency virus. *J Virol 67:601*.

34. Hohdatsu, T., H. Yamada, Y. Ishizuka, and H. Koyama. 1993. Enhancement and neutralization of feline infectious peritonitis virus infection in feline macrophages by neutralizing monoclonal antibodies recognizing different epitopes. *Microbiol Immunol 37:499*.

35. Orandle, M. S., P. C. Crawford, J. K. Levy, R. Udoji, G. P. Papadi, T. Ciccarone, A. Mergia, and C. M. Johnson. 2000. CD8+ thymic lymphocytes express reduced levels of CD8beta and increased interferon gamma in cats perinatally infected with the JSY3 molecular clone of feline immunodeficiency virus. *AIDS Res Hum Retroviruses 16:1559*.

36. Paillot, R., S. Richard, F. Bloas, F. Piras, H. Poulet, S. Brunet, C. Andreoni, and V. Juillard. 2005. Toward a detailed characterization of feline immunodeficiency virus-specific T cell immune responses and mediated immune disorders. *Vet Immunol Immunopathol 106:1*.

37. Flynn, J. N., C. A. Cannon, J. A. Beatty, M. Mackett, M. A. Rigby, J. C. Neil, and C. Jarrett. 1994. Induction of feline immunodeficiency virus-specific cytotoxic T cells in vivo with carrier-free synthetic peptide. *J Virol 68:5835*.

38. Beatty, J. A., B. J. Willett, E. A. Gault, and O. Jarrett. 1996. A longitudinal study of feline immunodeficiency virus-specific cytotoxic T lymphocytes in experimentally infected cats, using antigen-specific induction. *J Virol 70:6199*.

39. Tompkins, M. B., P. D. Nelson, R. V. English, and C. Novotney. 1991. Early events in the immunopathogenesis of feline retrovirus infections. *J Am Vet Med Assoc 199:1311*.

40. Beatty, J., and O. Jarrett. 1996. FIV monitoring project. *Vet Rec 139:243*.

41. Burkhard, M. J., C. K. Mathiason, T. Bowdre, and E. A. Hoover. 2001. Feline immunodeficiency virus Gag- and Env-specific immune responses after vaginal versus intravenous infection. *AIDS Res Hum Retroviruses 17:1767*.

42. Bucci, J. G., R. V. English, H. L. Jordan, T. A. Childers, M. B. Tompkins, and W. A. Tompkins. 1998. Mucosally transmitted feline immunodeficiency virus induces a CD8+ antiviral response that correlates with reduction of cell-associated virus. *J Infect Dis 177:18*.

43. Gebhard, D. H., J. L. Dow, T. A. Childers, J. I. Alvelo, M. B. Tompkins, and W. A. Tompkins. 1999. Progressive expansion of an L-selectin-negative CD8 cell with anti-feline immunodeficiency virus (FIV) suppressor function in the circulation of FIV-infected cats. *J Infect Dis 180:1503*.

44. Hohdatsu, T., M. Okubo, and H. Koyama. 1998. Feline CD8+ T cell non-cytolytic anti-feline immunodeficiency virus activity mediated by a soluble factor(s). *J Gen Virol 79 (Pt 11):2729*.

45. Bucci, J. G., D. H. Gebhard, T. A. Childers, R. V. English, M. B. Tompkins, and W. A. Tompkins. 1998. The CD8+ cell phenotype mediating antiviral activity in feline immunodeficiency virus-infected cats is characterized by reduced surface expression of the CD8 beta chain. *J Infect Dis 178:968*.

46. Hohdatsu, T., A. Yamazaki, M. Yamada, H. Kusuhara, T. Kaneshima, and H. Koyama. 2003. Ability of CD8(+) T cell anti-feline immunodeficiency virus activity correlated with peripheral CD4(+) T cell counts and plasma viremia. *Microbiol Immunol 47:765*.

47. Jeng, C. R., R. V. English, T. Childers, M. B. Tompkins, and W. A. Tompkins. 1996. Evidence for CD8+ antiviral activity in cats infected with feline immunodeficiency virus. *J Virol 70:2474*.

48. Joshi, A., T. W. Vahlenkamp, H. Garg, W. A. Tompkins, and M. B. Tompkins. 2004. Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. *Virology 321:307*.

49. Batten, C. J., R. De Rose, K. M. Wilson, M. B. Agy, S. Chea, I. Stratov, D. C. Montefiori, and S. J. Kent. 2006. Comparative evaluation of simian, simian-human, and human immunodeficiency virus infections in the pigtail macaque (Macaca nemestrina) model. *AIDS Res Hum Retroviruses 22:580*.

50. Amado, R. G., R. T. Mitsuyasu, and J. A. Zack. 1999. Gene therapy for the treatment of AIDS: animal models and human clinical experience. *Front Biosci 4:D468*.

51. Friedman H., S. S., Bendinelli M. 2006. Infectious Agents and Pathogenesis. In *In Vivo Models of HIV Disease and Control*. Springer Scjience and Business Media, New York, NY.

52. Lifson, J. D., M. A. Nowak, S. Goldstein, J. L. Rossio, A. Kinter, G. Vasquez, T. A. Wiltrout, C. Brown, D. Schneider, L. Wahl, A. L. Lloyd, J. Williams, W. R. Elkins, A. S. Fauci, and V. M. Hirsch. 1997. The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. *J Virol 71:9508*.

53. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science 283:857*.

54. McClure, J., A. M. Schmidt, M. A. Rey-Cuille, J. Bannink, L. Misher, C. C. Tsai, D. M. Anderson, W. R. Morton, and S. L. Hu. 2000. Derivation and characterization of a highly pathogenic isolate of human immunodeficiency virus type 2 that causes rapid CD4+ cell depletion in Macaca nemestrina. *J Med Primatol 29:114*.

55. Barnett, S. W., K. K. Murthy, B. G. Herndier, and J. A. Levy. 1994. An AIDS-like condition induced in baboons by HIV-2. *Science 266:642*.

56. Wallace, V. C., J. Blackbeard, A. R. Segerdahl, F. Hasnie, T. Pheby, S. B. McMahon, and A. S. Rice. 2007. Characterization of rodent models of HIV-gp120 and anti-retroviral-associated neuropathic pain. *Brain*.

57. Reddy, M. M., A. Englard, D. Brown, E. Buimovici-Klien, and M. H. Grieco. 1987. Lymphoproliferative responses to human immunodeficiency virus antigen in asymptomatic intravenous drug abusers and in patients with lymphadenopathy or AIDS. *J Infect Dis 156:374*.

58. Wahren, B., L. Morfeldt-Mansson, G. Biberfeld, L. Moberg, A. Sonnerborg, P. Ljungman, A. Werner, R. Kurth, R. Gallo, and D. Bolognesi. 1987. Characteristics of the specific cell-mediated immune response in human immunodeficiency virus infection. *J Virol 61:2017*.

59. Clerici, M., N. I. Stocks, R. A. Zajac, R. N. Boswell, D. R. Lucey, C. S. Via, and G. M. Shearer. 1989. Detection of three distinct patterns of T helper cell dysfunction in asymptomatic, human immunodeficiency virus-seropositive patients. Independence of CD4+ cell numbers and clinical staging. *J Clin Invest 84:1892*.

60. Davidson, M. G., J. B. Rottman, R. V. English, M. R. Lappin, and M. B. Tompkins. 1993. Feline immunodeficiency virus predisposes cats to acute generalized toxoplasmosis. *Am J Pathol 143:1486*.

61. Dean, G. A., J. A. Bernales, and N. C. Pedersen. 1998. Effect of feline immunodeficiency virus on cytokine response to Listeria monocytogenes in vivo. *Vet Immunol Immunopathol 65:125*.

62. Galati, D., and M. Bocchino. 2007. New insights on the perturbations of T cell cycle during HIV infection. *Curr Med Chem 14:1920*.

63. Meyaard, L., E. Hovenkamp, I. P. Keet, B. Hooibrink, I. H. de Jong, S. A. Otto, and F. Miedema. 1996. Single cell analysis of IL-4 and IFN-gamma production by T cells from HIV-infected individuals: decreased IFN-gamma in the presence of preserved IL-4 production. *J Immunol 157:2712*.

64. Klein, S. A., J. M. Dobmeyer, T. S. Dobmeyer, M. Pape, O. G. Ottmann, E. B. Helm, D. Hoelzer, and R. Rossol. 1997. Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokine detection on single cell level by flow cytometry. *Aids 11:1111*.

65. Graziosi, C., G. Pantaleo, K. R. Gantt, J. P. Fortin, J. F. Demarest, O. J. Cohen, R. P. Sekaly, and A. S. Fauci. 1994. Lack of evidence for the dichotomy of TH1 and TH2 predominance in HIV-infected individuals. *Science 265:248*.

66. Meroni, L., D. Trabattoni, C. Balotta, C. Riva, A. Gori, M. Moroni, M. Luisa Villa, M. Clerici, and M. Galli. 1996. Evidence for type 2 cytokine production and lymphocyte activation in the early phases of HIV-1 infection. *Aids 10:23*.

67. Than, S., R. Hu, N. Oyaizu, J. Romano, X. Wang, S. Sheikh, and S. Pahwa. 1997. Cytokine pattern in relation to disease progression in human immunodeficiency virus-infected children. *J Infect Dis 175:47*.

68. Heeney, J. L. 2002. The critical role of CD4(+) T-cell help in immunity to HIV. *Vaccine 20:1961*.

69. Liang, Y., L. C. Hudson, J. K. Levy, J. W. Ritchey, W. A. Tompkins, and M. B. Tompkins. 2000. T cells overexpressing interferon-gamma and interleukin-10 are found in both the thymus and secondary lymphoid tissues of feline immunodeficiency virus-infected cats. *J Infect Dis 181:564*.

70. Ritchey, J. W., J. K. Levy, S. K. Bliss, W. A. Tompkins, and M. B. Tompkins. 2001. Constitutive expression of types 1 and 2 cytokines by alveolar macrophages from feline immunodeficiency virus-infected cats. *Vet Immunol Immunopathol 79:83*.

71. Levy, J. K., Y. Liang, J. W. Ritchey, M. G. Davidson, W. A. Tompkins, and M. B. Tompkins. 2004. Failure of FIV-infected cats to control Toxoplasma gondii correlates with reduced IL2, IL6, and IL12 and elevated IL10 expression by lymph node T cells. *Vet Immunol Immunopathol 98:101*.

72. Dean, G. A., and N. C. Pedersen. 1998. Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. *J Virol 72:9436*.

73. Dean, G. A., A. LaVoy, J. Yearley, and C. Stanton. 2006. Cytokine modulation of the innate immune response in feline immunodeficiency virus-infected cats. *J Infect Dis 193:1520*.

74. Sarween, N., A. Chodos, C. Raykundalia, M. Khan, A. K. Abbas, and L. S. Walker. 2004. CD4+CD25+ cells controlling a pathogenic CD4 response inhibit cytokine differentiation, CXCR-3 expression, and tissue invasion. *J Immunol 173:2942*.

75. Groux, H., G. Torpier, D. Monte, Y. Mouton, A. Capron, and J. C. Ameisen. 1992. Activation-induced death by apoptosis in CD4+ T cells from human immunodeficiency virus-infected asymptomatic individuals. *J Exp Med 175:331*.

76. Gougeon, M. L., A. G. Laurent-Crawford, A. G. Hovanessian, and L. Montagnier. 1993. Direct and indirect mechanisms mediating apoptosis during HIV infection: contribution to in vivo CD4 T cell depletion. *Semin Immunol 5:187*.

77. Holznagel, E., R. Hofmann-Lehmann, C. M. Leutenegger, K. Allenspach, S. Huettner, U. Forster, E. Niederer, H. Joller, B. J. Willett, U. Hummel, G. L. Rossi, J. Schupbach, and H. Lutz. 1998. The role of in vitro-induced lymphocyte apoptosis in feline immunodeficiency virus infection: correlation with different markers of disease progression. *J Virol 72:9025*.

78. Bull, M. E., T. W. Vahlenkamp, J. L. Dow, E. W. Collisson, B. J. Winslow, A. P. Phadke, M. B. Tompkins, and W. A. Tompkins. 2004. Spontaneous T cell apoptosis in feline immunodeficiency virus (FIV)-infected cats is inhibited by IL2 and anti-B7.1 antibodies. *Vet Immunol Immunopathol 99:25*.

79. Vahlenkamp, T. W., M. E. Bull, J. L. Dow, E. W. Collisson, B. J. Winslow, A. P. Phadke, W. A. Tompkins, and M. B. Tompkins. 2004. B7+CTLA4+ T cells engage in

T-T cell interactions that mediate apoptosis: a model for lentivirus-induced T cell depletion. *Vet Immunol Immunopathol 98:203*.

80. Farmer, J. L., A. A. Gottlieb, and T. Nishihara. 1986. Inhibition of interleukin 2 production and expression of the interleukin 2 receptor by plasma from acquired immune deficiency syndrome patients. *Clin Immunol Immunopathol 38:235*.

81. Murray, J. L., J. Dowd, and E. M. Hersh. 1986. In vitro inhibition of interleukin-2 production by peripheral blood lymphocytes from stage III melanoma patients by prostaglandin E2: enhancement of lymphocyte proliferation by exogenous interleukin-2 plus indomethacin. *J Biol Response Mod 5:12*.

82. Lawn, S. D., S. T. Butera, and T. M. Folks. 2001. Contribution of immune activation to the pathogenesis and transmission of human immunodeficiency virus type 1 infection. *Clin Microbiol Rev 14:753*.

83. Anderson, P. O., A. Sundstedt, Z. Yazici, S. Minaee, E. J. O'Neill, R. Woolf, K. Nicolson, N. Whitley, L. Li, S. Li, D. C. Wraith, and P. Wang. 2005. IL-2 overcomes the unresponsiveness but fails to reverse the regulatory function of antigen-induced T regulatory cells. *J Immunol 174:310*.

84. Coleman, J. K., R. Pu, M. Martin, E. Sato, and J. K. Yamamoto. 2005. HIV-1 p24 vaccine protects cats against feline immunodeficiency virus infection. *Aids 19:1457*.

85. Muralidhar, G., S. Koch, M. Haas, and S. L. Swain. 1992. CD4 T cells in murine acquired immunodeficiency syndrome: polyclonal progression to anergy. *J Exp Med 175:1589*.

86. Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins. 2005. Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. *J Virol 79:4965*.

87. Carbonneil, C., V. Donkova-Petrini, A. Aouba, and L. Weiss. 2004. Defective dendritic cell function in HIV-infected patients receiving effective highly active antiretroviral therapy: neutralization of IL-10 production and depletion of CD4+CD25+ T cells restore high levels of HIV-specific CD4+ T cell responses induced by dendritic cells generated in the presence of IFN-alpha. *J Immunol 172:7832*.

88. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest 112:1437*.

89. Fantini, M. C., C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol 172:5149*.

90. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med 198:1875*.

91. Rouse, B. T., and S. Suvas. 2004. Regulatory cells and infectious agents: detentes cordiale and contraire. *J Immunol 173:2211*.

92. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol 174:3143*.

93. Kornfeld, C., M. J. Ploquin, I. Pandrea, A. Faye, R. Onanga, C. Apetrei, V. Poaty-Mavoungou, P. Rouquet, J. Estaquier, L. Mortara, J. F. Desoutter, C. Butor, R. Le Grand, P. Roques, F. Simon, F. Barre-Sinoussi, O. M. Diop, and M. C. Muller-Trutwin. 2005. Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J Clin Invest 115:1082*.

94. Weiss, L., V. Donkova-Petrini, L. Caccavelli, M. Balbo, C. Carbonneil, and Y. Levy. 2004. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood 104:3249*.

95. Ruprecht, C. R., M. Gattorno, F. Ferlito, A. Gregorio, A. Martini, A. Lanzavecchia, and F. Sallusto. 2005. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J Exp Med 201:1793*.

96. Bayer, A. L., A. Yu, and T. R. Malek. 2007. Function of the IL-2R for thymic and peripheral CD4+CD25+ Foxp3+ T regulatory cells. *J Immunol 178:4062*.

97. Davidson, T. S., R. J. DiPaolo, J. Andersson, and E. M. Shevach. 2007. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol 178:4022*.

98. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med 188:287*.

99. Thornton, A. M., E. E. Donovan, C. A. Piccirillo, and E. M. Shevach. 2004. Cutting edge: IL-2 is critically required for the in vitro activation of CD4+CD25+ T cell suppressor function. *J Immunol 172:6519*.

100. de la Rosa, M., S. Rutz, H. Dorninger, and A. Scheffold. 2004. Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. *Eur J Immunol 34:2480*.

101. Barthlott, T., H. Moncrieffe, M. Veldhoen, C. J. Atkins, J. Christensen, A. O'Garra, and B. Stockinger. 2005. CD25+ CD4+ T cells compete with naive CD4+ T cells for IL-2 and exploit it for the induction of IL-10 production. *Int Immunol 17:279*.

102. Kohm, A. P., J. S. McMahon, J. R. Podojil, W. S. Begolka, M. DeGutes, D. J. Kasprowicz, S. F. Ziegler, and S. D. Miller. 2006. Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol 176:3301*.

103. Mahnke, K., K. Schonfeld, S. Fondel, S. Ring, S. Karakhanova, K. Wiedemeyer, T. Bedke, T. S. Johnson, V. Storn, S. Schallenberg, and A. H. Enk. 2007. Depletion of CD4+CD25+ human regulatory T cells in vivo: kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. *Int J Cancer 120:2723*.

104. Coffer, P. J., and B. M. Burgering. 2004. Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol 4:889*.

105. Fontenot, J. D., and A. Y. Rudensky. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol 6:331*.

106. Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paeper, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet 27:68*.

107. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol 4:330*.

108. Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science 299:1057*.

109. Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol 6:152*.

110. Nomura, T., and S. Sakaguchi. 2007. Foxp3 and Aire in thymus-generated Treg cells: a link in self-tolerance. *Nat Immunol 8:333*.

111. Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A. D. Lapan, J. C. Stroud, D. L. Bates, L. Guo, A. Han, S. F. Ziegler, D. Mathis, C. Benoist, L. Chen, and A. Rao. 2006. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell 126:375*.

112. Ono, M., H. Yaguchi, N. Ohkura, I. Kitabayashi, Y. Nagamura, T. Nomura, Y. Miyachi, T. Tsukada, and S. Sakaguchi. 2007. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature 446:685*.

113. Bodor, J., Z. Fehervari, B. Diamond, and S. Sakaguchi. 2007. ICER/CREM-mediated transcriptional attenuation of IL-2 and its role in suppression by regulatory T cells. *Eur J Immunol 37:884*.

114. Li, B., A. Samanta, X. Song, K. Furuuchi, K. T. Iacono, S. Kennedy, M. Katsumata, S. J. Saouaf, and M. I. Greene. 2006. FOXP3 ensembles in T-cell regulation. *Immunol Rev 212:99*.

115. Nakamura, K., A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, and W. Strober. 2004. TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice. *J Immunol 172:834*.

116. Petty, C. S. 2006. Feline Lentivirus Enhanced CD4+CD25+ T regulatory Conversion of CD4+CD25- T cells to Phenotypic and Functional Treg Cells Via TGF-beta/TGF-betaRII Signaling Pathway. In *PhD Thesis in Immunology*. North Carolina State University, Raleigh, NC.

117. Morgan, M. E., J. H. van Bilsen, A. M. Bakker, B. Heemskerk, M. W. Schilham, F. C. Hartgers, B. G. Elferink, L. van der Zanden, R. R. de Vries, T. W. Huizinga, T. H. Ottenhoff, and R. E. Toes. 2005. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol 66:13*.

118. Zelenay, S., T. Lopes-Carvalho, I. Caramalho, M. F. Moraes-Fontes, M. Rebelo, and J. Demengeot. 2005. Foxp3+ CD25- CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proc Natl Acad Sci U S A 102:4091*.

119. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol 2:E198*.

120. Gleizes, P. E., J. S. Munger, I. Nunes, J. G. Harpel, R. Mazzieri, I. Noguera, and D. B. Rifkin. 1997. TGF-beta latency: biological significance and mechanisms of activation. *Stem Cells 15:190*.

121. Feng, X. H., and R. Derynck. 2005. Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol 21:659*.

122. Becker, C., M. C. Fantini, and M. F. Neurath. 2006. TGF-beta as a T cell regulator in colitis and colon cancer. *Cytokine Growth Factor Rev 17:97*.

123. Marie, J. C., J. J. Letterio, M. Gavin, and A. Y. Rudensky. 2005. TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med 201:1061*.

124. Estes, J. D., Q. Li, M. R. Reynolds, S. Wietgrefe, L. Duan, T. Schacker, L. J. Picker, D. I. Watkins, J. D. Lifson, C. Reilly, J. Carlis, and A. T. Haase. 2006. Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection. *J Infect Dis 193:703*.

125. Novotney, C., R. V. English, J. Housman, M. G. Davidson, M. P. Nasisse, C. R. Jeng, W. C. Davis, and M. B. Tompkins. 1990. Lymphocyte population changes in cats naturally infected with feline immunodeficiency virus. *Aids 4:1213*.

126. Huber, S., C. Schramm, H. A. Lehr, A. Mann, S. Schmitt, C. Becker, M. Protschka, P. R. Galle, M. F. Neurath, and M. Blessing. 2004. Cutting edge: TGF-beta signaling is required for the in vivo expansion and immunosuppressive capacity of regulatory CD4+CD25+ T cells. *J Immunol 173:6526*.

127. Zheng, S. G., J. H. Wang, W. Stohl, K. S. Kim, J. D. Gray, and D. A. Horwitz. 2006. TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J Immunol 176:3321*.

128. Sojka, D. K., A. Hughson, T. L. Sukiennicki, and D. J. Fowell. 2005. Early kinetic window of target T cell susceptibility to CD25+ regulatory T cell activity. *J Immunol 175:7274*.

129. de Parseval, A., C. K. Grant, K. J. Sastry, and J. H. Elder. 2006. Sequential CD134-CXCR4 interactions in feline immunodeficiency virus (FIV): soluble CD134 activates FIV Env for CXCR4-dependent entry and reveals a cryptic neutralization epitope. *J Virol 80:3088*.

130. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med 194:629*.

131. Li, M. O., Y. Y. Wan, S. Sanjabi, A. K. Robertson, and R. A. Flavell. 2006. Transforming growth factor-beta regulation of immune responses. *Annu Rev Immunol 24:99*.

132. Chichester, J. A., M. A. Feitelson, and C. E. Calkins. 2006. Transient inhibition of Th1-type cytokine production by CD4 T cells in hepatitis B core antigen immunized mice is mediated by regulatory T cells. *Immunology 118:438*.

APPENDIX

List of protocols used in acute phase infection studies

1. Making CTL media
2. Washing and counting cells
3. Percoll Gradient Cell Centrifugation
4. Ficoll- Histopaque cell centrifugation
5. Growing FIV in E cells
6. Treg cell suppression assays

List of commercial products used in acute phase infection studies

1. Intracellular cytokine staining for IL2 and IFN-γ was performed using BD Pharmingen's Cytofix/Cytoperm Kit and feline specific antibodies from Serotec.

2. Intracellular cytokine staining for FoxP3 was performed using eBiosciences FoxP3 Staining buffer set (cat. 00-5523) and anti-mouse FoxP3 antibodies

3. Qiagen's RNeasy mini and Viral mRNA isolation kits were used for cell and viral mRNA isolation

4. Promega's Reverse Transcription System

5. For RT-PCR protocol: Qiagen's QuantiTect Sybr Green PCR, or Applied Biosystems Taqman PCR Mastermix

6. For Treg cell suppression of IL2 production by target cells, R&D Systems' Feline IL-2 ELISPOT kits were purchased and used according the manufacturers instructions

Making CTL Media

To make one 500 ml bottle of cell culture media, combine and filter the following using sterile technique under the vacuum hood:

500 ml of RPMI cell culture media (Gebco)
10% Fetal Bovine Serum (heat inactivated)
2µl of stock 2-Mercaptoethanol (old bottle, no conc. given)
1% L-Glutamine
1% HEPES
1% Sodium Bicarbonate
One aliquot of Pen/Strep
500 mg of Ciprofloxacin

Media is stored at 4° C.
When desired add IL-2 at T cell maintenance levels (0.5 µl per ml of media)

Washing and counting live cells

1. Collect the cells to be washed into separate 15 or 50 ml conical bottom centrifugation tubes
2. Fill tubes with prepared Hanks with EDTA salt solution
3. Centrifuge for 10 minutes at 1200 RPM
4. Discard fluid supernate and keep cell pellet in tube (if pellet is not well formed, aspirate the fluid out with a syringe or pipet).
5. Resuspend the cell pellet in small amount of fluid (if needed combine the cells from all tubes at this time)
6. Fill tube again with Hanks with EDTA salt solution
7. Centrifuge again for 10 minutes at 1200 RPM
8. Repeat steps 4-7

To count the live cells using a hemocytometer and trypan blue exclusion:

1. Determine the desired concentration of cells to trypan solution
2. In general: add 20µl of cells to 180µl of Trypan blue to make 1:10
3. Use 10µl of the cell suspension in Trypan in the clean and dry hemocytometer slide
4. Cells that are not blue, concave or irregular are counted in the 4 outer quadrants of the hemocytometer slide
5. Average the four counts to obtain an average measure of cells in one quadrant
6. This number is multiplied by 10^4 and the dilution factor to obtain a cell number per ml
7. To obtain a total cell count, multiply the result by the number of mls of cell suspension you started from

Percoll Gradient Cell Centrifugation

Purpose: To isolate peripheral blood mononuclear cells from the blood.

Perform at room temperature using sterile technique

Steps:

2. Obtain blood sample
3. Centrifuge blood at 1500 RPM for 5 minutes
4. Discard or save plasma layer separately
5. Dilute remaining cell suspension using Hank's sterile soln. to twice the starting volume (i.e. for 20mls of whole blood, dilute to 40 mls).
6. Prepare:
    a. 100% Percoll: (for 4 tubes)
        i. 16.2 ml of stock Percoll soln.
        ii. 1.8 ml of 10X Saline

    b. 60% Percoll:
        i. 9.6 ml of 100% Percoll (from above)
        ii. 6.4 ml of 1X Saline

    c. 43% Percoll:
        i. 6.9 ml of 100% Percoll (from above)
        ii. 9.1 ml of 1X Saline

7. For each centrifugation tube:
    a. Add 4 ml of 43% Percoll solution
    b. Carefully layer 4 mls of 60% Percoll to the BOTTOM
    c. Carefully layer 4-5 mls of dilute blood sample to the TOP

8. Centrifuge at 400 G for 5 minutes and then 800 G for 20 minutes

9. Isolate white cell layer after centrifugation and wash cells 2-3 times

Ficol-Histopaque 1077 Gradient Cell Centrifugation

Purpose: To isolate peripheral blood mononuclear cells from the blood.

Perform at room temperature using sterile technique

Steps:

1. Obtain blood sample
2. Centrifuge blood at 1500 RPM for 5 minutes
3. Discard or save plasma layer separately
4. Dilute remaining cell suspension using Hank's sterile soln. to twice the starting volume (i.e. for 20mls of whole blood, dilute to 40 mls).

5. For each centrifugation tube:
   a. Add 3 ml of room temperature Ficoll
   b. Carefully layer 8 mls of the dilute blood sample to the TOP

6. Centrifuge at 300 G for 30 minutes

7. Isolate white cell layer after centrifugation and wash cells 2-3 times, centrifuging at 200-300 G for 10 minutes each time to lift platelets from cell layer.

Growing FIV in E cells

E cells were grown in 6 well plates for 2-3 days until growth is normal and readily apparent. Cells from one well are transferred to a tube, centrifuged and counted by trypan blue exclusion method. Total number of cells is calculated by multiplying number of cells in one well counted by the total number of wells E cells are growing in.

Calculate the amount of virus for MOI desired:

Ex: for MOI= 0.1   0.1=1vpu/10 cells

Determine virus stock concentration per 100µl

Ex: for 40 million cells at MOI 0.1
  You need 4 million vpu
  If stock virus is at 10^8 vpu/100µl, calculate how many µl for 4x10^6

Frozen virus is kept in liquid nitrogen freezer. Each thawing lowers virus titer by one tenth. If starting concentration is 10^8 vpu/100µl next time it is thawed it will be at 10^7 vpu/100µl.

Incubate the collected and centrifuged E cells with the determined amount of stock virus in conical 50ml tube (small volume of suspended cells) at 37°C and 7% CO_2 incubator for 2 hours rotating and mixing the tube manually every 30 minutes during incubation time.

Centrifuge at 1200 RPM for 10 minutes and wash cells 2-3 times.

Resuspend cells in media to plate in 6 well plates (4ml/well) at 2-5 million cells per ml.

Add IL2 (2x maintenance, or 1µl per ml of cells) to each well to ensure cell division and virus replication occurs

Monitor cell cultures for synctitia formation and contamination

Harvest virus at 5 days of culture
1. When synctitia are easily visualized in the cell cultures and before cells begin to dye off (usually 5-7 days post inoculation), collect infected cell suspensions into centrifuge tubes and spin at 1200 RPM for 10 minutes.
2. Supernate is the virus stock solution (= cell free virus)

3. Immediately collect into freezer tubes and place on ice without disturbing the cell pellet.
4. Aliquot cell-free virus into pre-labeled cryotubes (500µl/tube)
5. Quick freeze in dry ice and place at -70°C for 24 hours before transferring to the liquid nitrogen freezer for long term storage.

To determine virus titer:

Plate 2million E cells/ 100µl in each well of a 96 well plate making sure to have at least 5 replicate wells for each concentration.

Each column is labeled as follows:

1. No virus
2. Undiluted virus
3. 10^-1
4. 10^-2
5. 10^-3
6. 10^-4
7. 10^-5
8. 10^-6
9. 10^-7
10. 10^-8
11. 10^-9
12. 10^-10

To dilute the virus add, on ice, in eppendorf tubes: 660µl of virus stock in first tube. Add 600µl of sterile media to each other tube. Transfer 60µl of virus stock from first to second tube. Complete serial dilutions through the rest of the tubes.

Add 100µl of diluted virus solutions to each corresponding well in 96 well plate

Incubate plate at 37°C monitoring for contamination, synctitia, and cell death

Feed cells by removing 100µl of media and adding fresh media plus IL2 at day 3 of culture. Begin charting +/- synctitia per well on day 6 or 7 and repeat until day 28 of culture. Every 7 days, remove 100µl of media and store in new 96 well plates for ELISA.

When the number of well with synctitia remains stable for 2-3 days, collect media samples for ELISA and chart synctitia. Perform FIV p24 antigen capture ELISA and calculate virus titer (TCID 50) by Read and Muench or Karber methods.
Compare results of ELISA with synctitia formation.

SUPPRESSION ASSAYS

Lymphocytes from FIV-infected and sham inoculated cats were isolated from the peripheral lymph nodes or blood using Percoll or Ficoll Gradient Centrifugation techniques. Cells were sorted by MoFlo high speed cell sorter into four T cell subsets:

1. CD4+ CD25- Target cells
2. CD4+CD25+ Treg cells
3. CD8+ T cells
4. NOT CD4+ or CD8+ APC cells (used for antigen presentation)

Target cells were labeled or activated as follows as described for each experiment:

1. CFSE staining (Molecular probes, Invitrogen)
    a. Collect, wash and centrifuge cells to be stained
    b. Incubate cells for 10 minutes at room temperature with 1ml of a 1µmol/L solution of CFSE in PBS
    c. Centrifuge, remove supernatant and wash three times in PBS

2. Con A stimulation
    a. Collect and wash freshly isolated or CFSE stained target cells
    b. Resuspend at 2-5 million cells /ml of CTL media
    c. Incubate at 37°C with 5µg/ml of cells of Concanavalin A for 1-3 hours
    d. Wash cells three times in CTL media

Target cells were resuspended at 2-5 x10^6/ml in order to obtain desired number of cells per well in 100µl. Treg cells were washed and resuspended so 100 µl would contain the number of cells needed for an effector to target (E:T) ratio of 0.5 and 100 µl of these was added to designated wells in a 96 well plate containing target cells. 20µl of the Treg cell suspension was added to wells designated to have an E:T of 0.1 Some wells were left to contain target cells alone.

Cells were incubated for 3-6 days at 37°C and 7% CO2 prior to analysis by flow cytometry of CFSE staining, RT by tritiated thymidine incorporation, or ELISPOT development as desired for each experiment.