**ABSTRACT**

PETTY, CHRISTOPHER SAMUEL. FELINE LENTIVIRUS ENHANCED $CD4^+CD25^+$ T REGULATORY CONVERSION OF $CD4^+CD25^-$ T CELLS TO PHENOTYPIC AND FUNCTIONAL T REG CELLS VIA THE TGF-β/TGF-βR SIGNALING PATHWAY. (Under the direction of Wayne A. Tompkins.)

The human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) pandemic continues to escalate with over 40 million people now living with HIV/AIDS and 5 million new infections reported in 2003. Development of an effective vaccine is crucial for the prevention and possible eventual eradication of the disease. However, vaccines do not alleviate the need for new treatment strategies for individuals already infected and living with HIV/AIDS. Currently, the most effective treatment employs highly active anti-retroviral therapy (HAART) which is effective in decreasing plasma virus load, but infected cells producing virus remain. Modulation of the host immune response may prove to be an effective and long-term solution to control established HIV infection. To use this strategy, a more thorough understanding of the pathology of HIV infected cells is needed. Cell types permissive to HIV and feline immunodeficiency virus (FIV) infection include CD4 and CD8 T cells, macrophages, and dendritic cells. Interestingly, a subset of CD4 T cells, $CD4^+CD25^+$ T regulatory cells, is productively infected with both HIV in humans and feline immunodeficiency virus (FIV) in cats and may serve as a long-term virus reservoir.

In this study, we investigated the possibility of peripheral generation of Treg-like cells from $CD4^+CD25^-$ T cells in a TGF-β/TGF-βRII signaling dependent manner mediated by $CD4^+CD25^+$ T regulatory cells. While $CD4^+CD25^-$ T cells from both control and FIV-infected animals are subject to $CD4^+CD25^+$ T regulatory cell mediated conversion to a Treg-like phenotype, freshly isolated Treg cells from FIV-infected and not control cats exerted this conversion. Further understanding of the mechanisms involved in the homeostasis of $CD4^+CD25^+$ T regulatory cells in the periphery may provide a strategy to control expansion of HIV/FIV virus reservoirs and immune suppression and, therefore, control disease progression.

**Feline Lentivirus enhanced $CD4^+CD25^+$ T regulatory conversion of $CD4^+CD25^-$ T cells to phenotypic and functional T reg cells via the TGF-β/TGF-βR signaling pathway**

by

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## BIOGRAPHY

Christopher S. Petty was born on February 27th, 1967, in Charlotte, North Carolina. He graduated from East Gaston High School and then attended North Carolina State University, where he received a Bachelor of Science in Chemistry. After working in both environmental and pharmaceutical industries he decided to pursue an advanced degree. During his search for the field in which he would seek his training, he discovered his interest in immunology. In January of 2002 he was accepted into the Immunology graduate program at North Carolina State University to study the acute phase trafficking of Feline Immunodeficiency Virus infection under the guidance of Dr. Mary Jo Burkhard. Dr. Burkhard subsequently took a position at The Ohio State University, and Chris transferred to the lab of Dr. Wayne Tompkins to study the immunological interaction of T regulatory cells during chronic Feline Immunodeficiency Virus infection. He received his PhD in Immunology in 2006 under the excellent mentorship of Dr. Wayne Tompkins. He plans to pursue a research career in industry.

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# 1. INTRODUCTION

The human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) pandemic continues to escalate with over 38 million people now living with HIV/AIDS and 5 million new infections reported in 2003, the highest incidence of new infections in a single year since the beginning of the epidemic. In the same year (2003), almost three million AIDS related deaths were reported, with a total of over 20 million lives lost since the first cases of AIDS were identified in 1981. Development of an effective vaccine is crucial for the prevention and possible eventual eradication of the disease. However, preventive vaccines do not alleviate the need for new treatment strategies for individuals already infected and living with HIV/AIDS. Currently, the most effective treatment employs highly active anti-retroviral therapy (HAART) which is effective in decreasing plasma virus load, but infected cells producing virus remain. Modulation of the host immune response may prove to be an effective and long-term solution to control established HIV infection. To use this strategy, a more thorough understanding of the pathology of HIV infected cells is needed.

Both human immunodeficiency virus (HIV) and feline immunodeficiency virus (FIV) are retroviruses of the family Retroviridae and genus *Lentivirus*. Lentiviruses typically cause immune dysfunction characterized by an acute flu-like illness followed by a chronic and gradual loss in total $CD4^+$ T-helper cell (Th) numbers and therefore dysregulated lymphocyte homeostasis. In addition to disrupting $CD4^+$ T cell homeostasis, there is a loss of responsiveness of Th cells to mitogens or pathogenic antigen stimulation (Clerici, Stocks et al. 1989; van Noesel, Gruters et al. 1990;

Komanduri, Feinberg et al. 2001; Iyasere, Tilton et al. 2003; Gamberg, Barrett et al. 2004). CD4 T cell loss during HIV-infection has been attributed to several mechanisms including direct cell lysis by lentivirus specific $CD8^+$ cytotoxic T lymphocytes (CTL) (Kalams and Walker 1994; Yamamura, Rodriguez et al. 1995) and activation induced apoptosis of CD4 T cells (Lyerly, et al., 1987, Finkel, et al., 1995, Muro-Cacho, et al., 1995). Although these mechanisms may play a part in the late stages of HIV infection and the eventual onset of AIDS due to extreme loss of CD4 T cells, early in HIV infection even before significant losses in total cell number, CD4 T cells lose their ability to proliferate and exist in a state of anergy characterized by inability to produce IL-2 in response to antigenic stimulation (Gruters, Terpstra et al. 1990; Iyasere, Tilton et al. 2003).

Cell types permissive to HIV and feline immunodeficiency virus (FIV) infection include CD4 and CD8 T cells, macrophages, and dendritic cells (Datema 1990; Meltzer and Gendelman 1992; Toth, Mosborg-Petersen et al. 1993; Riley, Levine et al. 1998; Simonitsch, Geusau et al. 2000; Tomaras, Lacey et al. 2000). Interestingly, a subset of CD4 T cells, $CD4^+CD25^+$ T regulatory cells, is productively infected with both HIV (Chou, Ramilo et al. 1997; Oswald-Richter, Grill et al. 2004) in humans and feline immunodeficiency virus (FIV) (Joshi, Vahlenkamp et al. 2004) in cats and may serve as a long-term virus reservoir. T regulatory cells are responsible for the peripheral maintenance of self-tolerance (McHugh, Shevach et al. 2001; Shevach, McHugh et al. 2001; Takahashi and Sakaguchi 2003) and also affect the pathology of some viral bacterial, and parasitic infections (Belkaid 2003; Sakaguchi 2003; Xu, Liu et al. 2003; Hisaeda, Maekawa et al. 2004). $CD4^+CD25^+$ T regulatory cells display constitutively

high levels of CD25, the alpha chain of the IL-2 receptor, and produce high levels of TGF-ß and IL-10 (Bach and Francois Bach 2003; Baecher-Allan, Brown et al. 2003; Battaglia, Ferrandina et al. 2003; Fisson, Darrasse-Jeze et al. 2003).

Currently, there is controversy over whether fully functional $CD4^+CD25^+$ T regulatory cells are generated in the thymus exclusively or if a mechanism of peripheral generation exists. Recent publications support the hypothesis that $CD4^+CD25^+$ T regulatory cells are generated in the periphery (Chen, Jin et al. 2003; Cozzo, Larkin et al. 2003). Furthermore, the anergic properties of T regulatory cells and the atrophy of the thymus following maturity, reduce the possibility of continued thymic generation, pointing to the possibility of an extra-thymic generation mechanism. Further understanding of the mechanisms involved in the homeostasis of $CD4^+CD25^+$ T regulatory cells in the periphery may provide a strategy to control expansion of HIV/FIV virus reservoirs and immune suppression and, therefore, control disease progression.

## 2. BACKGROUND AND SIGNIFICANCE

### A. FIV as a model for HIV pathogenesis

A feline T-lymphotropic virus was first isolated from domestic cats in 1986 by Pedersen et al (Pedersen, Ho et al. 1987). The cats from which the virus was isolated were housed in a single pen in a cattery and all showed clinical signs including wasting and the occurrence of rare bacterial infections reminiscent of an immunodeficiency syndrome such as HIV induced AIDS. Domestic cats can be infected with multiple retroviruses including feline leukemia virus (FeLV) (Pedersen, Ho et al. 1987); however this new virus was distinct from other feline retroviruses. The virus was subsequently classified as a Lentivirus and because of its remarkable similarity to HIV in both structure and clinical manifestation it was later named Feline Immunodeficiency Syndrome (FIV) (Pedersen, Ho et al. 1987).

Due to the pandemic status of HIV, the development of an animal model is extremely important to study the pathogenesis of HIV. Although the Simian Immunodeficiency Virus (SIV) is more phylogenetically similar to HIV than is FIV, SIV does not cause disease in its natural host (Norley 1996). However, FIV does cause disease in the domestic cat. In addition, both FIV and HIV follow nearly identical patterns of disease progression with a marked loss of CD4 T cell numbers during the acute stage of infection followed by an incomplete recovery in CD4 T cell numbers and loss of function during the asymptomatic phase (Gaines, von Sydow et al. 1990; English, Johnson et al. 1993; Jeng, English et al. 1996). The progressive loss of $CD4^+$ T cells

during the asymptomatic stage of infection leads to a decrease in the CD4:CD8 ratio followed by the complete immunodeficiency characteristic of AIDS and eventually death. FIV in the domestic cat may be the most appropriate animal model for the study of HIV immunopathogenesis as both viruses follow a similar disease course and cause pathology in their natural hosts.

**B. $CD4^+CD25^+$ T Regulatory Cells**

Suppression of $CD4^+CD25^-$ T helper cells by T regulatory cells is crucial for both tolerance to self-antigens and control of immune responses to foreign antigen. Naturally occurring $CD4^+CD25^+$ cells are a unique subpopulation of T regulatory cells phenotypically defined by constitutively high expression of CD25, the IL-2Rα-chain, CTLA-4, and GITR (Levings, Sangregorio et al. 2002; Li, Mahesh et al. 2003). Functionally, Treg cells remain anergic to stimulation via their TCR by not producing IL-2 (Maloy and Powrie 2001; Walker 2004); however addition of exogenous IL-2 is able to rescue Treg cells from their anergic state promoting a proliferative response (Walker 2004). Additionally, co-culture with activated antigen presenting dendritic cells abrogates $CD4^+CD25^+$ Treg cells anergic phenotype and induces proliferation comparable to that seen in $CD4^+CD25^-$ T cells, while their suppressor function remains intact (Yamazaki, Iyoda et al. 2003). Further, thymus-derived self-reactive CD1d-restricted $CD4^+$ natural killer T cells ($CD4^+$ NKT cells) secrete substantial amounts of IL-2 after stimulation with dendritic cells (DC) and promote proliferation of $CD4^+CD25^+$ Treg cells. The expanded

Treg cells remain anergic to TCR stimulation and retain their potent suppressive properties (Jiang, Game et al. 2005).

$CD4^+CD25^+$ regulatory cells mediate contact-dependent active suppression of $CD4^+CD25^-$ T cells (Nakamura, Kitani et al. 2001; Annunziato, Cosmi et al. 2002; Nakamura, Kitani et al. 2004). Activation of suppressor function is antigen specific; however, once activated, suppression is independent of antigenic stimulation (Thornton and Shevach 2000). Suppression of $CD4^+CD25^-$ cells is mediated independently of cytokine production by Treg cells (Nakamura, Kitani et al. 2001; Annunziato, Cosmi et al. 2002; Nakamura, Kitani et al. 2004). These cells comprise 5-10% of the peripheral $CD4^+$ T cell population and are phenotypically and functionally homologous in humans, cats, and mice (Maloy and Powrie 2001; Vahlenkamp, Tompkins et al. 2004). However, these same cell surface markers are up-regulated on activated $CD4^+CD25^-$ cells, emphasizing the need for a specific marker to distinguish T regulatory cells.

Self-antigen presentation by thymic epithelium is believed to be responsible for generating $CD4^+CD25^+$ T regulatory cells capable of suppressing $CD4^+CD25^-$ T cells specific for the same self antigens and, therefore, generation should be limited to the thymus (Apostolou, Sarukhan et al. 2002). This is supported by Kyewski et al. and Derbinski et al., who found mRNA for multiple tissue specific proteins that are expressed by thymic medullary epithelial cells and could be instrumental in generating self antigen specific T regulatory cells (Derbinski, Schulte et al. 2001). These self antigen specific $CD4^+CD25^+$ T regulatory cells are functionally mature upon exit from the thymus into the periphery, and actively suppress immune responses of aberrant self-specific CD4 and

CD8 T cells that escape negative selection in the thymus (Suri-Payer, Amar et al. 1998; Thornton and Shevach 1998).

$CD4^+CD25^+$ Treg cells have also been implicated in suppressing inflammation mediated by innate immune responses. Innate suppression, in contrast to cell mediated immune suppression, is both IL-10 and TGF-β dependent (Maloy, Salaun et al. 2003), establishing a role for Treg cell production of soluble TGF-β and IL-10. Both TGF-β and IL-10 cytokines are produced by one or another T regulatory cell population ($CD4^+CD25^+$,Tr1, Th3) which emphasizes a likely role in the suppression by or maintenance of the T regulatory population.

**C. FoxP3 as a Molecular Marker of $CD4^+CD25^+$ Treg Cells**

As CD25 is currently accepted as the most reliable surface marker for Treg cells, a better more exclusive marker is needed due to the up-regulation of CD25 and other Treg markers such as GITR and OX40 during activation of $CD4^+CD25^-$ Th cells. FoxP3 may fill the need as a potential molecular marker for $CD4^+CD25^+$ regulatory cells. The FoxP3 gene encodes a member of the *forkhead*/winged helix family of proteins that act as a transcriptional regulators (Walker, Kasprowicz et al. 2003; Hori and Sakaguchi 2004). The first forkhead-box (FOX) was discovered in the fruit fly Drosophila melanogaster and is required for the terminal pattern formation in the embryo (Weigel, Jurgens et al. 1989). Since then over 100 members of the family have been identified and are associated with developmental regulation. FoxP3 was discovered in Scurfy mice as the gene responsible for hyperproliferation of $CD4^+CD8^-$ T cells, which produced large

amounts of various cytokines (Khattri, Kasprowicz et al. 2001; Kasprowicz, Smallwood et al. 2003; Khattri, Cox et al. 2003; O'Garra and Vieira 2003; Ramsdell 2003). The lethal mutation in the FoxP3 gene causes disease reminiscent of the uncontrolled immune responses seen after depletion of $CD4^+CD25^+$ T cells in mice (Maloy, Salaun et al. 2003) and is similar to that seen in CTLA-4 and TGF-β knockout mice (Bjorses, Halonen et al. 2000; Kyewski, Derbinski et al. 2002). The FoxP3 gene encodes a transcriptional repressor which has been reported to be exclusively expressed in and required for the development of $CD4^+CD25^+$ T regulatory cells in mice (Chen, Jin et al. 2003; Fontenot, Gavin et al. 2003). Furthermore, forced expression by vectors encoding FoxP3 in purified $CD4^+CD25^-$ murine cells confers T regulatory function to this population (Khattri, Kasprowicz et al. 2001).

In humans, FoxP3 mutations are responsible for a genetic autoimmune disease characterized by immune-mediated polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX), which is usually lethal within the first year of life in affected males (Bennett, Christie et al. 2001; Bennett and Ochs 2001; Kobayashi, Shiari et al. 2001; Gambineri, Torgerson et al. 2003; Owen, Jennings et al. 2003; Bakke, Purtzer et al. 2004). Mutations invariably map to the forkhead domain of the protein, which is highly conserved among Fox family members. The forkhead domain is uniquely located in the carboxyl terminus and affects the DNA binding function of the protein (Bennett and Ochs 2001). Human FoxP3 is also stably expressed in $CD4^+CD25^+$ T regulatory cells and can be induced in $CD4^+CD25^-$ cells upon activation (Walker, Kasprowicz et al. 2003). If these activated $CD4^+CD25^-$ cells are further separated based on FoxP3 expression, the

$CD4^+CD25^-$ cells expressing FoxP3 also have the ability to suppress naïve $CD4^+CD25^-$ T cell proliferation (Walker, Kasprowicz et al. 2003; Fantini, Becker et al. 2004).

FoxP3 could act to maintain T regulatory function/anergy by repressing IL-2 transcription, as a potential binding site has been identified in the IL-2 promoter next to the nuclear factor of activated T cells (NFAT) binding site and FoxP3 binding would block NFAT recruitment to the IL-2 promoter (Schubert, Jeffery et al. 2001). More recently, due to the effect of FoxP3 on production of multiple cytokines derived from both NFAT and NF-κB activity, the effect was investigated upstream of DNA binding. The results show that FoxP3 suppresses the production of cytokines by physically associating with NFAT and NF-κB thereby suppressing their transcriptional activation activity (Bettelli, Dastrange et al. 2005). Additionally, analysis of generated FoxP3 deletion mutants in mice revealed that the first 200 amino acids in the N terminus are critical for the ability to suppress IL-2 production (Bettelli, Dastrange et al. 2005).

Expression of a FoxP3 isoform missing exon 2 (FOXP3Δ2) has been identified during analysis of cDNA generated from human $CD4^+$ CD25+ T cell mRNA (Allan, Passerini et al. 2005). The deletion results in approximately a 4 kDa decrease in the molecular weight of FoxP3. Agarose gel analysis readily revealed both isoforms in all donors tested in the study and correlated with double bands seen during Western blot analysis of lysates. In Jurkat T cell transfection assays, both proteins suppressed IL-2 promoter activity and the effect was significantly greater when they were co-expressed suggesting a cooperative relationship.

FoxP3 has also been implicated in down-regulation of SMAD-7 (Fantini, Becker et al. 2004). SMAD-7 is normally up-regulated after T cell activation and functions to

down-regulate TGF-β mediated SMAD-2,3,4 signaling (Coffer and Burgering 2004). As Treg cells constitutively express both FoxP3 and surface TGF-β, it is reasonable that FoxP3 may play a key role in the maintenance of Treg cell function by interrupting the SMAD-7 autoregulatory negative feedback loop and promoting constitutive TGF-β signaling. Taken together, data supports the hypothesis that FoxP3 is a molecular marker for Treg cell suppressor function, and it is possible that FoxP3 specifically directs T regulatory cell maintenance and/or development just as the transcription factors T-bet and GATA-3 direct development of Th1 and Th2 cells, respectively (Finotto and Glimcher 2004).

**D. TGF-β expression by $CD4^+CD25^+$ Treg Cells**

TGF-β family members including activins, inhibins, bone morphogenetic proteins and TGF-β isoforms affect a variety of cell types by regulating cell growth, apoptosis, tissue repair, modulation of the immune system, differentiation and cell phenotype (Massaous and Hata 1997). TGF-β and TGF-β receptors can be expressed by almost any cell type, indicating the pleiotropic nature of this cytokine. There are three isoforms TGF-β1,2,3 which are highly homologous among mammalian species. TGF-β1 is the predominate isoform and responsible for the majority (~90%) of the anti-inflammatory effects. TGF-β is normally secreted as one of two types of latent complexes, latency associated peptide (LAP) or a larger protein consisting of LAP disulfide linked to latent TGF-β binding protein (LTBP). In either case, proteolysis or low pH degradation is

needed to release active TGF-β. Among the multiple effects of this cytokine are the anti-proliferative effects on $CD4^+$ T cells by inhibition of IL-2 production and up-regulation of cell cycle inhibitors (Levings, Bacchetta et al. 2002).

TGF-β signaling is mediated through TGF-β receptor 1 (TGF-βR1) and TGF-β receptor 2 (TGF-βRII) interaction with SMADS 2, 3, and 4 (Nakao, Imamura et al. 1997). SMAD proteins are restricted to the cytoplasm in resting cells and translocate into the nucleus on activation by receptor stimulation with its ligand (Heldin, Miyazono et al. 1997). On binding TGF-β, two molecules of TGF-βRI and two molecules of TGF-βRII are crosslinked to form a heterotetramer complex (Yamashita, ten Dijke et al. 1994) and the complex is stabilized by cytoplasmic receptor interaction (Feng and Derynck 1996). TGF-βRII has constitutive serine/threonine kinase activity which functions solely to phosphorylate TGF-βRI and activate its kinase (Chen and Derynck 1994; Wrana, Attisano et al. 1994). Subsequently, SMAD2 and 3 are activated by transient, direct interaction with phosphorylated TGF-βRI and dissociate to form a complex with SMAD 4 followed by translocation to the nucleus (Nakao, Imamura et al. 1997). The nuclear SMAD 2, 3, 4 complex then mediates transcription of multiple target genes, including FoxP3 in $CD4^+$ T cells (Fu, Zhang et al. 2004; Peng, Laouar et al. 2004; Schramm, Huber et al. 2004; Marie, Letterio et al. 2005; Walther, Tongren et al. 2005).

TGF-ß is expressed on the surface $CD4^+CD25^+$ T regulatory cells in both the latent and active form (Nakamura, Kitani et al. 2001; Chen and Wahl 2003). Both TGF-β RII and TGF-β1 surface expression are up-regulated upon engagement of CTLA-4, which is expressed constitutively on the surface of Treg cells (Takahashi, Tagami et al. 2000), further enhancing their suppressor function (Nakamura, Kitani et al. 2001; Chen

and Wahl 2002). This suggests that Treg suppressor function is mediated by TGF-β and enhanced by CTLA-4 engagement. In support, Nakamura et al. found that anti- TGF-β antibody treatment abolished cell contact-dependent Treg suppressor activity (Nakamura, Kitani et al. 2001). The finding that CTLA-4 engagement up-regulates surface TGF-β may serve to reconcile opposing reports that CTLA-4 and not TGF-β is responsible for Treg contact-dependent suppressor function (Takahashi, Tagami et al. 2000; Tang, Boden et al. 2004). Additionally, in another study either anti-CTLA-4 or anti-TGF-β1 partially inhibited suppressor activity $CD4^+CD25^+$ Treg cell isolated from postnatal human thymuses, while a combination of both antibodies completely blocked Treg function (Annunziato, Cosmi et al. 2002). These data suggest that surface bound, active TGF-β on Treg cells could interact with TGF-βRI and RII on Th cells and provide a mechanism(s) 1) to induce FoxP3 expression and promote the conversion to T regulatory phenotype or 2) down-regulate IL-2 production, therefore effectively anergizing the helper T cells.

### E. $CD4^+CD25^+$ Treg Cells in Infectious Diseases

Natural Treg cells develop in the thymus during negative and positive selection and are responsible for maintaining tolerance to self-antigen in the periphery. The adaptive immune system responds to foreign antigen and effectively eliminates pathogens in healthy individuals. As natural Treg cells in healthy individuals constantly survey the periphery and abrogate immune responses to self-antigen before damage occurs, it is reasonable to conclude that Treg cells also play a role in down-regulating immune responses to foreign pathogens and maintain lymphocyte homeostasis. In order for this to occur there must be natural Treg TCR cross-reactivity with foreign antigen, or

alternatively peripheral generation of adaptive Treg that respond specifically to the foreign antigen, or a combination of the two. Recent data supports the hypothesis that adaptive Treg cells are generated extra-thymically and function to control the intensity and duration of adaptive immune responses.

$CD4^+CD25^+$ Treg cells play an important role during Leishmania major infection by preventing complete sterilizing immunity to the parasites and therefore allowing for the maintenance of a memory T cell population (Belkaid, Piccirillo et al. 2002). Additionally, Hisaeda et al (Hisaeda, Maekawa et al. 2004) showed that $CD4^+CD25^+$ Treg cells activated during malaria infection may contribute to protection against additional infections in a similar fashion. Treg cells also function in immune responses to bacterial infections such as Listeria monocytogenes by controlling the size of the CD8 memory response (Kursar, Bonhagen et al. 2002) and Pneumocystis carinii pneumonia by preventing $CD4^+CD25^-$ T cell induced disease (Hori, Carvalho et al. 2002). Long-lasting protective immunity to fungal infections such as Candida albicans is also mediated by Treg cells (Montagnoli, Bacci et al. 2002). Cumulatively, these data suggest that adaptive Treg cells function to control the duration of immune responses and to aid in the acquisition and maintenance of T cell memory pools during parasitic, bacterial, and fungal infections. Treg cells have also been implicated in viral infections and will be discussed below.

**F. Treg Cells and Retroviral Infection**

Treg cells function as immunosuppressor cells to control responses to both self-antigen and pathogenic stimulus. HIV subverts the normal function of the immune

system and initiates a state of chronic immune suppression through yet undefined mechanisms. It has been suggested that retroviral infection induces a chronic aberrant Treg cell activation. If this is the case, Treg cell mediated suppressor activity may contribute to the chronic immune suppression observed during retroviral infections such as HIV.

Friend virus (FV) is a murine retrovirus that chronically infects its host and has been employed as a model of chronic retroviral infection. Most FV-infected mice live out a normal life span without overt adverse effects of disease; however, a small percentage of infected mice are susceptible to relapse and succumb to FV-induced erythroleukemia. Further examination of apparently healthy FV-infected mice revealed a consistent 20-30% decrease in CTL activity which was induced by increased numbers of $CD4^+CD25^+CD69^+CD38^+$ Treg cells, suggesting a viral induced $CD4^+$ regulatory population. The Treg population was shown to suppress the function of $CD8^+$ CTL cells and the suppression was mediated by both TGF-β and CTLA-4 mechanisms as blocking with either anti-TGF-β or anti-CTLA-4 monoclonal antibodies reduced the suppressive effect and the inhibition was amplified when both TGF-β and CTLA-4 were blocked (Iwashiro, Messer et al. 2001).

In light of the FV-infection study in mice, it is reasonable to hypothesize that retroviruses in general may affect Treg homeostasis and therefore contribute to the immune suppression observed in retrovirus-infected individuals. In support of this theory, there is recent evidence, specifically in HIV-infected individuals, that an imbalance in Treg homeostasis may adversely affect virus specific immune function.

Weiss et al. (Weiss, Donkova-Petrini et al. 2004) found an expanded population of $CD4^+CD25^+$ T cells in HIV-infected persons receiving HAART therapy. Patients included in this study had CD4 counts above 500 cells/$mm^3$ and a plasma viral load below 50 copies/mL, characteristic of individuals in the asymptomatic phase of infection. The phenotype of the $CD4^+CD25^+$ cell population in HIV-infected individuals ($CD45RO^+$, $CD69^-$, cytoplasmic CTLA-$4^+$, FoxP3 $mRNA^+$) was similar to that of healthy controls. These cells were anergic to stimulation and suppressed $CD4^+CD25^-$ T cell responses in a dose-dependent manner similar to $CD4^+CD25^+$ Treg cells from control subjects. In support of the theory that extra-thymic $CD4^+CD25^+$ Treg cells are generated in the periphery of HIV-infected patients in response to chronic HIV associated antigenimia, TGF-β mRNA was induced specifically in response to HIV p24 antigen. As $CD4^+CD25^+$ Treg cells from HIV-infected patients are responsive to p24 antigen by inducing TGF-β mRNA and activating their suppressor function, it seems more likely that HIV specific $CD4^+$ effector cells are being suppressed by the adaptive Treg cells rather than a defect in the generation/expansion of HIV specific $CD4^+$ effector cells early in the response to HIV infection. To support this both $CD4^+$ Th and $CD8^+$ CTL HIV specific cells exist in HIV-infected individuals; however, they are ineffective in mounting an immune response, existing in an anergic unresponsive state. In separate studies, depletion of $CD4^+CD25^+$ Treg cells from PBMC of HIV-infected subjects resulted in restoration of proliferative responses and cytokine production in both $CD4^+$ and $CD8^+$ T cells (Kinter, Hennessey et al. 2004; Weiss, Donkova-Petrini et al. 2004; Eggena, Barugahare et al. 2005). The response was antigen specific to HIV p24 stimulation,

suggesting that p24 specific Treg cells were involved in suppressing HIV specific immune responses.

Andersson et al (Andersson, Boasso et al. 2005), report that FoxP3 levels are decreased in the circulation of HIV-infected individuals not undergoing anti-retroviral treatment, while at the same time FoxP3 mRNA levels were increased in the lymphoid tissue when compared to uninfected controls. Increased FoxP3 mRNA levels in the tonsils were highly correlated with the patient's plasma viral load. In addition, increased expression of GITR and CTLA-4 were also observed. Anti-retroviral treatment restored the homeostasis of FoxP3 expressing cells distribution to near uninfected healthy controls. The same trend was observed when TGF-β mRNA was analyzed for the same samples. Together these findings suggest that HIV-infection induces an alteration in the distribution of Treg cells which may be due to migration to or expansion in the lymphoid tissue. This increase in Treg cells in the LN correlates with an increase in the viral load.

The data suggest that Treg cells are likely to play an important role in the pathogenesis of chronic retroviral infection. To date, the exact mechanism of Treg action and the eventual effect Treg cells have on disease progression have not been fully defined. One possibility is that Treg cells in HIV-infected individuals suppress virus-specific $CD4^+$ and $CD8^+$ immune responses and may be partly responsible for disease progression. Alternatively, as suggested by Kinter et al Treg cells may serve to squelch HIV specific immune responses and protect the individual from AICD, CTL mediated cell destruction, and limit $CD4^+$ T cell activation thereby decreasing the potential pool of targets for productive viral infection (Kinter, Hennessey et al. 2004). The latter seems

less likely, as restoration of both $CD4^+$ and $CD8^+$ T cell function could provide a means for controlling disease progression and possibly eradicating the pool of infected cells.

## G. Natural Mechanisms for Overriding $CD4^+CD25^+$ Treg Cell Suppressor Activity

For Treg cells to play a dual role in maintaining homeostatic immune function by suppressing autoreactive immune responses and pathogen originated responses, there must exist a mechanism(s) to control the suppressive ability, expansion and/or differentiation of Treg cells. Recently, several mechanisms that abrogate Treg function, both in vitro and in vivo, have been identified and are discussed below.

### a) GITR

The glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR or TNFRSF18) exhibits high homology with the cytoplasmic signaling domains of CD40 (TNFRSF5), CD134 (OX40, TNFRSF4), CD137 (4-1BB, TNFRSF9) and CD27 (TNFRSF7), other TNFRSF members(Ronchetti, Nocentini et al. 2002). Members of this family are generally involved in regulating cell survival and differentiation in lymphocyte and other cell lineages (MacEwan 2002; Wajant, Pfizenmaier et al. 2003; Younes and Kadin 2003). Interestingly, GITR is constitutively expressed at high levels on naïve $CD4^+CD25^+$ regulatory T cells in both humans and mice (Levings, Sangregorio et al. 2002; Gavin and Rudensky 2003; Heikkinen, Mottonen et al. 2004), while it is expressed only at low, basal levels on resting $CD4^+CD25^-$ T cells (Shimizu, Yamazaki et al. 2002;

Ji, Liao et al. 2004; Ronchetti, Zollo et al. 2004). However, upon TCR and costimulatory molecule activation, GITR expression is strongly but transiently induced on both $CD4^+$ and $CD8^+$ T cells (Gurney, Marsters et al. 1999; Kwon, Yu et al. 1999; Ronchetti, Nocentini et al. 2002; Li, Mahesh et al. 2003).

The gene encoding the natural ligand of murine glucocorticoid-induced tumor necrosis factor receptor (mGITR) is composed of 173 amino acids and is similar to other type II membrane proteins with 51% homology to the human counterpart, activation-inducible TNF receptor (AITR) ligand (Kim, Choi et al. 2003). The expression of GITRL is confined to APC, including immature and mature dendritic cells, macrophages and B cells (Ermann and Fathman 2003). Further, LPS stimulation of dendritic cells via surface TLR-4 leads to a transient up-regulation of surface GITRL (Ermann and Fathman 2003).

Recent reports suggest that engagement of GITR on Treg cells may serve as a counterbalance of Treg mediated suppression (McHugh, Whitters et al. 2002; Shimizu, Yamazaki et al. 2002). This is accomplished through the interaction of GITR on Treg cells with its natural ligand (GITRL) on APC. Ligation of GITR on the T regulatory cells triggers NFκB activation and as a consequence induces IL-2 production (Ji, Liao et al. 2004). In addition, SMAD2/3 mediated TGF-β1/TGF-βRII signaling is inhibited by GITR ligation, which functions to abrogate the suppressor activity of Treg cells (Shimizu, Yamazaki et al. 2002). The interaction of GITR on T regulatory cells with GITRL on antigen presenting cells (APC) could play an important role during naïve $CD4^+CD25^-$ T helper cell activation by allowing an antigen specific expansion of effector cells.

Ronchetti et al. have shown that GITR functionally protects T cells from activation induced apoptosis which correlates well with expression on Treg cells, as they are resistant to activation induced death (Ronchetti, Nocentini et al. 2002). Additionally, GITR up-regulation after TCR stimulation protected T cells from glucocorticoid-induced cell death (Zhan, Funda et al. 2004). Conversely, GITR has also been linked to increased apoptosis, as GITR signaling and consequential activation of the pro-apoptotic protein SIVA slightly increased the rate of apoptosis (Spinicelli, Nocentini et al. 2002).

GITR also seems to play a role in both viral and tumor immune responses. To investigate how GITR might affect CD8 antiviral responses, transgenic $CD8^+$ T cells specific for the H-$2D^b$ MHC I-restricted GagL epitope of FV from B6 mice were adoptively transferred into chronically FV-infected mice. Results from the study revealed that $CD8^+$ T cells transferred into FV-infected hosts readily proliferated in response to their cognate antigen; however, CTL effector function in these cells was impaired as they failed to produce IFN-γ and reduce viral burden (Dittmer, He et al. 2004). Further, a non-depleting anti-GITR antibody in combination with adoptive transfer treatment was successful in significantly reducing chronic viral load (Dittmer, He et al. 2004) suggesting that engagement of GITR on Treg cells by GITRL may facilitate an effective CTL response during chronic retroviral infection.

FIV-mediated interruption of the GITR/GITRL interaction could facilitate immediate suppression of naïve $CD4^+CD25^-$ during priming in the LN, thereby preventing the expansion of antigen-specific T helpers cells and contributing to the immune suppression seen in HIV and FIV. Conversely, the GITR/GITRL interaction could allow simultaneous activation of both $CD4^+CD25^+$ regulatory T cells and

$CD4^+CD25^-$ T helper cells while abrogating the suppressive activity of the Treg cells, therefore allowing surface TGF-β on Treg cells to interact with TGF-βRII on the $CD4^+CD25^-$ cells undergoing activation and induce FoxP3 expression. The increase in virus specific Treg cells could then mediate suppression of virus specific $CD8^+$ CTL thereby promoting chronic viral infection rather than eradication, even with a robust virus-specific $CD8^+$ T cell expansion.

**b) OX40**

OX40 (CD134) is another member of the glucocorticoid-induced tumor necrosis factor receptor family. OX40 is expressed on both naïve and activated $CD4^+CD25^+$ Treg cells (McHugh, Whitters et al. 2002; Takeda, Ine et al. 2004) and is transiently up-regulated on $CD4^+CD25^-$ Th and $CD8^+$ T cells upon activation (Gramaglia, Weinberg et al. 1998). The natural ligand for OX40, OX40L, is expressed on APC, including DC, macrophages, and B cells and endothelial cells when activated (Stuber, Neurath et al. 1995; Imura, Hori et al. 1996; Brocker, Gulbranson-Judge et al. 1999; Sato, Ishii et al. 2002). The OX40/OX40L interaction functions to regulate Th1 cytokine production and T cell expansion and memory development by up-regulating anti-apoptotic proteins Bcl-$X_L$ and Bcl-2 and promoting long-term survival (Pippig, Pena-Rossi et al. 1999; Evans, Prell et al. 2001; Rogers, Song et al. 2001). As the expression profile of OX40 on Treg cells and OX40L on APC closely parallels that of GITR and GITRL and as the signaling domains are highly homologous, it follows that they may function in a similar mechanistic manner.

In a study comparing the roles of GITR and OX40 on the function of $CD4^+CD25^+$ Treg cells, OX40 signaling mediated the inhibition of Treg function by acting directly on Treg cells and not $CD4^+$ Th cells(Valzasina, Guiducci et al. 2005). This was affected by restoring IL-2 production in the Th cells, as previously reported for GITR. In support of this finding, in vivo OX40 triggering was associated with overcoming tolerance to tumor antigen and promoting tumor cell rejection (Bansal-Pakala, Jember et al. 2001). Contrary to the action of GITR, OX40 did not induce Treg cell proliferation nor was OX40 able to inhibit suppressor activity of in vitro expanded Treg cells (Valzasina, Guiducci et al. 2005). These results suggest that mechanisms exist that can regulate Treg cell suppressor activity at various stages of activation to allow for a robust adaptive immune response while maintaining peripheral tolerance.

**c) IL-12**

IL-12 is a heterodimer (IL-12p70) formed by a 35-kDa light chain p35 or IL-12α and a 40-kDa heavy chain p40 or IL-12β (Kobayashi, Fitz et al. 1989). IL-12 is produced mainly by dendritic cells (DCs) in response to stimulation through TLR signaling (D'Andrea, Rengaraju et al. 1992). The IL-12 receptor is composed of two chains IL-12Rβ1 and IL-12Rβ2 which signal through activation of the Janus kinase (JAK)–STAT (signal transducer and activator of transcription) pathway (Presky, Yang et al. 1996), mainly through activation of STAT4 (Thierfelder, van Deursen et al. 1996). Expression of IL-12R is primarily restricted to activated T cells and NK cells (Presky, Yang et al. 1996) with some expression reported on DCs (Grohmann, Belladonna et al.

1998). Resting naïve T cells do not express IL-12R, however both transcription and expression of IL-12Rβ1 and IL-12Rβ2 are up-regulated on T cells by TCR activation, with a positive auto-regulatory relationship with IL-12 and CD28 co-stimulation (Rogge, Barberis-Maino et al. 1997; Szabo, Dighe et al. 1997).

Recently, King et al. observed that IL-12, when added to a standard in vitro suppressor assay restored $CD4^+$ Th proliferation in response to anti-CD3 and syngeneic APC stimulation even at high Treg:Th ratios (King and Segal 2005). The effects of IL-12 were dose-dependent, however suppression was significantly reduced by concentrations lower than typically used for in vitro measurement of the effects of IL-12. Furthermore, IL-12 acted directly with $CD4^+CD25^-$ T cells and not via IL-12R to induce proliferation. The exclusive IL-12/ IL-12R interaction on $CD4^+CD25^-$ T cells was confirmed using $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from both IL-12Rβ2$^{-/-}$ and wild-type mice in suppressor assays (King and Segal 2005). Thus, IL-12 signaling on Th cells that over-rides the effect of Treg cells in addition to GITR- and OX40-mediated down-regulation of Treg suppressor activity may be important mechanisms that allow for an effective immune response to pathogens within the LN microenvironment where both Treg and Th cells exist together.

**H. The role of LN in Generating Peripheral Treg Cells**

As the individual TCRVβ repertoire is determined intrathymically during negative and positive selection, thymic-derived Treg cells responsible for maintaining self-tolerance should have a limited self-antigen specific TCRVβ expression profile. To

the contrary, Battaglia, et al. in a study of human LN and PB found that while PB Treg cells were similar in TCRVβ expression, LN Treg cells expressed higher diversity and there was no correlation of TCRVβ expression between LN and PB Treg cells (Battaglia, Ferrandina et al. 2003). This finding suggests that peripheral Treg cells may be generated in the LN microenvironment during responses to pathogenic stimuli thereby contributing to the observed increase in TCRVβ diversity in the LN compared to PB Treg cells. Further, as activated DC populate the LN, this also suggests possible involvement of DC in the development of pathogen specific Treg cells. To support this Ochando et al found that LN occupancy was required for the peripheral development of $CD4^+CD25^+FoxP3^+$ Treg cells in mice (Ochando, Yopp et al. 2005). Alloantigen-specific Treg cells expressing FoxP3 were expanded in the LN of cardiac allograft recipients but not other peripheral sites. Expansion of peripheral Treg cells was inhibited by anti-CD62L treatment, while the total $CD4^+$ population increased (Ochando, Yopp et al. 2005), suggesting CD62L-mediated LN homing was necessary for generating peripheral Treg in these mice. Further, another study found that both anti-tumor effector T cells and Treg cells capable of suppressing the anti-tumor reactivity of the effector T cells are primed in the same tumor draining LN (Hiura, Kagamu et al. 2005). Taken together, these data indicate the possible involvement of the LN microenvironment in generating peripheral antigen specific adaptive Treg cells.

## I. TGF-β Mediated Induction of Treg Function in $CD4^+CD25^-$ T cells

It is established that $CD4^+CD25^+$ T regulatory are produced in the thymus and exit into the periphery to regulate immune responses. However, this may not be the only mechanism of generating $CD4^+CD25^+$ regulatory cells. Recently, Chen et al. showed that TGF-β in combination with anti-CD3 stimulation and anti-CD28 co-stimulation was able to induce $CD4^+CD25^-$ T cells to attain T regulatory characteristics (Chen, Jin et al. 2003). These TGF-ß anergized cells were phenotypically identical to $CD4^+CD25^+$ regulatory T cells (surface $CD45RB^{lo}$ $CD25^+$ $CTLA\text{-}4^+$ and cytoplasmic CTLA-4 positive). Regulatory function in the $CD4^+CD25^-$ converted population was conferred by the induction of FoxP3, consistent with previous findings in the mouse. In addition, the cells were functionally similar to naturally occurring T regulatory cells in that they were anergic to TCR stimulation and able to suppress $CD4^+CD25^-$ T cell proliferation and cytokine production both in vitro and in vivo in an antigen specific manner (Chen, Jin et al. 2003). .

Recently it was reported that $CD4^+CD25^-$ T cells were converted to FoxP3 expressing regulatory T cells after exposure to TGF-β during anti-CD3 and anti-CD28 stimulation. The up-regulation of FoxP3 was transient when either TGF-β or anti-CD3 and anti-CD28 co-stimulation was removed, however FoxP3 levels were restored in the $CD4^+CD25^-$ T cells during stimulation even in the absence of TGF-β indicating the requirement for TGF-β only during establishment of the Treg phenotype (Park, Paik et al. 2004). The increase in $CD4^+CD25^+$ T regulatory cells seen after Friend virus infection in mice coupled with immunosuppression seen in FV, FIV, SIV, and HIV may indicate an

alteration in the converted T regulatory long-term function by maintaining what is normally a transient function.

### J. $CD4^+CD25^+$ Treg Cell -Mediated Induction of Treg Function in $CD4^+CD25^-$ T cells

One of the functional aspects of Treg cells in the induction of anergy in $CD4^+CD25^-$ Th cells responding to their cognate antigen. Although there are few studies involved with the function of Treg anergized Th cells, Jonuleit et al (Jonuleit, Schmitt et al. 2002) analyzed these cells for suppressor activity over activated Th cells that were not previously exposed to Treg cells. In the study Treg suppressor function was found to be independent of TGF-β, although there was a significant increase in surface TGF-β on Treg cells compared to Th cells. There was no evidence of soluble TGF-β production by either Treg or Th cells regardless of their activation state. These results are contradictory with other reports that Treg suppressor function was dependent on surface TGF-β, and in addition activated Treg cells produced soluble TGF-β (Jonuleit, Schmitt et al. 2002). Interestingly, the anergized $CD4^+$ Th cells exerted suppressor activity over freshly activated $CD4^+$ Th cells that was comparable to the suppressor activity of natural Treg cells. Further examination of the anergized suppressor cells revealed that they did produce biologically active TGF-β immediately after polyclonal stimulation. Additionally, the observed suppressor function of the anergized Th cells was contact independent as suppression was inhibited by transwell separation (Jonuleit, Schmitt et al. 2002).

Recently in support, activated human $CD4^+CD25^+$ T cells were found to not only suppress antigen-specific $CD4^+$ and $CD8^+$ T cells, but also induce a suppressive phenotype in $CD4^+$ T cells (Dieckmann, Plottner et al. 2005). The effect was dependent on prior activation of the Treg cells with anti-CD3 and anti-CD28 antibodies 24 hr prior to co-culture. To investigate the cytokine profile of anergized $CD4^+$ T cells, HLA mismatch Treg cells were used for co-cultures and the separated based on HLA expression. The anergized $CD4^+$ cells were restimulated after 4 days rest and analysis of the supernatants showed up-regulation of both TGF-β and IL-10. In addition the anergized clones suppressed other T cells proliferation after antigen specific activation and suppressor function was dependent on TGF-β and IL-10. Although Treg cells suppressed proliferation and IFN-γ production by $CD8^+$ cells, neither TGF-β nor IL-10 production was induced in the $CD8^+$ T cells. Suppressor function was not detected in anergized $CD8^+$ T cells.

Regardless of the mechanism of suppression, data suggest that activated $CD4^+CD25^+$ T cells can induce suppressor function in $CD4^+CD25^-$ T cells. The possibility of in vivo generation of adaptive Treg cells is particularly interesting in the case of Lentiviral AIDS infection, given that both FIV and HIV preferentially and productively infect $CD4^+CD25^+$ T cells (Joshi, Vahlenkamp et al. 2004; Kinter, Hennessey et al. 2004; Oswald-Richter, Grill et al. 2004). Because these cells are both anergic and resistant to clonal deletion, they could provide an expanded long-lived reservoir for virus even during aggressive treatment strategies such as HAART. The peripherally generated regulatory population would be subject to long-term productive FIV/HIV infection and suppressor function could be recalled on exposure to cognate

antigen whether it originates from viral, bacterial, parasitic or fungal pathogens. These results may offer an explanation for immune suppression seen in FIV/HIV without drastic decreases in the total $CD4^+$ population. An increase in $CD4^+CD25^+$ T regulatory cells coupled with a loss of $CD4^+CD25^-$ T and increased immunosuppression is observed during FIV/HIV infection and could possibly be explained by $CD4^+CD25^+$ Treg anergized/converted $CD4^+CD25^-$ adaptive T regulatory cells.

**K. Based on the above literature, the present study was undertaken with the following specific aims**

**Specific Aim 1:** Can TGF-β1 with ConA stimulation convert $CD4^+CD25^-$ feline T cells to Treg-like T cells?

**Specific Aim 2:** Can we inhibit TGF-β conversion of $CD4^+CD25^-$ T cells with blocking antibodies?

**Specific Aim 3:** Is there an increase in TGF-β surface expression on T cells from FIV-infected cats?

**Specific Aim 4:** Are Treg cells from cats capable of converting $CD4^+CD25^-$ T cells to Treg-like cells? Is expression of TGF-β associated with the conversion?

**Specific Aim 5:** Can we inhibit $CD4^+CD25^+$ conversion of $CD4^+CD25^-$ T cells with blocking antibodies?

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# 3. CHAPTER-1

## TGF-β plus Con A stimulation converts feline $CD4^{\pm}CD25^{-}$ T cells to phenotypic and functional T regulatory cells through a TGF-β1/TGF-βRII dependent signaling pathway

## ABSTRACT

We have shown previously that $CD4^+CD25^+$ T regulatory (Treg) cells exist in cats and that these cells are preferentially and productively infected with FIV. As FIV infection in cats closely parallels HIV infection in humans, it is important to more fully define the Treg cell, which may play a critical role in $CD4^+$ T cell function and homeostasis, and ultimately disease progression. Currently there is considerable debate over the origin of Treg cells, whether they are solely of thymic origin, so called natural Treg cells, or if there exists a mechanism of peripheral, extra-thymic generation. Data suggest that TGF-β may play a role in maintaining peripheral Treg homeostasis and function in humans and mice. We initially asked if peripheral feline $CD4^+CD25^-$ cells could be converted via soluble TGF-β to a Treg-like phenotype. We found, in addition to up-regulation of CD25, TGF-β plus Con A treatment of FACS purified feline $CD4^+CD25^-$ T cells induced TGF-β surface expression another key Treg cell marker. To further refine the definition of feline Treg cells we identified a 385 bp fragment from feline MYA-1 cells ($CD4^+CD25^+$), fFoxP3, with greater than 90% homology to both human and macaque FoxP3, that was expressed in $CD4^+CD25^+$ T cells but not $CD4^+CD25^-$ feline T cells. FoxP3 was also up-regulated in $CD4^+CD25^-$ T cells after TGF−β and Con A stimulation, that correlated with up-regulation of surface expression of CD25 and TGF−β, suggesting a conversion to a Treg-like phenotype. To confirm this, we asked if TGF−β and Con A converted $CD4^+CD25^-$ T cells were able to suppress the proliferation of Con A-activated $CD4^+CD25^-$ T cells. We found that Con A

plus TGF−β treatment of feline $CD4^+CD25^-$ T cells conferred the ability to suppress the proliferation of activated $CD4^+CD25^-$ T cells, the functional hallmark of Treg cells.

## INTRODUCTION

Naturally occurring, thymic-derived $CD4^+CD25^+$ Treg cells, as well as pathogen-induced Treg cells, are phenotypically defined by constitutive cell surface expression of CD25 (IL-2Rα-chain), GITR and CTLA-4, as well as the transcription factor FoxP3. When activated Treg cells also express biologically active TGF-β on their surface (Nakamura, Kitani et al. 2001; Annunziato, Cosmi et al. 2002). Self-antigen presentation by thymic epithelium is currently believed to be responsible for generating $CD4^+CD25^+$ T regulatory cells capable of suppressing peripheral $CD4^+CD25^-$ and $CD8^+$ T cells specific for the same self antigens and therefore maintaining peripheral self tolerance (Apostolou, Sarukhan et al. 2002). This is supported by Kyewski et al. (Kyewski, Derbinski et al. 2002) who found mRNA for multiple tissue specific proteins that are expressed by thymic medullary epithelial cells and could be instrumental in generating self-antigen specific T regulatory cells (Derbinski, Schulte et al. 2001). These $CD4^+CD25^+$ T regulatory cells are functionally mature upon exit from the thymus into the periphery and exert an active mechanism of immune suppression of activated $CD4^+$ Th and $CD8^+$ effector cells (Suri-Payer, Amar et al. 1998; Thornton and Shevach 1998; Suvas, Kumaraguru et al. 2003; Trzonkowski, Szmit et al. 2004), once activated by TCR engagement. It is now well established that, in addition to maintaining peripheral self-tolerance $CD4^+CD25^+$ Treg cell play a major role in modulating immune responses to infectious agents (Suvas, Kumaraguru et al. 2003; Rouse and

Suvas 2004; Bach 2005; Chattopadhyay, Mehrotra et al. 2006; Pelletier, Transue et al. 2006). These Treg cells are activated by a number of pathogen-associated antigens and cytokines, including LPS, TGFβ1 and IL-2, and negatively modulate protective immune responses (Maloy, Salaun et al. 2003). Whether pathogen-induced Treg cells are derived from natural Treg generated in the thymus, or are peripherally generated Treg, or both has yet to be elucidated. Treg cell homeostatis is essential, as they comprise a small fraction (5-10%) of the total T cell population in the blood and are anergic and cannot expand by themselves. Recently, Chen et al. reported that $CD4^+CD25^-$ T helper (Th) cells can be induced to attain Treg characteristics in vitro by stimulation with TGF-β in combination with TCR engagement, suggesting a mechanism of Treg peripheral homeostasis independent of the thymus (Chen, Jin et al. 2003). These TGF-ß anergized cells were phenotypically identical to naturally occurring $CD4^+CD25^+$ Treg cells (surface $CD45RB^{lo}$ $CD25^+$ $CTLA\text{-}4^+$ and cytoplasmic CTLA-4 positive). Regulatory function in the $CD4^+CD25^-$ converted population was conferred by the induction of FoxP3, a regulatory transcription factor, that is essential for Treg homeostasis and suppressor function, consistent with previous findings in the mouse (Schubert, Jeffery et al. 2001; Chen, Jin et al. 2003; Gavin and Rudensky 2003; Hori, Nomura et al. 2003; Powrie and Maloy 2003; Walker, Kasprowicz et al. 2003; Fantini, Becker et al. 2004; Fehervari and Sakaguchi 2004; Hori and Sakaguchi 2004; Peng, Laouar et al. 2004; Verhasselt, Vosters et al. 2004). The possibility of peripheral generation of Treg cells is particularly interesting in the case of FIV/HIV infections, given that both viruses productively infect $CD4^+CD25^+$ T cells (Joshi, Vahlenkamp et al. 2004; Kinter, Hennessey et al. 2004; Oswald-Richter, Grill et al. 2004) and in both infections Treg cells are chronically activated in vivo (Grossman, Feinberg et al. 1998; Vahlenkamp, Tompkins et al.

2004). As HIV/FIV-infected T cells are prone to lysis, and as activated T cells are highly susceptible to apoptosis, survival of Treg cells in HIV/FIV infection could be problematic. However, in both HIV and FIV infections the numbers of Treg cells in the circulation do not decrease or may actually increase as a percentage of total $CD4^+$ T cells throughout the course of the infection. Thus, peripherally generated $CD4^+CD25^+$ Treg-like cells from the pool of Th cells may be of particular importance in the case of AIDS lentivirus infection. As discussed above, Treg conversion of Th cells to a Treg-like phenotype is mediated by the TGF-β1 signaling pathway. Recruitment of Treg cells from the Th pool requires that both the Treg cell and the Th cell be activated. These conditions are met in both HIV and FIV infection as CD4 Th cells and $CD4^+CD25^+$ Treg cells are characterized by chronic immune hyperactivation throughout the course of infection. The state of chronic T cell hyperactivation seen in these infections would facilitate conversion of Th cells to Treg-like phenotype and contribute to the Treg pool at the expense of the Th pool. The theory of peripheral generation of and immune suppression by Treg is supported in the mouse Friend retrovirus (FV) infection, in which mice chronically infected with FV develop two times the normal number of splenic $CD4^+CD25^+$ T regulatory cells (Iwashiro, Messer et al. 2001; Beilharz, Sammels et al. 2004). Furthermore, FV-infected mice lose their ability to reject certain tumor transplants, which suggests a mechanism of active suppression. Immunosuppression in FV infection by the expanded population of T regulatory cells was implicated both *in vivo* and *in vitro* (Iwashiro, Messer et al. 2001). A role for TGF-β in recruitment of Treg cells in lentivirus infection was recently suggested in the SIV African green monkey model. Kornfeld et al. (Kornfeld, Ploquin et al. 2005) reported an early induction of TGF-β1 and FoxP3 expression that correlated with increased levels of $CD4^+CD25^+$ and

$CD8^+CD25^+$ T cells in acutely SIV-infected African green monkeys. We recently reported that functionally activated Treg cells from FIV-infected cats express TGF-β1, as well as TGF-βRII on their surface, and a small but significant fraction of $CD4^+CD25^-$ Th cells express TGF-βRII on their surface, suggesting the potential for in vivo interaction with Th cells and Th to Treg cells. To explore the possibility of $CD4^+CD25^-$ Th cell conversion to a Treg-like phenotype in FIV-infection, we initially further characterized the phenotype of feline Treg cells and examined the role of TGF-β1 and TGF-βRII in the Treg conversion process. We report that in vitro conversion of $CD4^+CD25^-$ Th cells to phenotypic and functional Treg-like cells requires stimulation by mitogen plus TGF-β1 and hypothesize that specific antigen plus TGF-b1 would facilitate this conversion in vivo. Further we report that Treg conversion can be inhibited by anti-TGF-βRII antibody, while Treg immunosuppressor function can be inhibited by anti-TGF-β1 antibody.

## RESULTS

### TGF-β plus Con A stimulation induces regulatory function in $CD4^+CD25^-$ T cells

Recent reports demonstrate that both human and rodent $CD4^+CD25^-$ T cells are converted to functional and phenotypic Treg cells by stimulation with anti-CD3 and anti-CD28 in the presence of TGF-β1 (Chen, Jin et al. 2003; Fantini, Becker et al. 2004). We were therefore interested to determine whether TGF-β also induces feline $CD4^+CD25^-$ T cells to acquire the characteristic T regulatory suppressor activity. As suppressor activity is the hallmark of Treg

cells, we assessed suppressor function of TGF-β converted FACS purified $CD4^+CD25^-$ T cells using a standard 72 h in vitro proliferation assay with Con A-stimulated $CD4^+CD25^-$ T cells as target cells. Con A plus TGF−β (Con A/TGFβ) stimulated $CD4^+CD25^-$ T cells isolated from PLN of SPF control cats suppressed the proliferative response of heterologous Con A-stimulated $CD4^+CD25^-$ target cells in a dose dependent manner (Fig. 1a). Suppressor activity of FACS purified Con A/TGF-β1 induced Treg-like cells was comparable to that of IL-2/LPS activated $CD4^+CD25^+$ Treg cells obtained from a SPF control cat (Fig. 1a). It is possible that expansion of the residual 1-2% of $CD4^+CD25^+$ T cells in the Con A/TGF-β stimulated $CD4^+CD25^-$ population was responsible for the observed suppressor activity. To rule out this possibility, we also tested the suppressor activity of autologous $CD4^+CD25^-$ T cells that were cultured in medium supplemented with IL-2, which is known to activate $CD4^+CD25^+$ Treg suppressor function (Thornton, Donovan et al. 2004). As expected, cells supplemented only with IL-2 were unable to suppress the proliferation of mitogen stimulated $CD4^+CD25^-$ T cells at any ratio tested (Fig. 1a). Further, neither TGF−β alone nor Con A alone were able to induce suppressor function in $CD4^+CD25^-$ T cells (Fig. 1a), verifing that both TGF−β and mitogen stimulation are required for induction of suppressor function in feline $CD4^+CD25^-$ T cells.

**FIV infection does not affect Con A/TGF-β1 converesion of $CD4^+CD25^-$ cells**

In this experiment, we asked whether chronic FIV infection of cats impacted the ability of $CD4^+CD25^-$ T cells to attain regulatory function after Con A/TGF−β stimulation. This is an important question, as we previously reported that $CD4^+CD25^-$ T cells harbor a latent FIV

infection that becomes replication competent when stimulated by Con A (Joshi, Vahlenkamp et al. 2004). $CD4^+CD25^-$ T cells were FACS purified from PLN cells from FIV-infected cats and treated as described for control cats (Fig. 1a). Our results indicate that $CD4^+CD25^-$ T cells isolated from asymptomatic, chronically FIV-infected cats are also capable of being converted to Treg cells by Con A/TGF-β1 treatment. $CD4^+CD25^-$ T cells from FIV-infected cats also required both TGF-β and Con A for acquisition of suppressor activity, as neither Con A nor TGF-β1 alone induced any suppressor function (Fig. 1b). As shown, $CD4^+CD25^+$ natural Treg cells from FIV-infected cats suppressed Con A-induced $CD4^+CD25^-$ cells proliferation comparable to converted cells (Fig. 1b) These experiments demonstrate TGF-β treatment of Con A stimulated feline $CD4^+CD25^-$ T cells from either control or FIV-infected cats, confers suppressor activity in this population that compares to that seen in *in vitro* activated $CD4^+CD25^+$ T cells from control cats or freshly isolated unstimulated $CD4^+CD25^+$ T cells from FIV-infected cats. The TGF-β/Con A converted cells from both SPF control and FIV-infected cats also displayed an anergic phenotype characteristic of Treg cells, as they failed to proliferate in response to Con A stimulation as opposed to $CD4^+CD25^-$ T cells when treated with Con A (data not shown).

**Con A/TGF-β stimulation of feline $CD4^+CD25^-$ T cells induces a $CD25^+TGF\text{-}\beta^+FoxP3^+$ Treg-like phenotype**

Peripheral generation of Treg cells may be essential in maintaining a continuing pool of Treg cells. Recent studies in mice and humans have shown that TGF-β coupled with TCR stimulation of $CD4^+CD25^-$ T cells confers Treg-like function in this population through the induction of

FoxP3 (Dieckmann, Plottner et al. 2005). Therefore we investigated the possibility that TGF-β plus Con A converted feline $CD4^+CD25^-$ T cells also displayed an up-regulation of FoxP3 mRNA. In addition, as surface TGF−β1 is a marker associated with activated Treg cells from mice (Nakamura, Kitani et al. 2001) and FIV-infected cats (Emani, 2006), cell surface TGF−β on converted cells was also assayed by flow cytometry. Culturing of $CD4^+CD25^-$ T cells in IL-2 alone for 48 h caused no significant changes with regard to expression of cell surface CD25 or TGF-β1 (Fig. 3a). Treatment of $CD4^+CD25^-$ T cells with Con A alone or Con A plus TGF-β1 for 48 h, caused a marked up-regulation in surface CD25 (Fig. 3a), whereas TGF-β1 treatment alone had no effect on CD25 expression (Fig. 3a). In the case of cell surface TGF-β1 expression, only Con A plus TGF-β stimulation significantly increased the number of TGF-β positive T cells, which were almost exclusively $CD4^+CD25^+$ T cells (Fig. 3a). In this experiment, we investigated the association of Treg immunosuppressor function and Foxp3 expression. Preliminary to assessing Con A/TGFβ stimulated $CD4^+CD25^-$ for Foxp3, it was necessary to develop and validate primers for feline Foxp3 mRNA. To do this, total RNA was isolated from MYA-1 cells, a feline $CD4^+CD25^+$ $CD3^+CD8^-$, IL-2 dependent T lymphoblastoid cell line established from PBMC (Miyazawa, Furuya et al. 1989; Miyazawa, Toyosaki et al. 1992). After reverse transcription, a 385bp fragment from the resulting cDNA was amplified by PCR using primers designed from the published human FoxP3 sequence. Sequence analysis of a purified 385 bp feline product, fFoxP3 (Fig. 2a,b) showed high homology to several published mammalian FoxP3 sequences. We used the feline FoxP3 primers developed from the MYA-1 cell line, and assessed Foxp3 expression in FACS purified primary feline T cells including

$CD4^+CD25^+$ T cells with demonstrable Treg function (Vahlenkamp, Tompkins et al. 2004) and $CD4^+CD25^-$ Th cells freshly isolated from PBMC from a normal cat. Primary feline $CD4^+CD25^+$ T cells expressed high levels of FoxP3, while minimal expression was also detected in $CD4^+CD25^-$ Th cells (Fig. 2c) indicating that FoxP3 is preferentially, but not exclusively confined to $CD4^+CD25^+$ Treg cells in cats. In addition, ethidium bromide agarose gel analysis of the fFoxP3 cDNA revealed two bands, which indicates the possibility of at least two isoforms of fFoxP3 and closely resembles the expression profile reported for human T cells (Allan, Passerini et al. 2005; Morgan, van Bilsen et al. 2005; Roncador, Brown et al. 2005).

Using the feline specific FoxP3 primers, we next assessed FoxP3 expression in Con A/TGF-β-stimulated $CD4^+CD25^-$ T cells. Similar to cell surface TGF-β up-regulation, Con A/TGF-β treatment of the purified $CD4^+CD25^-$ T cells resulted in marked up-regulation of FoxP3 message (Fig. 3b). FoxP3 expression was minimally increased when cells were treated with Con A but not with TGF–β alone (Fig. 3b). These data suggest that TGF-β1 plus mitogen activation is required for the induction of a $CD4^+CD25^+FoxP3^+TGF\text{-}\beta1^+$ Treg-like phenotype, whereas mitogen treatment alone will up-regulate CD25 on $CD4^+CD25^-$ T cells. The presence of surface TGF-β and CD25, in addition to FoxP3 mRNA expression suggests that TGF-β1 plus Con A treatment converts $CD4^+CD25^-$ T cells a functional Treg phenotype and this is confirmed by the observed suppressor activity in the converted cells (Fig. 1).

**Con A induces TGF-βRII surface expression on $CD4^+CD25^-$ T cells**

As both mitogen and TGF-β1 treatment of $CD4^+CD25^-$ T cells is required for the acquisition of a Treg-like phenotype, and as both the homeostasis and function of peripheral Treg cells is dependent on the TGF-β1/TGF-βR signaling pathway (Green, Gorelik et al. 2003; Peng, Laouar et al. 2004; Marie, Letterio et al. 2005), it follows that the TGF-βRII must be up-regulated on the surface of mitogen stimulated $CD4^+CD25^-$ T cells. This is supported by the observation that Con A treatment, but not TGF-β treatment alone upregulates CD25 on $CD4^+CD25^-$ T cells (Fig.3). To more directly investigate the TGF-βRII expression profile on $CD4^+CD25^-$ T cells, FACS purified $CD4^+CD25^-$ T cells from control cats were stimulated with Con A and cell surface TGF-βRII expression was assessed by flow cytometry. TGF-β was significantly up-regulated on $CD4^+CD25^-$ T cells when compared to freshly purified cells or cells cultured in medium not supplemented with Con A (Fig. 4a,b,c).

**Blockade of TGF−βRII abrogates conversion of $CD4^+CD25^-$ T cells**

To more fully address the role of TGFβ-RII on Con A/TGFβ conversion of $CD4^+CD25^-$ T cells, FACS purified naïve PLN $CD4^+CD25^-$ T cells from a normal cat were stimulated with Con A for 18 hrs then washed and incubated with anti-TGF−βRII mAb for 30 min and washed again before addition of TGF−β to the culture medium. TGF−β surface expression was completely abrogated when anti-TGF-βRII receptor was added to Con A stimulated $CD4^+CD25^-$ T cells prior addition of TGF-β1 (Fig. 5), whereas TGF-β surface expression was elevated if no blocking antibody was

used before addition of TGF-β1 (Fig. 5). In addition, we assessed the functional conversion of Con A stimulated $CD4^+CD25^-$ T cells when treated with anti-TGF-βRII before addition of TGF-β and found that the suppressor function was also abrogated (Fig. 6a) and correlated with the absence of surface TGF-β1 and diminished FoxP3 mRNA.

**Anti-TGF-β1 treatment of converted $CD4^+CD25^-$ T cells abrogates suppressor function**

While expression and cell surface TGF-β is a constant feature of activated Treg cells, the role of TGF-β in mediating immunosuppression has been controversial. While Nakamura et al. (Nakamura, Kitani et al. 2001; Nakamura, Kitani et al. 2004) has reported that cell surface TGF-β mediates immunosuppression, others (Piccirillo, Letterio et al. 2002; Tang, Boden et al. 2004) have suggested it is not involved. As we found that anti-TGF-βRII interrupted the conversion of $CD4^+CD25^-$ T cells to a Treg-like cell, we further investigated if blockade of TGF−β1 after conversion could inhibit the suppressor function of Con A/TGF-β converted cells. To address this question we converted $CD4^+CD25^-$ T cells FACS purified from PLN of normal cats to $CD25^+$TGF-$\beta1^+$ Treg-like suppressor cells as described above. The converted cells were then incubated with anti-TGF-β1 for 30 min before assaying their anti-proliferative effect on Con A-stimulated $CD4^+CD25^-$ T cells purified from PBMC of a normal cat. As shown in Figure 6b, addition of anti-TGF-β1 after conversion to a Treg-like phenotype blocked the suppressor activity of the converted cells, as demonstrated by uninhibited proliferation of the Con A-stimulated Th target cells. Further, in support of the immunosuppressor role of TGF-β, we found diminished suppressor activity from freshly purified $CD4^+CD25^+$ Treg cells purified from PLN

of a chronically FIV-infected cat when incubated with anti-TGF-β1 for 30 min before assaying their anti-proliferative effect on autologous Con A-stimulated $CD4^+CD25^-$ T cells when compared to untreated $CD4^+CD25^+$ Treg cells from the same cat (Fig.6c). Together, these experiments suggest that TGF−βRII signaling plays a critical role in the development of peripheral Treg cells, and that the function of these cells is TGF−β dependent, as reported for naturally occurring thymic-derived $CD4^+CD25^+$ Treg cells.

## DISSCUSSION

$CD4^+CD25^+$ regulatory T cells (Treg) have emerged as a unique distinct lineage of T cells thought to function in maintenance of peripheral self-tolerance and modulate immune responses to pathogens (Iwashiro, Messer et al. 2001; Belkaid, Piccirillo et al. 2002; Hori, Carvalho et al. 2002; Kursar, Bonhagen et al. 2002; Montagnoli, Bacci et al. 2002; Long, Nakazawa et al. 2003; Hisaeda, Maekawa et al. 2004). Although the immunoregulatory property of these cells is no longer questioned, where and how these cells are generated and the scope and mechanism of their immunoregulatory function remain areas of active investigation.

Major current research questions whether Treg cells are generated only in the thymus from a distinct $CD4^+$ lineage or whether they represent a phenotypic and functional stage that different $CD4^+$ T cell subsets can acquire in different immune compartments in response to specific stimuli. While early studies indicate that the thymus is the sole reservoir of Treg cells, recent evidence suggest that Treg cells may also be induced in the periphery, particularly under conditions of chronic immune stimulation such as chronic infectious disease. The mechanism(s)

regulating the survival and expansion of the Treg cell pool in the periphery is poorly understood. However, a number of studies indicate that peripheral Treg homeostasis is independent of the thymus. $CD4^+CD25^+$ Treg cells express a number of Toll-like receptors, including TLR4 and can be activated with LPS (Caramalho, Lopes-Carvalho et al. 2003). Also, IL-2 has been shown to induce a proliferative response by Treg cells, as well as increase their immunosuppressor function (Vahlenkamp, Tompkins et al. 2004; Setoguchi, Hori et al. 2005; Zhang, Chua et al. 2005). Marie et al. (Marie, Letterio et al. 2005) reported that FoxP3 expression and the size of the Treg cell compartment, as well as suppressor function are dependent on signals induced by TGF-β in the periphery and are not thymus dependent. In this regard, Dieckmann, et al. demonstrated that activated $CD4^+CD25^+$ Treg cells are capable of inducing a Treg phenotype from activated Th cells in a contact-dependent manner (Dieckmann, Plottner et al. 2005). Chen et al. also reported that TGF-β converted TCR-stimulated $CD4^+CD25^-$ Th cells into FoxP3 positive $CD4^+CD25^+$ Treg cells with potent immunosuppressive function (Chen, Jin et al. 2003). For conversion of $CD4^+CD25^-$ T cells to a Treg phenotype both the Treg cell and the Th target cell must be activated (Jonuleit, Schmitt et al. 2002). Herein, we report that Con A/TGF-β1-stimulated feline $CD4^+CD25^-$ T cells can be converted to $CD4^+CD25^+TGF\text{-}\beta1^+FoxP3^+$ anergic, immunosuppressive Treg-like cells. Moreover, we report that treatment of Con A-stimulated $CD4^+$ $CD25^-$ with anti-TGFβ-RII antibody abrogates conversion to a Treg phenotype and that treatment of converted Treg-like cells with anti-TGF-β1 antibody abrogates immunosuppressor function. These data suggest that both a source of TGF-β1 and expression of TGFβ-RII on

$CD4^+CD25^-$ Th cells is required for conversion of $CD4^+CD25^-$ T cells to a Treg phenotype, while TGF-β1 is required for immuosuppressor function of the converted Treg cell.

Conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+FoxP3^+$ T suppressor cells by anti-CD3 and anti-CD28 antibody treatment with exogenous TGF-β1 has been reported in both humans (Fantini, Becker et al. 2004)and rodents (Chen, Jin et al. 2003). Here we report that Con A plus exogenous TGF-β1 converts feline $CD4^+CD25^-$ T cells to functional $CD4^+CD25^+TGF\beta1^+FoxP3^+$ Treg cells. We confirmed that TGF-β signaling is required for the conversion of $CD4^+CD25^-$ T cells and demonstrated this requirement first by examining the surface expression of TGF-βRII. As TGF-βRII was not expressed on naïve $CD4^+CD25^-$ T cells we speculated that activation may induce TGF-βRII expression. Our data indicate, that Con A treatment is responsible for inducing significant TGF-βRII expression on $CD4^+CD25^-$ T cells, as TGF-βRII expression was not induced by exogenous rhTGF-β1 alone or cells cultured in unsupplemented medium. These findings correlate with the observed requirement for both Con A and TGF-β1 to convert $CD4^+CD25^-$ T cells to $CD4^+CD25^+FoxP3^+$ Treg cells. To further confirm the requirement for TGF-β1/TGF-βRII interaction in the Treg conversion mechanism, we induced TGF-βRII expression on $CD4^+CD25^-$ T cells with Con A and then blocked the receptor with anti- TGF-βRII mAb before adding TGF-β1 to the cultures. Blocking TGF-βRII completely abrogated the TGF-β1 conversion of $CD4^+CD25^-$ T cells to Treg-like cells. In addition blocking TGF-β1 on the converted cells blocked the acquired suppressor function, further emphasizing the critical role of TGF-β1/ TGF-βRII signaling in the conversion and function of peripherally generated adaptive Treg cells.

Our data suggest that TGF-β1 mediates both conversion and function in extra-thymically generated Treg cells. As we are inducing TGF-βRII in vitro before adding exogenous TGF-β1, it is possible that we are merely loading TGF-β1 into available receptors. It is impossible to distinguish between rhTGF-β1 introduced into our in vitro conversion system and TGF-β1 produced by converted cells and therefore we can not rule out this possibility. We are currently developing PCR and IHC methods for detecting up-regulation of TGF-β1 message and and protein production to better address this question. Although it is important to determine whether the converted cells produce TGF-β1 that could act in an autocrine manner, it may be more significant that these cells are functional suppressors and whether they are merely loaded with TGF-β1 or not may be irrelevant for this function.

Although this study did not address the role of $CD4^+CD25^+TGF\text{-}\beta^+FoxP3^+$ Treg cells in converting $CD4^+CD25^-$ T cells to Treg-like cells, we have provided evidence that suggests surface bound TGF-β on Treg cells could interact with TGF-βRII on activated $CD4^+CD25^-$ T cells thereby initiating TGF-β signaling and the peripheral Treg conversion process. We are currently investigating the role of chronic immune hyperactivation observed during FIV infection on peripheral conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ Treg-like cells. We expect that TGF-β associated with natural Treg cells will facilitate the conversion of peripheral Treg cells.

In summary, we have provided evidence that feline $CD4^+CD25^-$ T cells can be converted to suppressor T cells and that TGF-β1 interaction with TGF-βRII is an essential component for the development of suppressor function. Our current work demonstrates that Con A plus TGF-β1

not only converts $CD4^+CD25^-$ T cells to suppressor T cells, but also induces a phenotype similar to natural Treg cells including CD25 and TGF-β surface expression and expression of the transcription factor FoxP3. Peripheral generation of Treg cells may contribute to the imbalance of the Treg to Th ratio and the eventual immunosuppression seen in chronic Lentiviral infections, such as FIV and HIV. Therefore, therapeutically manipulating the hosts Treg response to FIV/HIV may lead to a means of controlling or eliminating the virus from the host.

## MATERIALS AND METHODS

### *Cats*

Specific pathogen-free cats were obtained from Liberty Labs (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA) and housed at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Cats were inoculated with the $NCSU_1$ isolate of FIV, a pathogenic clade A virus (English, Nelson et al. 1994), as described by Bucci et al (Bucci, English et al. 1998). FIV-infection was confirmed by immunoblot analysis and provirus detection by PCR using primers specific for the FIV-p24 GAG sequence. At the time samples were taken, cats had been infected with FIV for at least 5 years and were clinically asymptomatic. Uninfected control cats ranged in age from 3 to 6 years and were housed separately from FIV-infected cats.

*Sample Collection and Preparation*

Whole blood was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). Subsequently, PBMC were isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as previously described (Tompkins, Ogilvie et al. 1987). Single-cell suspensions were prepared from LN obtained from biopsies by gently and repeatedly injecting sterile PBS into the tissue using 18G needle until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion. Viability was always >90%.

*Reagents and antibodies*

Recombinant human IL-2 were was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Maurice Gately, Hoffmann - La Roche Inc. LPS and Concanavalin A (Con A) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IgG coated magnetic Dynabeads® M-450 were purchased from Dynal (Great Neck, NY). Streptavidin–PerCP was purchased from BD Biosciences PharMingen (San Diego, CA). Anti-TGF-β1 (MAB240) was purchased from R&D Systems (Minneapolis, MN) and conjugated to allophycocyanin (APC), PE-conjugated anti-TGF-βRII (FAB241P), anti-TGF-βRII (AF-241-NA) and recombinant human TGF-β1 (240-B) were also purchased from R&D Systems. Mouse anti-feline CD25 (mAb 9F23) was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan). Anti-CD21 was purchased from P. Moore, (University of California, Davis). Mouse anti-feline CD4 (mAb 30A) and CD8 (mAb 3.357) (Tompkins, Gebhard et al. 1990) were developed in our lab.

*Flow cytometric analysis*

At least $5x10^5$ PBMC were stained for surface expression of CD25, CD4, and TGF-β1 using FITC conjugated anti-CD25, biotin-conjugated anti-CD4, and APC-conjugated anti-TGF-β1 mAbs. T cell preparations were stained in 5% FBS/PBS for 30 min on ice. When necessary, T cells also were washed and incubated with streptavidin PerCP secondary antibodies for 30 min on ice. Flow cytometry data were acquired using 3-color flow cytometry and analyzed (FACSCalibur; CellQuest software; respectively, BD Biosciences, Mountain View, CA). Lymphocytes were gated based on forward vs side scatter, and 20,000 gated events were acquired and stored list-mode fashion for analysis using CellQuest software.

*Purification of T cells*

For FACS purification, LN cells were stained with anti-CD4 biotin/streptavidin PerCP and anti-CD25 FITC; $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets were purified using a high-speed high-purity fluorescence activated cell sorter (MoFlo, DakoCytomation). The purity of FACS sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ cell populations was always > 95%.

$CD4^+$ $CD25^-$ T cells for use as target cells in proliferation assays were enriched using biomagnetic bead separation performed using goat anti-mouse IgG-coated beads as described by Bucci et al. (Bucci, English et al. 1998). Briefly, $CD21^+$ B cells, $CD8^+$ and $CD25^+$ T cells were depleted in successive steps using magnetic beads coated with anti-CD21, anti-CD8 and anti-CD25 antibody respectively. Purity of the magnetic bead enriched $CD4^+CD25^-$ T cells was > 90%, as verified by flow cytometric analysis.

*Reverse Transcription PCR Analysis*

Total RNA was isolated from $2x10^{6}$-$5x10^{6}$ $CD4^{+}CD25^{+}$ or $CD4^{+}CD25^{-}$ T cells using RNeasy Protect Mini Kit (Qiagen, Valencia, CA) samples were treated on column with RNAse free DNAse to eliminate contaminating DNA. Reverse transcription was carried out using (reverse transcription system kit from Promega) as per the manufacturer's protocol, followed by PCR using HotStar Taq polymerase. FoxP3 message was detected using feline specific primers. GAPDH expression was determined and used as a normalizing control. PCR consisted of a 15 min 95 °C denaturing step followed by 35 cycles of 1min at 94 °C, 45 s at 60 °C, and 45 s at 72 °C with a final 5 min at 72 °C extension. Primers used were as follows:

FoxP3, 5'-ATTTCATGCACCAGCTCTCAACGG-3' and 5'-ACCATCTTCCTGGATGAGAAGGGCA-3';

GAPDH, 5'-CCTTCATTGACCTCAACTCCAT-3' and 5'-GGTCATCCATGACCACTTCGG-3'.

*In vitro T cell suppression assay*

Enriched $CD4^{+}CD25^{-}$ target cells ($10^{6}$ cells/ml) were stimulated for 4 h with 5 µg/ml Con A, washed twice in RPMI 1640, and plated at 5 x $10^{4}$ viable cells/well in 96-well U bottom plates. $CD4^{+}$ effector cells were then added to the wells at various E:T ratios ranging from 0.125:1 to 1:1. $CD4^{+}CD25^{+}$ effector cells were added as: 1) freshly isolated, untreated cells, 2) after treatment in 24-well plates at $1x10^{6}$ cells/ml for 4 days with 10 µg/ml LPS and 100 U/ml rhIL-2, or 3) after conversion from $CD4^{+}CD25^{-}$ T cells by culture in 24-well plates at $1x10^{6}$ cells/ml for 4 days with 10 ng/mL rhTGF-β1 and 5µg/mL Con A. The cells were then washed, counted, and added to the target cells. Assays were run in triplicate. Effector and target cells were cocultured

for 72 hrs and pulsed with 1 μCi of [3H]TdR/well for the last 18 h and harvested using a Filtermake Harvester (Packard Bioscience, Meriden, CT). [$^3$H]thymidine incorporation was measured using a Top Count NXT Microplate scintillation counter (Packard Bioscience). Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ target cells alone and calculated as follows:

percent inhibition = {[($CD4^+CD25^-$ alone - $CD4^+CD25^+$) cpm]/[($CD4^+CD25^-$ alone) cpm]} x100.

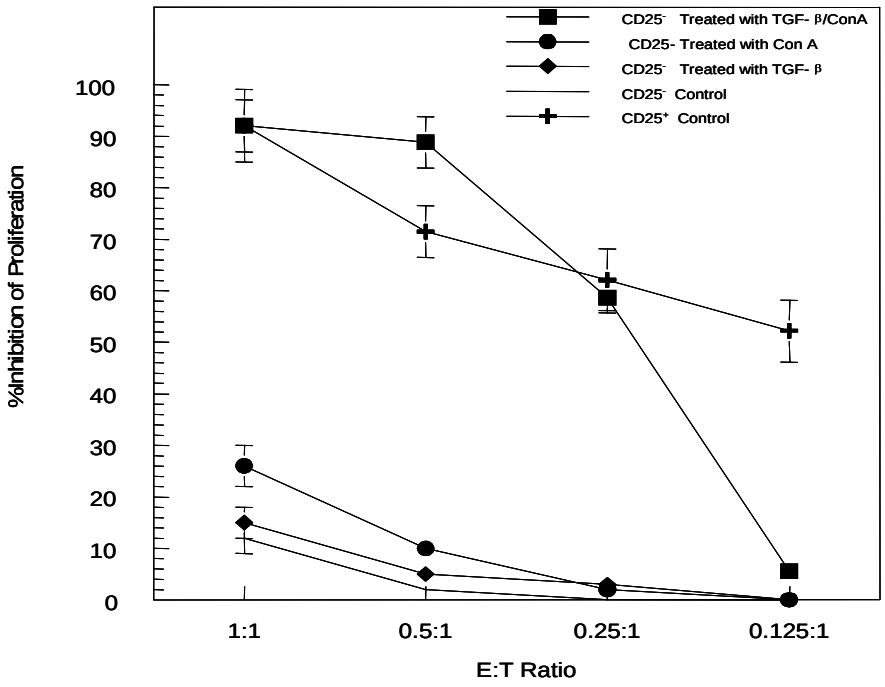
*Statistical analysis*

The Mann-Whitney U test (t test-like for nonparametric data) was used for pairwise comparison of surface molecule expression. Differences were considered to be significant at $p < 0.01$.

**FIGURE 1 TGF-β Treatment of $CD4^+CD25^-$ T cells induces regulatory function.** FACS purified PLN $CD4^+CD25^-$ T cells (2 x $10^6$) from both control and FIV-infected cats were cultured at 37 °C with 5% $CO_2$ for 4 days in medium containing IL-2 (10 U/mL) and supplemented with TGF-β1 (10 ng/mL) plus Con A (5 μg/mL) ■, Con A (5 μg/mL) alone ●, TGF-β (10 ng/mL) alone ♦, or unsupplemented —. After 4 days, cells were washed extensively and plated in 96-well U bottom plates at the indicated ratios with 1x$10^5$ with heterologous magnetic bead enriched $CD4^+CD25^-$ T cells from PBMC that were activated with Con A for 4 h and washed before plating. Effector and target cells were cocultured for 3 days and pulsed with 1 μCi of [3H]TdR/well for the last 18 h. Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ target cells alone and calculated as follows:
percent inhibition = {[($CD4^+CD25^-$ alone - $CD4^+CD25^+$) cpm]/[($CD4^+CD25^-$ alone) cpm]} x100. Assays were run in triplicate.

TGF-β converted cells from both control and FIV-infected cats were able to suppress Con A-stimulated expansion of $CD4^+CD25^-$ T cells in a dose-dependent manner that closely resembles unstimulated $CD4^+CD25^+$ Treg cells from FIV-infected cats and LPS/IL-2 stimulated Treg cells from control cats. Cells supplemented with IL-2 only, Con A only or TGF-β1 only were unable to suppress the proliferation of mitogen stimulated $CD4^+CD25^-$ T cells at any ratio tested. Bars represent the mean ± standard deviation of three separate experiments.

FIV-infected Cat




Control Cat

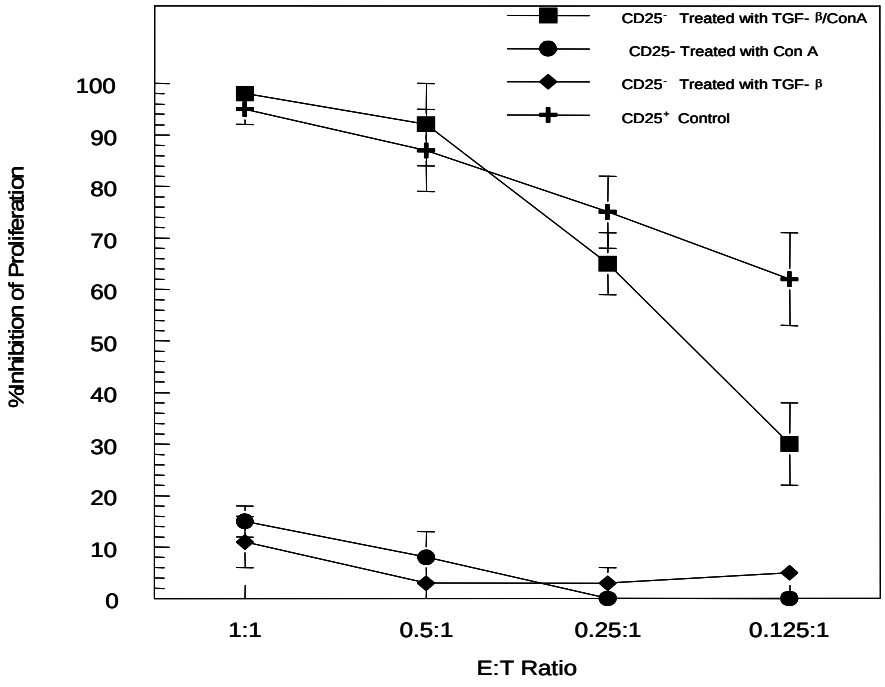
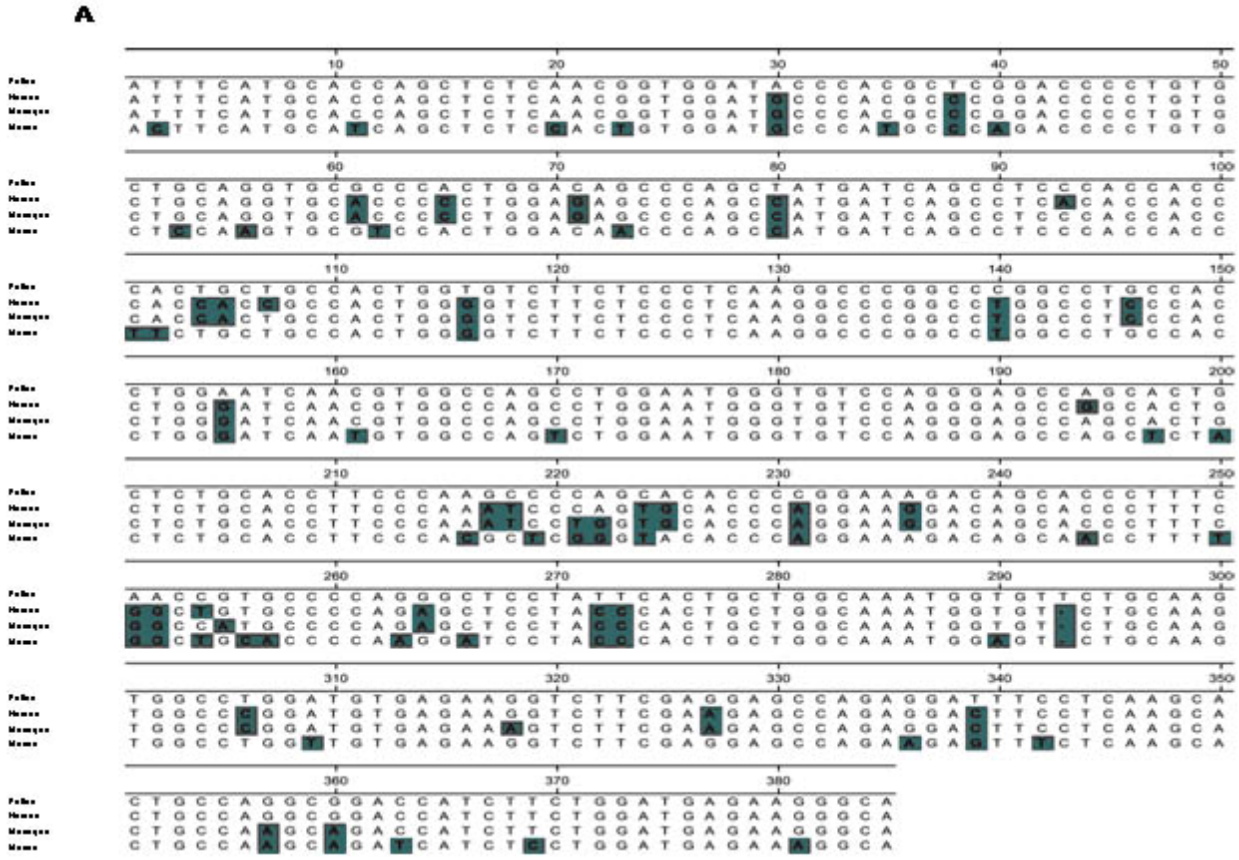


**Figure 2 Feline FoxP3 is highly homologous to human, macaque and mouse FoxP3.** (A) Sequence alignment of FoxP3 top to bottom feline, human, macaque, and mouse. RNA was isolated from 5 x $10^6$ MYA-1 cells and cDNA was prepared. Using primers designed from the human FoxP3 sequence, a region was amplified from the feline MYA-1 cDNA and submitted for sequence analysis. A sequence alignment with FoxP3 sequences from human, macaque, and mouse was done using DNAStar MegAlign. Shaded residues shown are substitutions when compared to the feline FoxP3 sequence. (B) High percent identity and low divergence of the sequences was found using Lasergene's DNAStar. Percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign. Percent Similarity compares sequences directly, without accounting for phylogenetic relationships. (C) **Purified feline $CD4^+$ T cell subsets express FoxP3 differentially.** Single cell suspensions of feline peripheral lymph nodes (PLN) were FACS sorted into $CD4^+CD25^-$ and $CD4^+CD25^+$ populations with > 98% purity as verified by flow cytometric analysis. RNA was then extracted from the purified populations and then reverse-transcribed as described in Materials and Methods. PCR was carried out on the resulting cDNA using primers designed based on the published human sequence (748 bp) and feline specific primers (385 bp). Expression of FoxP3 in freshly isolated $CD4^+CD25^+$ T cells was significantly greater (lane 3, human) and (lane 5, feline) than in freshly isolated $CD4^+CD25^-$ T cells (lane 2, human) and (lane 4, feline) using both human and feline specific primers. Lane 1 shows 100 bp ladder for comparison of product sizes.




B

| Percent Identity | | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | | |
| 1 | | 90.1 | 90.1 | 84.4 | 1 | Feline |
| 2 | 8.4 | | 97.4 | 84.9 | 2 | Human |
| 3 | 9.0 | 2.7 | | 85.9 | 3 | Macaque |
| 4 | 14.2 | 15.2 | 14.2 | | 4 | Mouse |
| | 1 | 2 | 3 | 4 | | |

Divergence

Feline Human Macaque Mouse

C

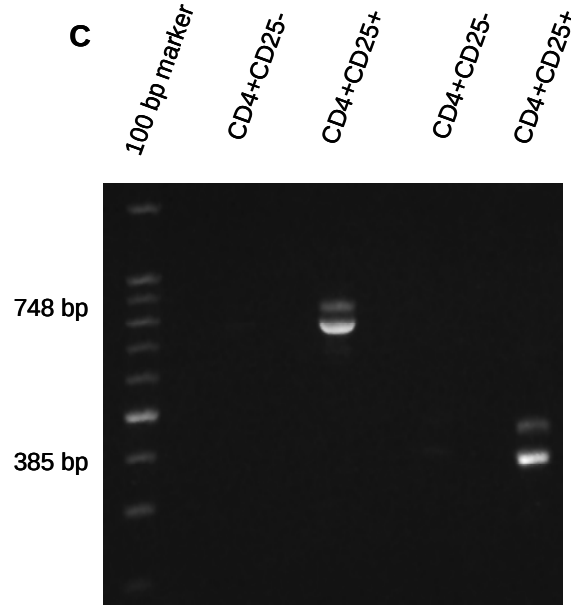
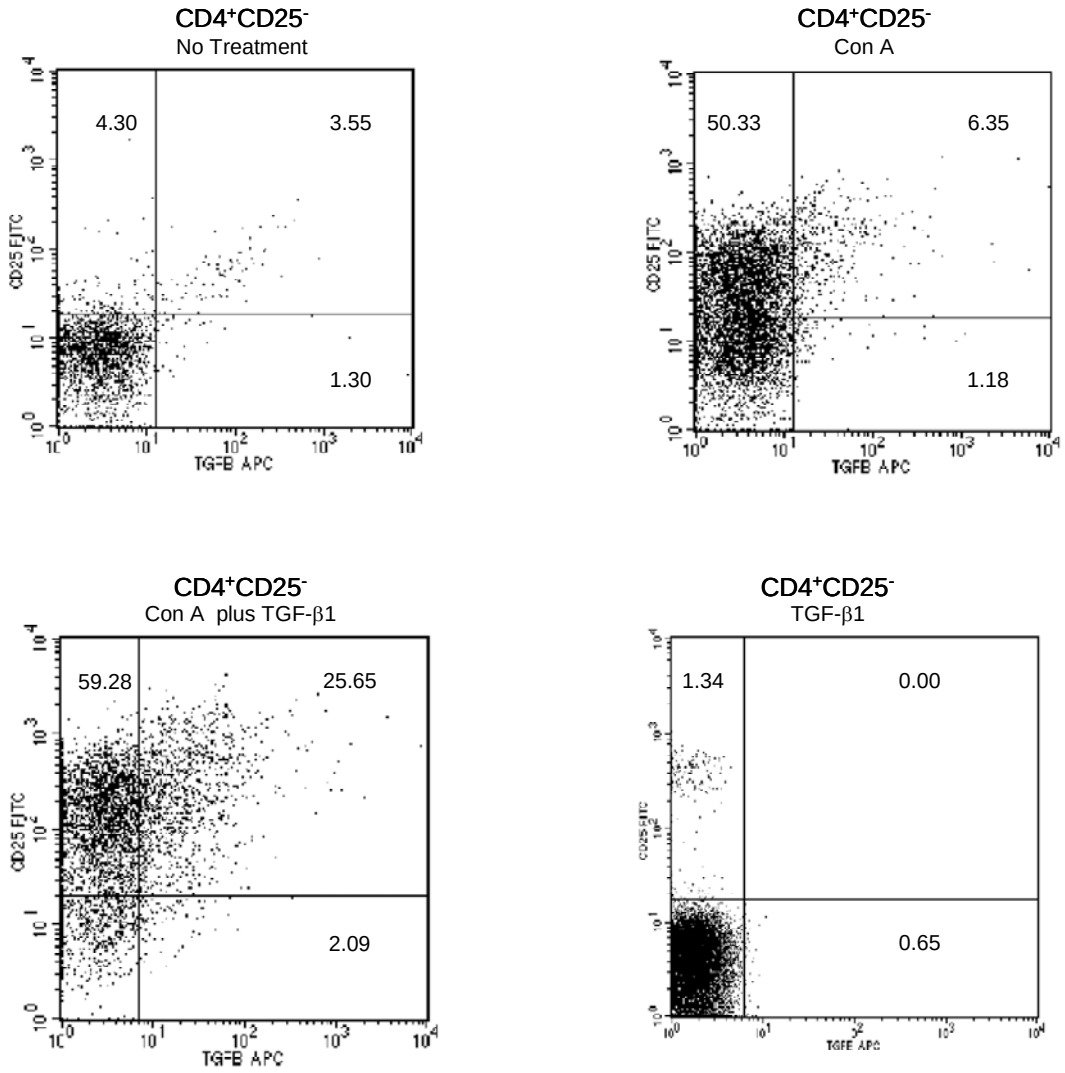
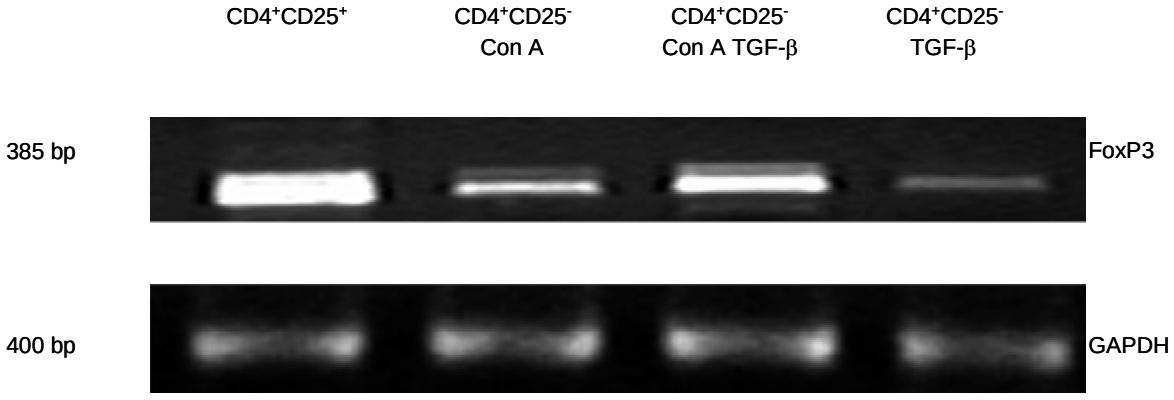


**FIGURE 3 TGF-β plus Con A treatment of $CD4^+CD25^-$ T cells induces surface CD25 and TGF-β and FoxP3 mRNA expression.** FACS purified PLN $CD4^+CD25^-$ T cells from normal cats were plated for 48 hours at a concentration of $2x10^6$/mL in medium alone, supplemented with Con A (5 μg/mL), supplemented with TGF-β1 (10 ng/mL), or Con A (5 μg/mL) plus TGF-β1 (10 ng/mL). (A) The cultured cells were then washed and stained for surface expression of CD4, CD25, and TGF-β as described in Materials and methods. Dot plots of flow cytometric analysis of $CD4^+$ gated cells from a representative experiment shows upregulation of CD25 after Con A (56.68%) or Con A plus TGF-β1 (84.93%) stimulation. Surface TGF-β1 was upregulated only after Con A plus TGF-β1 treatment and only on the $CD4^+CD25^+$ cells. (B) RNA was also isolated from the washed 48 h cultures and reverse transcribed. PCR analysis of the cDNA revealed expression of FoxP3 mRNA in freshly isolated purified PLN $CD4^+CD25^+$ T cells (lane 1) using fFoxP3 specific primers. In addition FoxP3 expression was significantly up-regulated in purified PLN $CD4^+CD25^-$ cells treated with TGF-β plus Con A ( lane 3) to levels near that seen in freshly isolated $CD4^+CD25^+$ (lane 1). A slight up-regulation of FoxP3 mRNA expression was seen after treatment with Con A only (lane 2), while TGF-β alone had no effect on FoxP3 mRNA expression (lane 4). GAPDH mRNA expression was determined using feline specific primers and used as a normalizing control.

**A**


CD4+CD25-
No Treatment
4.30
3.55
1.30
CD25 FITC
TGFB APC
CD4+CD25-
Con A
50.33
6.35
1.18
CD4+CD25-
Con A plus TGF-β1
59.28
25.65
2.09
CD4+CD25-
TGF-β1
1.34
0.00
0.65

**B**


CD4+CD25+
CD4+CD25-
Con A
CD4+CD25-
Con A TGF-β
CD4+CD25-
TGF-β
385 bp
FoxP3
400 bp
GAPDH

**A** **B** **C**

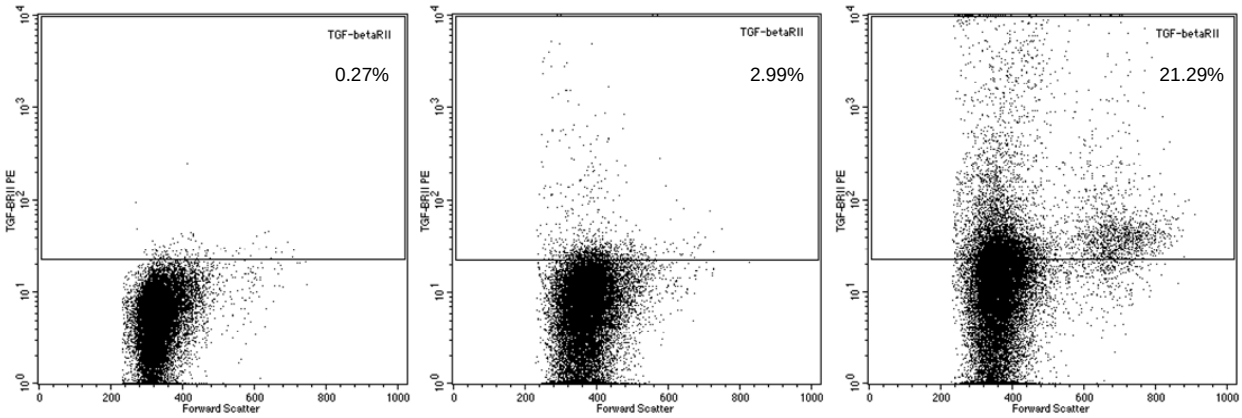



**Figure 4 CD4$^+$CD25$^-$ T cells express TGF-βRII on activation.** FACS purified CD4$^+$CD25$^-$ T cells (4 x $10^6$) were stimulated with 5 μg/mL Con A for 24 h and then thoroughly washed. Cells were then stained with PE conjugated anti-TGF-βRII mAb for 30 min on ice, then washed and analyzed for surface expression of TGF-βRII by flow cytometry. The dot plots show Con A stimulation of CD4$^+$CD25$^-$ T cells induces significant TGF-βRII surface expression (21.29%, MFI=339) (C) when compared to cells cultured in medium without the addition of Con A (2.99%, MFI=234) (B). TGF-βRII expression on freshly isolated CD4$^+$CD25$^-$ T cells is shown in (A).

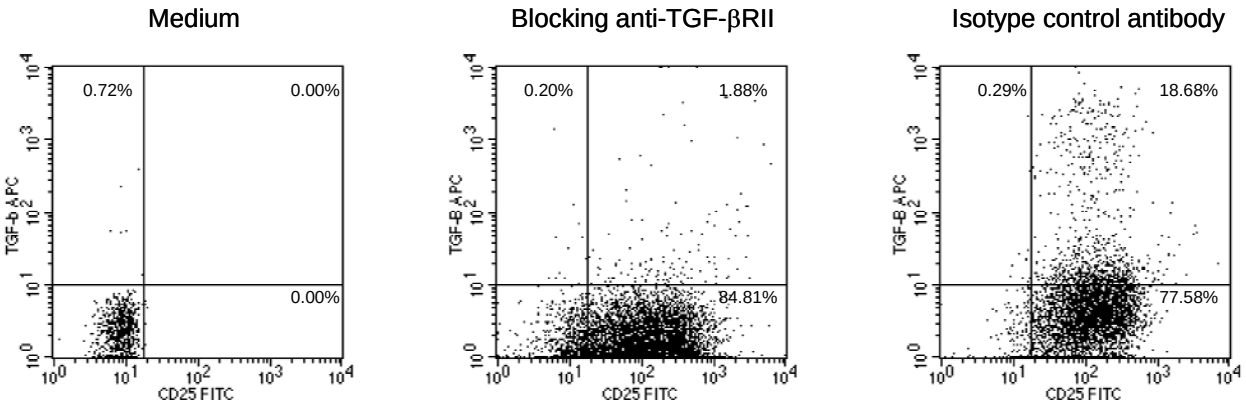



**Figure 5 Blockade of TGF-βRII on activated $CD4^+CD25^-$ T cells inhibits the surface TGF-β expression on TGF-β treated cells.** FACS purified $CD4^+CD25^-$ T cells ($4 \times 10^6$) were stimulated with 5 μg/mL Con A for 18 h and then thoroughly washed. Subsequently, ($2 \times 10^6$) cells were incubated with or without 50 μg/mL anti-TGF-βRII mAb for 30 min prior to the addition of 10 ng/mL rhTGF-β1. After 48 h of culture, cells were washed and stained for surface expression of TGF-β1 and CD25 using APC labeled anti-TGF-β1 and FITC labeled anti-CD25 then analyzed by flow cytometry. Results shown in the dot plots above, revealed that anti-TGF-βRII mAb treatment before addition of TGF-β1 inhibited the expression of surface TGF-β1(1.88%, MFI=505) when compared to cells without anti-TGF-βRII treatment (18.68%, MFI=368). In addition essentially all $TGF\text{-}\beta^+$ cells were also $CD25^+$.

**Figure 6 Blockade of TGF-βRII before conversion abrogates the acquisition of regulatory function while blockade of TGF-β1 after conversion inhibits suppressor function.**

(A) FACS purified $CD4^+CD25^-$ T cells (4 x $10^6$) from pooled PLN of normal cats were stimulated with 5 μg/mL Con A for 4 h and then thoroughly washed. Subsequently, (2 x $10^6$) cells were incubated with or without 50 μg/mL anti TGF-βRII mAb for 30 min prior to the addition of 10 ng/mL rhTGF-β1. After 48 hrs, effector cells were washed extensively and plated in 96-well U bottom plates at a 0.5:1 ratio with 1x$10^5$ heterologous target $CD4^+CD25^-$ T cells FACS purified from PBMC that were previously stimulated with 5 μg/mL Con A for 4 h and washed before plating. Effector and target cells were cocultured for 3 days and pulsed with 1 μCi of [3H]TdR/well for the last 18 h. Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ target cells alone and calculated as follows: percent inhibition ={[(CD4+CD25- alone - CD4+CD25+) cpm]/[(CD4+CD25- alone) cpm]} x100.

(B) FACS purified $CD4^+CD25^-$ T cells (4 x $10^6$) were stimulated with 5 μg/mL Con A plus 10 ng/mL rhTGF-β1 for 48 h and then thoroughly washed. Converted cells (2 x $10^6$) were then incubated with or without 100 μg/mL anti TGF-β1 mAb for 30 min prior to being used as effector cells in a [$^3$H]TdR suppressor assay as described above. Data shows that treatment of Con A activated $CD4^+CD25^-$ T cells with anti-TGF-βRII before addition of rhTGF-β1 abrogated conversion to functional suppressor cells as determined by a standard [$^3$H]TdR T cell suppressor assay and correlates with inhibition of induction of surface TGF-β1. In addition, when anti-TGFβ1 mAb was added after the 48 h TGF-β1 plus Con A conversion of $CD4^+CD25^-$ T cells,

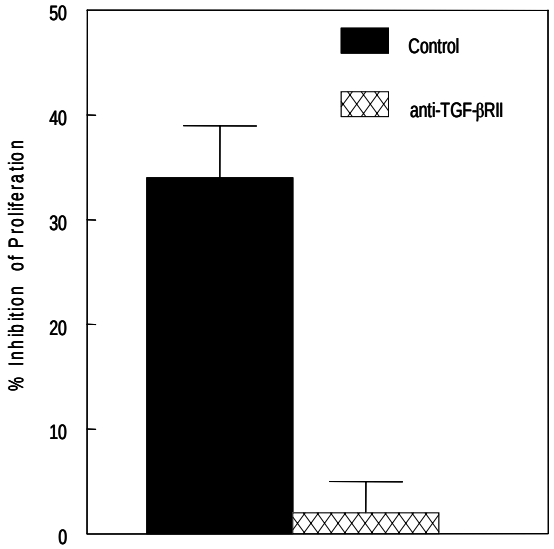
suppressor function of the converted cells was completely abrogated compared to converted cells without addition of TGF-β1 blocking antibody.

(Figure 6 continued)

(C) Freshly FACS purified natural $CD4^+CD25^+$ Treg cells from an FIV-infected cat were incubated with or without 100 μg/mL anti TGF-β1 mAb for 30 min prior to being used as effector cells in a [$^3$H]TdR suppressor assay as described in (A). Natural $CD4^+CD25^+$ Treg cell suppressor function is inhibited bytreatment with blocking anti-TGF-β1 mAb prior to suppressor assay coculture when compared to untreated control Treg cells.

Bars represent the mean ± standard deviation of three separate experiments.

A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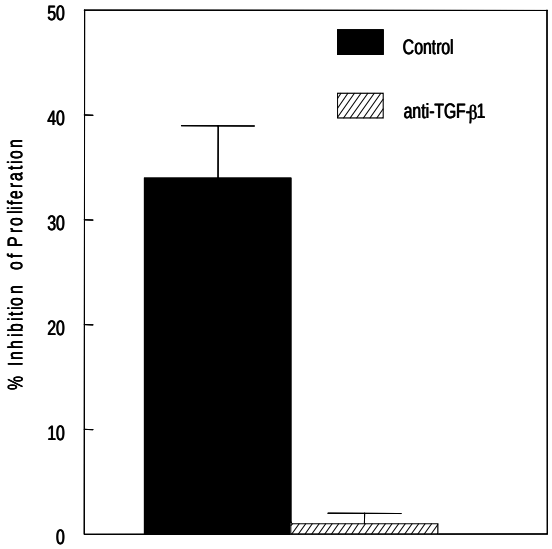
40
30
20
10
0
% Inhibition of Proliferation
Control
anti-TGF-βRII

B


50
40
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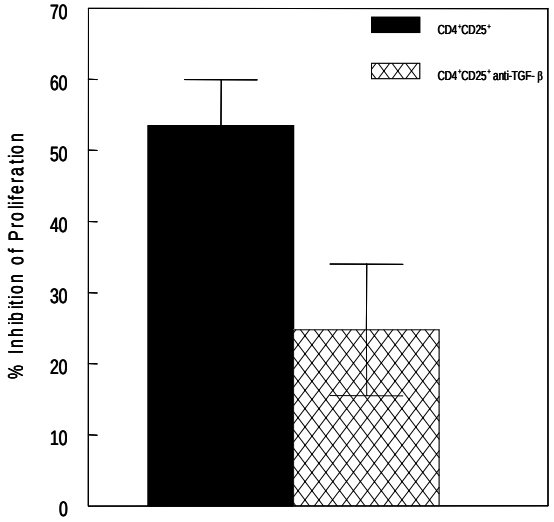
10
0
% Inhibition of Proliferation
Control
anti-TGF-β1

C


70
60
50
40
30
20
10
0
% Inhibition of Proliferation
CD4+CD25+
CD4+CD25+ anti-TGF- β

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# 4. CHAPTER-2

# $CD4^{+}CD25^{+}$ T regulatory cells from feline AIDS Lentivirus infection are chronically activated in vivo and capable of converting $CD4^{+}CD25^{-}$ T cells to phenotypic and functional Treg cells via the TGF-β/TGF-βR signaling pathway

## ABSTRACT

Suppression of $CD4^+CD25^-$ T helper cell proliferation by $CD4^+CD25^+$ T regulatory (Treg) cells is crucial for both maintenance of peripheral tolerance to self-antigens and control of immune responses to foreign antigen. Naturally occurring, thymic-derived $CD4^+CD25^+$ Treg cells, as well as pathogen-induced Treg cells, are phenotypically defined by constitutive expression of CD25, the IL-2Rα-chain, GITR, CTLA-4, and when activated express biologically active TGF-β1 on their surface. Treg cells selectively express FoxP3, a regulatory transcription factor, which is essential for homeostasis and suppressor function. $CD4^+CD25^-$ T helper (Th) cells can be induced to attain Treg characteristics in vitro by TGF-β in combination with TCR engagement, suggesting a mechanism of Treg peripheral homeostasis. Given that Treg cells express cell surface TGF-β when activated, we asked whether they could convert Th cells to Treg-like cells. We found that in vitro activated Treg cells purified from lymph node (LN) biopsies of normal cats can convert $CD4^+CD25^-$ Th cells to TGF-$\beta^+$ $FoxP3^+$ immunosuppressive Treg cells. As PBMC or LN Treg cells from FIV-infected cats are chronically activated in vivo and display cell surface TGF-β, we asked if Treg cells from FIV-infected cats could recruit additional Treg cells from the T helper pool in the absence of in vitro stimulation. Our results show that freshly isolated FACS purified Treg cells from chronically FIV-infected cats but not control cats convert $CD4^+CD25^-$ T cells to anergic, immunosuppressive $CD4^+CD25^+$TGF-$\beta^+$ Treg cells. Furthermore, we found the $CD4^+CD25^-$ Th conversion to a Treg phenotype was

mediated by a TGF-β-dependent mechanism, as either anti-TGF-β1 or anti-TGF-βRII abrogated the ability of Treg cells to convert Th cells.

Our results support the hypothesis that peripheral conversion of Th cells by Treg cells is a normal immune function that may play a role in maintaining Treg cell homeostasis. Further, our data suggest that chronic antigenemia associated with Lentivirus infections may persistently activate Treg cells and subvert normal Treg homeostasis by overriding a Treg "shut-off" signal. Chronic activation and expansion of pathogen-induced Treg cells could lead to unregulated conversion of Th cells to the Treg phenotype and a progressive loss of $CD4^+$ Th and $CD8^+$ effector cell numbers and/or function and ultimately the development of immunodeficiency, the hallmark of AIDS Lentivirus infections.

# INTRODUCTION

Thymic-derived $CD4^+CD25^+$ natural T regulatory (Treg) cells are phenotypically defined by constitutive surface expression of CD25, the IL-2Rα-chain, GITR, CTLA-4, and FoxP3 mRNA expression (Baecher-Allan, Brown et al. 2003). In addition, when $CD4^+CD25^+$ Treg cells are activated they express biologically active TGF-β on their surface (Chen and Wahl 2003). It is now well established that Treg cells, once activated, play a major role in modulating immune responses to a variety of infectious agents (Suvas, Kumaraguru et al. 2003; Rouse and Suvas 2004; Bach 2005; Chattopadhyay, Mehrotra et al. 2006; Pelletier, Transue et al. 2006). However, whether pathogen-induced Treg cells are derived from natural Treg cells generated in the thymus and specific for self-antigen, or are peripherally generated Treg, or both has yet to be elucidated. Treg cells act to maintain the homeostasis of circulating peripheral lymphocytes by controlling the expansion of self-antigen as well as pathogen-activated lymphocytes. In order to manage homeostasis there must be strict control over self-reactive T cells while allowing for an initial expansion of pathogen-induced Th cells in order to control infections followed by a Treg mediated weaning of the pathogenic response. Although there is probably some degree of TCR specificity cross reaction in self antigen specific thymic-derived natural Treg cells that could account for some Treg suppressor function to pathogenic responsive Th cells, more likely a specific TCR interaction would be required to effectively squelch the response. As the kinetics of pathogen specific peripheral Treg cell generation would provide time for a Th response to the particular infectious agent, this model better fits with cell mediated response to pathogens.

Peripheral Treg homeostasis, according to recent studies, is independent of thymic involvement, although a mechanism has yet to be defined. Evidence suggests that Treg cells, by expressing surface TGFβ, at least have a mechanism that would allow them to convert Th cells into Treg-like cells in rodents (Chen, Jin et al. 2003), and humans (Wahl and Chen 2005). Chen et al. (Chen, Jin et al. 2003) reported that TCR-engaged $CD4^+CD25^-$ T cells stimulated with TGF-β1 converted to a Treg phenotype. Jonuleit and Dieckmann have reported that purified $CD4^+CD25^+$ T cells anergize both purified $CD4^+CD25^-$ and $CD8^+CD25^-$ T cells when co-cultured (Jonuleit, Schmitt et al. 2002; Dieckmann, Plottner et al. 2005). Further, in addition to anergy, suppressor function is induced in $CD4^+CD25^-$ Th cells but not $CD8^+$ cells by $CD4^+CD25^+$ Treg cells. For this to occur both Treg cells and pre-target Th cells must be activated through the TCR or by a mitogen (Dieckmann, Plottner et al. 2005). We previously reported that feline $CD4^+CD25^-$ T cells could be induced to Treg phenotype (surface CD25 and TGF-β and FoxP3 mRNA expression) and function by immunosuppressor stimulation with Con A plus TGF-β1 (Petty, Chapter 1, Emani 2006). Further, we demonstrated that ConA treatment up-regulates TGF-βRII on $CD4^+CD25^-$ cells, and addition of either anti-TGFβ1 or anti-TGF-β-RII blocked their conversion to a Treg phenotype (Petty, Chapter 1) suggesting a role for TGF-β1/TGF-βRII signaling in this conversion process. These data collectively indicate that TGF-$\beta^+$ Treg cells, once activated could recruit additional Treg cells from $CD4^+CD25^+$ T cell pool. For this to happen, both the $CD4^+CD25^+$ Treg cell and the $CD4^+CD25^-$ target cells must be activated. Both of these conditions appear to be met in FIV-infected cats (Joshi, Garg et al. 2005) and HIV-infected humans (Orendi, Bloem et al. 1998; Cohen Stuart, Hazebergh et al. 2000; Deeks,

Kitchen et al. 2004). First, $CD4^+CD25^+$ Treg cells from FIV-infected hosts are chronically activated in vivo as evidenced by potent immunosuppressor function (Vahlenkamp, Tompkins et al. 2004) and up-regulation of TGF-β1 on their surface. Secondly, there is ample evidence of $CD4^+$ and $CD8^+$ T cell immune hyperactivation in chronic FIV infection (Gebhard, Dow et al. 1999; Tompkins, Bull et al. 2002; Shimojima, Nishimura et al. 2003; Bull, Vahlenkamp et al. 2004) and activation significantly induces expression of cell surface TGF-βRII (Petty, Chapter 1). We have demonstrated that in FIV-infected cats $CD4^+CD25^+$ Treg cells express TGF-β1 on their surface. This set of experiments will test the hypothesis that activated Treg cells in FIV-infected cats maintain homeostasis and expand with disease progression by conversion of activated $CD4^+$ Th cells into Treg cells. In both HIV and FIV infections, there is an early loss in $CD4^+$ and $CD8^+$ T cell immune function (Imami and Gotch 2002; Piazza, Fan et al. 2002). Data suggest that in untreated primary HIV infection or in patients where HAART is delayed, $CD4^+$ Th cell responses to HIV antigens develop early, as measured by lymphocyte proliferative responses, but are prematurely lost during the acute stage infection . This loss of proliferative response to HIV-specific epitopes is long lasting, as evidenced by similar HIV-epitope specific immune deficiency in long-term progressor patients with established infections (Reddy, Englard et al. 1987; Wahren, Morfeldt-Mansson et al. 1987; Kelker, Seidlin et al. 1992). Also, this $CD4^+$ T cell immunodeficiency is specific to HIV antigens, as $CD4^+$ T cells from these patients proliferate normally to other antigens such as PPD, TT and CMV (Reddy, Englard et al. 1987; Wahren, Morfeldt-Mansson et al. 1987; Kelker, Seidlin et al. 1992). However, these HIV-specific T cell clones appear not to be deleted, as $CD4^+$ and $CD8^+$ T cells from immunodeficient patients will produce IFN-γ in response to HIV peptide stimulation, despite the fact that there is

no proliferative response to peptide-APC stimulation (Caruso, Canaris et al. 1995; Boritz, Palmer et al. 2004). A role for $CD4^+CD25^+$ Treg cells on this $CD4^+$ Th and $CD8^+$ anergy to HIV peptide stimulation is implied by the observation that depletion of $CD4^+CD25^+$ T cells from PBMC from $HIV^+$ patients increases a cytokine response to HIV antigen stimulation in vitro (Aandahl, Michaelsson et al. 2004; Eggena, Barugahare et al. 2005). Collectively these data suggest that the mechanism(s) responsible for perturbation of HIV specific T cell responses during acute stage infection may involve epitope specific clonal anergy rather than clonal deletion, and this anergy may be mediated by activated Treg cells. Firstly, despite the fact that HIV-specific anergy is induced in both $CD4^+$ Th and $CD8^+$ immune compartments, paradoxically there is a generalized state of T cell immune hyperactivation (Sousa, Carneiro et al. 2002; Hazenberg, Otto et al. 2003; Eggena, Barugahare et al. 2005). A similar T cell hyperactivation occurs in FIV-infected cats as manifested by a progressive conversion of naïve $CD4^+$ and $CD8^+$ T cells to an activation phenotype with advancement of disease (Gebhard, Dow et al. 1999; Tompkins, Bull et al. 2002). As T cell hyperactivation in FIV and HIV infection is a strong predictor of $CD4^+$ T cell depletion and disease progression, activated Treg cells could suppress T cell immune activation and expansion. From the above discussion, it is possible that activated TGF-$\beta^+$ Treg cells in FIV/HIV infections could not only anergize antigen activated $CD4^+$ Th cells but convert them to a functional Treg phenotype, thus increasing the Treg pool at the expense of the Th pool and contributing to the immunodeficiency. Importantly in the case of FIV/HIV infection, as $CD4^+CD25^+$ Treg cells from Lentiviral AIDS-infected subjects display a constitutive activation phenotype which could include up-regulated surface TGF-β1, we hypothesized they may mediate peripheral conversion to a Treg-like phenotype. As both viruses preferentially and

productively infect $CD4^+CD25^+$ T cells and $CD4^+CD25^+$ Treg cells display a constitutive activation phenotype, peripheral generation of activated Treg-like cells could provide an expanded long-lived reservoir for Lentiviral replication.

To investigate questions regarding the generation of peripheral $CD4^+CD25^+$ T regulatory cells during FIV infection, specifically the TGF-β1/TGF-βRII-mediated $CD4^+CD25^+$ Treg cell conversion of peripheral $CD4^+CD25^-$ T cells to a Treg-like phenotype, we developed an in vitro model using heterologous activated membrane labeled Treg cells in a co-culture with unlabeled activated $CD4^+CD25^-$ T cells from cats. Suppressor function and Treg phenotype were induced in the $CD4^+CD25^-$ T cells in this system after 5 days of co-culture with activated $CD4^+CD25^+$ Treg cells. These results further support the hypothesis that peripheral Treg cells are generated during Lentiviral infection and that this may lead to reduction of Th cell numbers, inversion in the Treg: Th cell ratio and therefore contribute to the immune deficiency seen in Lentiviral infection.

## RESULTS

### Increased $CD4^+CD25^+$ T cells in FIV infected cats

Blood was collected in EDTA from both FIV-infected and SPF control cats by venipuncture. After isolation by density gradient centrifugation, PBMC were stained for CD4 and CD25 using feline monoclonal antibodies. Analysis of data collected by two color flow cytometry showed a significant increase in the percentage of the total CD4 T cells that expressed CD25 from PBMC

of chronically FIV-infected cats when compared to age matched SPF control cats (Fig. 1a). However, when CD25 expression on CD4 T cells was calculated as a percentage of total gated lymphocytes, there was a slight but not significant decrease in $CD4^+CD25^+$ T cells (Fig. 1a). As the $CD4^+CD25^+$:$CD4^+CD25^-$ ratio may be a better indicator of Lentiviral AIDS progression, we also analyzed our data to include this ratio. We found that chronic FIV-infection significantly increased the $CD4^+CD25^+$:$CD4^+CD25^-$ ratio when compared to SPF control cats (Fig. 1a), suggesting that chronic Lentiviral infection leads to a preferential loss or anergizing of Th cells. It is also possible that $CD4^+CD25^-$ T cells are being recruited to the Treg pool thereby contributing to the maintenance of Treg numbers and immune suppression of the Th cells while playing a key role in the observed inverted $CD4^+CD25^+$:$CD4^+CD25^-$ ratio.

**Increased Expression of TGF-β on $CD4^+CD25^+$ T cells from FIV infected cats**

As we observed an imbalance in the $CD4^+CD25^+$:$CD4^+CD25^-$ ratio of chronically-infected cats that could be due to recruitment of $CD4^+CD25^-$ to the Treg pool, we explored the possibility that $CD4^+CD25^-$ were being converted to $CD4^+CD25^+$ Treg-like cells in vivo. To address this possibility we initially explored the expression of surface TGF-β1, as a mediator of conversion, on $CD4^+CD25^+$ Treg cells from both chronically FIV-infected and SFP control cats. This was an important first step as we have previously demonstrated the requirement for TGF-β1 and ConA activation of $CD4^+CD25^-$ T cells for in vitro conversion of $CD4^+CD25^-$ Th cells to $CD4^+CD25^+$ Treg-like cells. PBMC were isolated from 6 SPF control and 6 chronically FIV-infected cats by density centrifugation. Cells were then stained with feline anti -CD4, -CD25, and human anti-TGF-β1 antibodies and data were collected by three color flow cytometry. Analysis of

$CD4^+CD25^-$ and $CD4^+CD25^+$ T cell subsets from SPF control cats showed no significant difference in surface TGF-β1 expression on $CD4^+CD25^+$ T cells when compared to $CD4^+CD25^-$ T cells from control cats (Fig. 2a). However, surface TGF-β1 was significantly up-regulated on $CD4^+CD25^+$ T cells from FIV-infected cats, which correlates with the chronic state of activation seen in $CD4^+CD25^+$ T cells from FIV-infected cats, when compared to their $CD4^+CD25^-$ counterparts and either $CD4^+CD25^-$ or $CD4^+CD25^+$ T cell from control cats (Fig. 2a). In addition, TGF-β expression on $CD4^+CD25^-$ T cells from FIV-infected cats although lower than $CD4^+CD25^+$ T cells from FIV-infected cats, was significantly higher than that observed from either $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells from control cats (Fig. 2a), suggesting that these cells could be in the process of conversion to a Treg-like phenotype.

**Activated $CD4^+CD25^+$ T cells from normal cats induce regulatory function in $CD4^+CD25^-$ T cells**

It has been clearly demonstrated that TGF-β treatment coupled with TCR stimulation/co-stimulation establishes a T regulatory phenotype and function in activated $CD4^+CD25^-$ T helper cells in humans, and mice. We have previously provided evidence that a similar conversion takes place in cats (Petty, Chapter 1, Emani 2006). Given that activated $CD4^+CD25^+$ T regulatory cells have the ability to provide co-stimulation via surface CD80 and CD86 (Vahlenkamp, Tompkins et al. 2004; Zheng, Manzotti et al. 2004) while at the same time expressing surface bound active TGF-β (Gregg, Jain et al. 2004), we asked whether or not these

cells were capable of converting recently activated $CD4^+CD25^-$ T cells into T cells with regulatory function. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were FACS purified from single cell suspensions of pooled PLN from SPF control cats. $CD4^+CD25^+$ Treg cells from a SPF control cat were in vitro activated by 4 days of co-culture in the presence of rhIL-2 and LPS and then washed thoroughly before membrane labeling with Vybrant DiD. To emphasize the requirement of Treg cells for conversion, $CD4^+CD25^-$ T cells were treated in a like manner and also labeled with Vybrant DiD. Labeled cells were then used as effector cells at a 1:2 ratio in a 5 day co-culture experiment to convert autologous, Con A-activated, membrane label negative $CD4^+CD25^-$ Th cells to Treg-like phenotype. After 5 days of co-culture, membrane negative cells from the co-cultures were FACS purified (Fig. 3) and their suppressor function and phenotype was subsequently analyzed. The suppressor capacity of the purified membrane negative converted cells over freshly purified $CD4^+CD25^-$ T cells isolated from PBMC of a control cat that were activated with Con A for 4h was assayed by a $^3$H-thymidine uptake. $CD4^+CD25^+$ T cells from control cats activated in vitro by LPS and IL-2 were able to promote regulatory function in autologous $CD4^+CD25^-$ T cells after 5 days of co-culture similar to that seen in $CD4^+CD25^+$ T cells purified from a SPF control cat activated for 4 days with IL-2 and LPS (Fig. 4,$CD25^+$ Control). $CD4^+CD25^+$ T cells stimulated with IL-2 alone for 4 days did not induce Treg function in $CD4^+CD25^-$ T cells during the 5 day co-culture similar to freshly isolated $CD4^+CD25^-$ T cells ($CD25^-$ Control). In addition, membrane labeled effector cells generated from $CD4^+CD25^-$ T cells treated with IL-2 and LPS or IL-2 alone did not induce suppressor function in autologous unlabeled $CD4^+CD25^-$ T cells. IL-2 treatment of $CD4^+CD25^-$ T cells for 5 days did not induce suppressor function.

**Freshly purified $CD4^+CD25^+$ T cells from FIV-infected cats induce regulatory function in activated $CD4^+CD25^-$ T cells**

We have demonstrated two requirements for the *in vitro* conversion of Th cells to Treg-like phenotype in cats. First Treg cells must be activated which up-regulates surface TGF-β, and secondly that Th cells are concomitantly activated. Both requirements are met *in vivo* in FIV-infected cats, in addition co-stimulatory molecules CD80 and CD86 are up-regulated on Treg cells from these cats. Therefore we furthered our investigation to include freshly purified $CD4^+CD25^+$ T cells from PLN of FIV-infected cats. Our data showed that membrane labeled $CD4^+CD25^+$ T cells freshly isolated from FIV-infected cats induced regulatory function in 5 day co-cultured unlabeled autologous $CD4^+CD25^-$ T cells (Fig. 5). Similar to what we observed for SPF control cats, IL-2 plus TGF-β or IL-2 alone treatment of $CD4^+CD25^-$ T cells did not generate effector cells capable of inducing suppressor function in autologous Con A-activated $CD4^+CD25^-$ T cells (Fig. 5).

**Anti-TGF-β1 or anti-TGF-βRII antibodies inhibit activated $CD4^+CD25^+$ Treg cell mediated up-regulation of surface TGF-β1 on $CD4^+CD25^-$ T cells**

We have previously demonstrated that TGF-β1 is constitutively expressed on $CD4^+CD25^+$ T cells from FIV-infected cats. We have also shown that blocking TGF-βRII abrogates in vitro TGF-β plus Con A conversion of activated $CD4^+CD25^-$ T cells to a Treg-like phenotype. To

confirm that a TGF-β1 on $CD4^+CD25^+$ Treg cells interaction with TGF-βRII on $CD4^+CD25^-$ Th cells is at least partially responsible for converting $CD4^+CD25^-$ Th cells to a peripheral Treg-like phenotype, we examined TGF-β1 surface expression on membrane negative cells from FIV-infected cats after the previously described 5 day co-culture with autologous membrane labeled $CD4^+CD25^+$ Treg cells . After FACS purification, membrane negative cells were stained with anti-CD25 and anti-TGF-β antibodies and data were collected by flow cytometry. Analysis revealed that either anti-TGF-β treatment of $CD4^+CD25^+$ T cells or anti-TGF-βRII treatment of $CD4^+CD25^-$ T cells before the 5 day co-culture, significantly reduced the up-regulation of TGF-β1 on the surface of the membrane negative CD4 T cells recovered after the 5 day co-culture when compared to untreated cell cultures (Fig. 6a), suggesting that both TGF-β1 on Treg cells and TGFβRII on Th cells are crucial for the Treg mediated up-regulation of TGF-β on $CD4^+CD25^-$ T cells.

**Anti-TGF-β1 or anti-TGFβRII antibodies abrogate suppressor function in $CD4^+CD25^+$ Treg converted $CD4^+CD25^-$ T cells**

As TGF-β is a key component of Treg cell suppressor function, we also assayed the suppressor function of membrane negative cells recovered from 5 day co-cultures after treatment with the blocking antibodies described above. In agreement with our previous finding that anti-TGF-βRII treatment of Con A-activated $CD4^+CD25^-$ T cells before addition of TGF-β1 blocked the conversion to a Treg-like suppressor cell, addition of blocking anti-TGF-βRII antibody added to

Con A-activated $CD4^+CD25^-$ T cells before a 5 day co-culture with autologous $CD4^+CD25^+$ Treg cells from an FIV-infected cat abrogated the conversion to Treg-like cells and therefore suppressor capacity when compared to untreated cells (Fig. 6b). Similarly, blocking TGF-β antibody treatment of $CD4^+CD25^+$ Treg cells from an FIV-infected cat prior to a 5 day co-culture with Con A-activated $CD4^+CD25^-$ T cells abrogated the conversion of the $CD4^+CD25^-$ T cells to Treg-like cells. In addition RNA was extracted from cells treated as described above. Agarose/ethidium bromide gel analysis of the RT-PCR products revealed both blocking anti-TGF-βRII treatment of $CD4^+CD25^-$ T cells (Figure 6d, Lane 3) and blocking TGF-β antibody treatment of $CD4^+CD25^+$ Treg cells (Figure 6d, Lane 2) prior to the 5 day co-culture significantly diminished FoxP3 expression which was comparable to expression seen in freshly isolated $CD4^+CD25^-$ T cells (Figure 6d, Lane 1). When no blocking antibody was used before co-culture, FoxP3 was significantly up-regulated (Figure 6d, Lane 4). The absence of suppressor activity in the converted Th cells correlated with the absence of surface TGF-β1 (Fig. 6a) and FoxP3 expression (Fig. 6d).

Nakamura et. al (Nakamura, Kitani et al. 2004) showed that treating $CD4^+CD25^+$ Treg cells with blocking TGF-β1 antibody diminished their ability to suppress proliferation of stimulated $CD4^+CD25^-$ T cells. We furthered our investigation to determine if we could block the suppressor activity of $CD4^+CD25^+$ Treg cell converted $CD4^+CD25^+$ Treg-like cells by the addition of blocking TGF-β1 antibody before $^3$H-thymidine assessment of suppressor function over Con A-stimulated heterologous $CD4^+CD25^-$ T cells purified from PBMC. Blocking TGF-β antibody completely blocked the suppressor function of converted cells whereas untreated cells

efficiently suppressed proliferation at a 0.5:1 ratio (Fig. 6b) and this data was supported by our previous findings that TGF-β blocking antibody abrogated the suppressor function of $CD4^+CD25^-$ Con A/TGF-β converted Treg-like cells as well as natural $CD4^+CD25^+$ Treg cells.

## DISCUSSION

The interaction between natural $CD4^+CD25^+$ Treg cells and pathogenic T cells in peripheral immunity has been well studied from the prospective of suppressor function of the regulatory cells. Recent studies are just beginning to shed light on the function of activated effector CD4 cells as suppressors after interaction with Treg cells. Phenotypically, Treg cells express surface CD25, CTLA-4, GITR, CD80, CD86 and surface expression of biologically active TGF-β1 on $CD4^+CD25^+$ Treg cells has been previously reported in both mice and humans. Naturally occurring $CD4^+CD25^+$ Treg cells represent 5-10% of the circulating $CD4^+$ T cell pool and are responsible for maintaining tolerance to self-antigen in a cell contact dependent manner. Interestingly, TGF-β signaling in T cells, in addition to down-regulating IL-2 production, induces regulatory phenotype and function in activated $CD4^+CD25^-$ T cells *in vitro* in mice and humans by inducing the expression of FoxP3 (Yamagiwa, Gray et al. 2001; Horwitz, Zheng et al. 2003; Peng, Laouar et al. 2004; Schramm, Huber et al. 2004).

We have previously shown that activated $CD4^+CD25^+$ T cells from cats are able to suppress activated T helper cell expansion in a dose dependent manner. In addition our research shows that $CD4^+CD25^+$ Treg cells from FIV-infected cats display constitutive activation

phenotype, which in turn confers constitutive suppressor activity in these $CD4^+CD25^+$ Treg cells (Vahlenkamp, Tompkins et al. 2004). Here we have further characterized the feline Treg cell by providing evidence that $CD4^+CD25^+$ Treg cells express surface TGF-β1 when activated and that the expression is constitutive in chronically FIV-infected cats. As we earlier demonstrated that in vitro rhTGF-β1 plus Con A treatment induces FoxP3 mRNA and suppressor function in $CD4^+CD25^-$ T cells (Petty, Chapter 1), we speculated that surface bound TGF-β on feline Treg cells may also play a conversion role in activated $CD4^+CD25^-$ T cells. Significantly, we report that feline Treg cells under certain conditions can convert Th cells to Treg-like cells in vitro as reported in humans (Jonuleit, Schmitt et al. 2002; Dieckmann, Plottner et al. 2005). We have demonstrated two requirements for *in vitro* Treg conversion of Th cells in cats. First that Treg cells are activated which up-regulates surface TGF-β1, and secondly that Th cells are concomitantly activated which up-regulates TGF-βRII providing a means for TGF-β signal transduction. Both requirements are met *in vivo* in FIV-infected cats. Further, we have implicated TGF-β signaling in the conversion process. We show that anti-TGF-β1 blocking antibody treatment of $CD4^+CD25^+$ Treg cells or anti-TGF-βRII blocking antibody treatment of $CD4^+CD25^-$ T cells prior to co-culture, abrogates the induction of FoxP3 and surface expression of TGF-β, both markers of Treg cells. Our current data suggests that TGF-β signaling is crucial for the peripheral development of Treg cells. The transcriptional activation of several genes including Foxp3 are targeted by TGF-β signaling, depending upon the combinations of SMAD complex interactions with co-activators and inhibitors of gene transcription (Shi and Massague 2003). Importantly Foxp3, which serves as a transcriptional repressor, down regulates SMAD 7

which in turn interrupts the negative auto-regulatory feedback loop of TGF-β mediated SMAD signaling allowing unchecked TGF-β signaling in naturally occurring Treg cells and TGF-β1 induced Treg-like cells in human and murine models (Fantini, Becker et al. 2004). The continued signaling through TGF-β1 and SMADS may be critical in the maintenance and homeostasis of the Treg population. The constitutive activation seen in chronic Lentiviral infections, such as FIV and HIV, may allow an abnormal continuing activation of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells and provide the environment needed to convert $CD4^+CD25^-$ cells in vivo.

In this paper we have addressed the role of $CD4^+CD25^+$ T regulatory cells from FIV-infected cats in generating peripheral T regulatory cells from $CD4^+CD25^-$ T cells and have established a potential link between surface expression/production of biologically active TGF-β1 by activated $CD4^+CD25^+$ T cells and the TGF-β mediated conversion of $CD4^+CD25^-$ T cells into phenotypic and functional T regulatory cells. We speculate that Lentiviral infection in cats alters either the $CD4^+CD25^+$ interaction with $CD4^+CD25^-$ cells or alters what we believe to be a transient Treg function seen in the activated $CD4^+CD25^-$ cells after exposure to natural Treg cells by direct infection of converted cells or chronic antigenemia associated with FIV-infection or opportunistic infections arising from compromised immune function. Our results may offer an explanation for immune suppression seen in FIV/HIV without drastic decreases in the total CD4 population. An increase in $CD4^+CD25^+$ T regulatory cells coupled with a loss of $CD4^+CD25^-$ T cells and increased immunosuppression seen during FIV/HIV infection could possibly be

explained by recruitment of $CD4^+CD25^-$ anergized/converted T regulatory cells to the Treg pool. Estes (Estes, Li et al. 2006), in a recent study, shows viral replication and immune activation in lymph nodes causes a premature immunosuppressive response, with dramatic increases in $CD4^+CD25^+FOXP3^+TGF\text{-}\beta^+$ Treg cells suggesting that the timing of the initial immunosuppressive response by Treg cells may be indicative of the disease course during chronic viral infection such as HIV/FIV. Induction of Treg suppressor activity too early during Lentiviral infection could lead to suppression of effector cells before viral clearance, leaving activated, yet anergic effector cells incapable of mounting an immune response as seen in SIV infection of rhesus macaques. In a nonpathogenic model of SIV infection, Kornfeld (Kornfeld, Ploquin et al. 2005) observed an early and strong TGF-β expression compared to the later response in the pathogenic response seen in macaques. Further, they correlated Treg responses with a protective role against SIV progression. Although the timing of the initial Treg response may affect the acute disease progression, our study of chronically FIV-infected cats suggests an expansion and/or preferential survival of Treg cells coupled with a loss of CD4 Th cells contributes to the overall immunosuppression, characteristic of late stage FIV and the onset of AIDS. Currently studies are underway in our lab to address the role of Treg cells in acute FIV infection.

As we have demonstrated the ability of Treg cells activated in vitro by IL-2 and LPS or in vivo by FIV-infection to convert $CD4^+$ Th cells to Treg-like cell phenotype and function, Lentiviral infection could provide an immunological microenvironment that is optimal for peripheral Treg cell development with chronic activation and surface TGF-β expression on Treg cells and chronic antigenemia associated Th activation.

Data from previous studies supports the conclusion that peripheral conversion of $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T regulatory is a normal occurrence in healthy individuals during bacterial, viral, or allergic challenge, and that $CD4^+CD25^+$ T cell associated TGF-ß may mediate this conversion process. While our data support the hypothesis that in vivo, TGF-β mediated conversion of Th cells to Treg-like cells may contribute to the increased frequency of $CD4^+CD25^+$TGF-$\beta^+$ $FoxP3^+$ T cells observed in PBMC of FIV-infected cats, it is possible that the expression of surface TGF-β in the converted cells is a result of loading available TGF-β receptors with TGF-β, rather than inducing production of TGF-β in the converted cells. In our experimental system, whether the source of TGF-β is from natural Treg cells or induced in the converted cells is unlikely to matter for acquisition of suppressor function, as we found FoxP3 induction and suppressor in the converted cells. The development of this in vitro model of Treg–Th cell interactions will allow us to further examine the mechanisms of TGF signaling interactions and to investigate the role of virus in the peripheral conversion of Th cells to Treg-like phenotype, as well as further investigate the source of surface TGF-β.

## MATERIALS AND METHODS

*Cats*

Specific pathogen-free cats were obtained from Liberty Labs (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA) and housed at the Laboratory Animal Resource Facility at the

College of Veterinary Medicine, North Carolina State University. Cats were inoculated with the $NCSU_1$ isolate of FIV, a pathogenic clade A virus (English, Nelson et al. 1994), as described by Bucci et al (Bucci, English et al. 1998). FIV-infection was confirmed by immunoblot analysis and provirus detection by PCR using primers specific for the FIV-p24 GAG sequence. At the time samples were taken, cats had been infected with FIV for at least 5 years and were clinically asymptomatic. Uninfected control cats ranged in age from 3 to 6 years and were housed separately from FIV-infected cats.

*Sample Collection and Preparation*

Whole blood was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). Subsequently, PBMC were isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as previously described (Tompkins, Ogilvie et al. 1987). Single-cell suspensions were prepared from LN obtained from biopsies by gently and repeatedly injecting sterile PBS into the tissue using 18G needle until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion. Viability was always >90%.

*Reagents and antibodies*

Recombinant human IL-2 were was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Maurice Gately, Hoffmann - La Roche Inc.

LPS and Concanavalin A (ConA) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IgG coated magnetic Dynabeads® M-450 were purchased from Dynal (Great Neck, NY). Streptavidin–PerCP was purchased from BD Biosciences PharMingen (San Diego, CA). Anti-TGF-β1 (MAB240) was purchased from R&D Systems (Minneapolis, MN) and conjugated to allophycocyanin (APC), PE-conjugated anti-TGF-βRII (FAB241P), anti-TGF-βRII (AF-241-NA) and recombinant human TGF-β1 (240-B) were also purchased from R&D Systems. Mouse anti-feline CD25 (mAb 9F23) was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan). Anti-CD21 was purchased from P. Moore, (University of California, Davis). Mouse anti-feline CD4 (mAb 30A) and CD8 (mAb 3.357) (Tompkins, Gebhard et al. 1990) were developed in our lab.

*Flow cytometric analysis*

At least $5x10^5$ PBMC were stained for surface expression of CD25, CD4, and TGF-β1 using FITC conjugated anti-CD25, biotin-conjugated anti-CD4, and APC-conjugated anti-TGF-β1 mAbs. T cell preparations were stained in 5% FBS/PBS for 30 min on ice. When necessary, T cells also were washed and incubated with streptavidin PerCP secondary antibodies for 30 min on ice. Flow cytometry data were acquired using 3-color flow cytometry and analyzed (FACSCalibur; CellQuest software; respectively, BD Biosciences, Mountain View, CA). Lymphocytes were gated based on forward vs. side scatter, and gated events were acquired and stored list-mode fashion for analysis using CellQuest software.

*Purification of T cells*

For FACS purification, LN cells were stained with anti-CD4 biotin/streptavidin PerCP and anti-CD25 FITC; $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets were purified using a high-speed, high-purity fluorescence activated cell sorter (MoFlo, DakoCytomation). The purity of FACS sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ cell populations was always > 95%.

$CD4^+$ $CD25^-$ T cells for use as target cells in proliferation assays were enriched using biomagnetic bead separation performed using goat anti-mouse IgG-coated beads as described by Bucci et al. (Bucci, English et al. 1998). Briefly, $CD21^+$ B cells, $CD8^+$ and $CD25^+$ T cells were depleted in successive steps using magnetic beads coated with anti-CD21, anti-CD8 and anti-CD25 antibody respectively. Purity of the magnetic bead enriched $CD4^+CD25^-$ T cells was > 90%, as verified by flow cytometric analysis.

*Effector Cell Pretreatments and Labeling*

T cells were cultured in RPMI 1640 which was supplemented with 10% heat-inactivated FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. For pretreatments, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells ($2 \times 10^6$/mL, $4 \times 10^6$ per well, 24-well plate) were stimulated for 4 days with LPS (10 ng/mL) plus IL-2 (100U/mL) or IL-2 alone. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells ($4 \times 10^6$ from each treatment) were then washed

extensively and labeled with Vybrant DiD fluorescent dye. Labeled cells were plated at a 1:2 ratio for 5 days with autologous or heterologous unlabeled $CD4^+CD25^-$ T cells that were stimulated with 5 μg/mL ConA and 100 U/mL IL-2 for 24 h or not and washed prior to co-culture. For conversion blocking experiments, anti–TGF-β, anti– TGF-βRII, or control IgG was added to the cultures. After 5 days, membrane negative cells were FACS purified for use in suppression assays, cell surface molecule analysis and mRNA analysis. The purity of membrane negative cells was >98% as verified by flow cytometric analysis.

*In vitro T cell suppression assay*

Enriched $CD4^+CD25^-$ target cells ($10^6$ cells/ml) were stimulated for 4 h with 5 μg/ml Con A, washed twice in RPMI 1640, and plated at 5 x $10^4$ viable cells/well in 96-well U bottom plates. $CD4^+$ effector cells were then added to the wells at various E:T ratios ranging from 0.125:1 to 1:1. $CD4^+CD25^+$ effector cells were added as: 1) freshly isolated, untreated cells, 2) after treatment in 24-well plates at $1x10^6$ cells/ml for 4 days with 10 μg/ml LPS and 100 U/ml rhIL-2, or 3) $CD4^+$ T cells that were sorted after conversion from $CD4^+CD25^-$ T cells cultured with pretreated $CD4^+$ subsets as discussed above. For suppressor activity blocking experiments, 100 μg/mL anti-TGF-β, 100 μg/mL anti-TGF-βRII, or 100 μg/ml control IgG was added to the cultures. The cells were then washed, counted, and added to the target cells. Assays were run in triplicate. Effector and target cells were co-cultured at 37°C for 72 hrs and pulsed with 1 μCi of [3H]TdR/well for the last 18 h and harvested using a Filtermate Harvester (Packard Bioscience, Meriden, CT). [$^3$H]thymidine incorporation was measured using a Top Count NXT Microplate

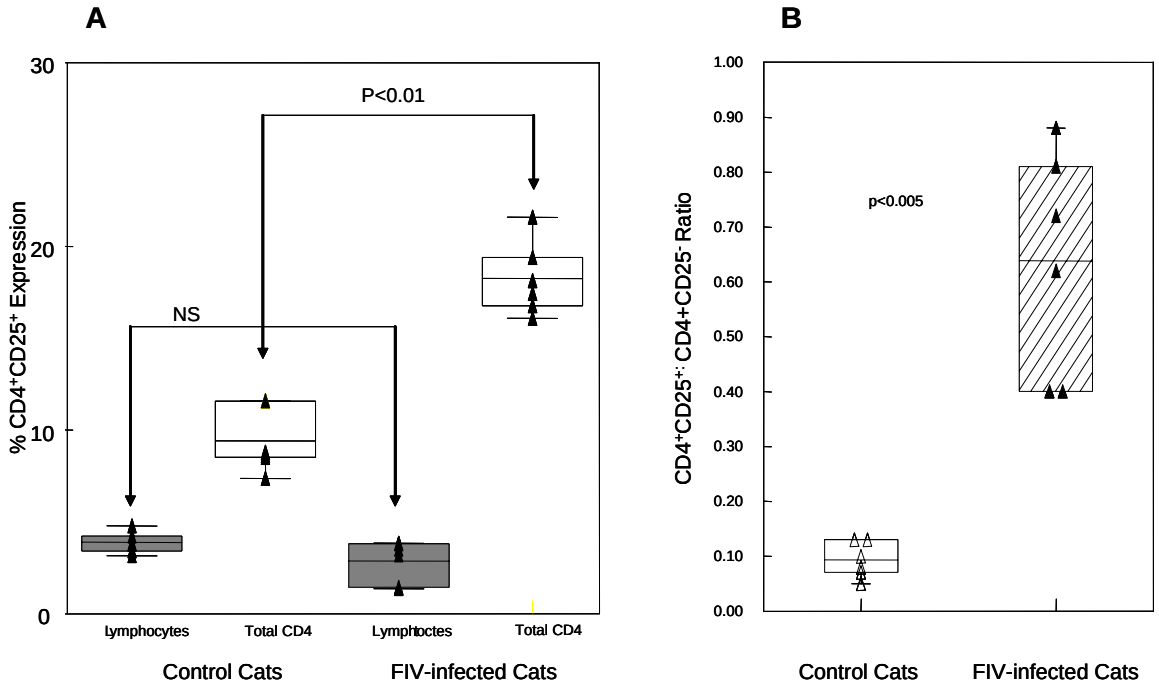
scintillation counter (Packard Bioscience). Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ target cells alone and calculated as follows:

percent inhibition ={[($CD4^+CD25^-$ alone - $CD4^+CD25^+$) cpm]/[($CD4^+CD25^-$ alone) cpm]} x100.

*Statistical analysis*

The Mann-Whitney U test (t test-like for nonparametric data) was used for pair wise comparison of expression of surface molecules. Differences were considered to be significant at $p < 0.01$.

**Figure 1.** Increased expression of CD25 on CD4 T cells in FIV-infected cats. Blood from both FIV-infected and SPF control cats was collected in EDTA tubes by venipuncture. PBMC isolated by density gradient centrifugation were stained with feline anti-CD4 and anti-CD25 antibodies and data were collected by two color flow cytometry. Box-whisker plots representing 5th and 95th percentiles (whisker), 25th and 75th percentiles (box), and median (A) Analysis of CD4 and CD25 surface expression shows no significant difference in CD4+CD25+ T cells in the total lymphocyte population from FIV-infected cats compared to control SPF cats. However, there was a significant increase in $CD4^{+}CD25^{+}$ T cells as a percentage of total CD4 T cells in PBMC from FIV-infected cats when compared to age matched SPF control cats. (B) The ratio of $CD4^{+}CD25^{+}$:$CD4^{+}CD25^{-}$ T cells, calculated from flow cytometric analysis, was significantly higher in PBMC from FIV-infected cats when compared to PBMC from SPF control cats. (C) Representative dot plot of CD4 and CD25 analysis. (D) Absolute CD4 T cell numbers obtained from CBC's of cats and calculated by multiplying %CD4 T cells of gated lymphocytes determined from flow cytometric analysis. Bars represent the mean ± standard deviation of six cats for each group.


A
30
20
10
0
% CD4+CD25+ Expression
P<0.01
NS
Lymphocytes
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Total CD4
Control Cats
FIV-infected Cats
B
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
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0.00
CD4+CD25+: CD4+CD25- Ratio
p<0.005
Control Cats
FIV-infected Cats

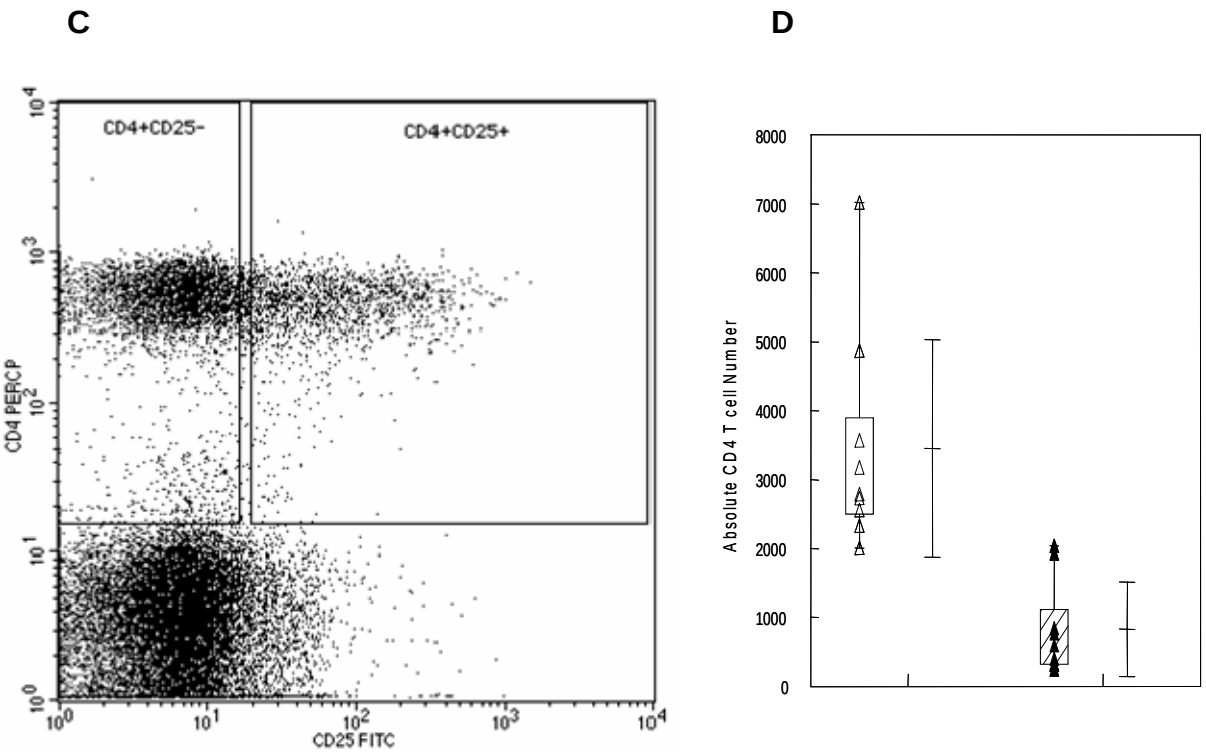
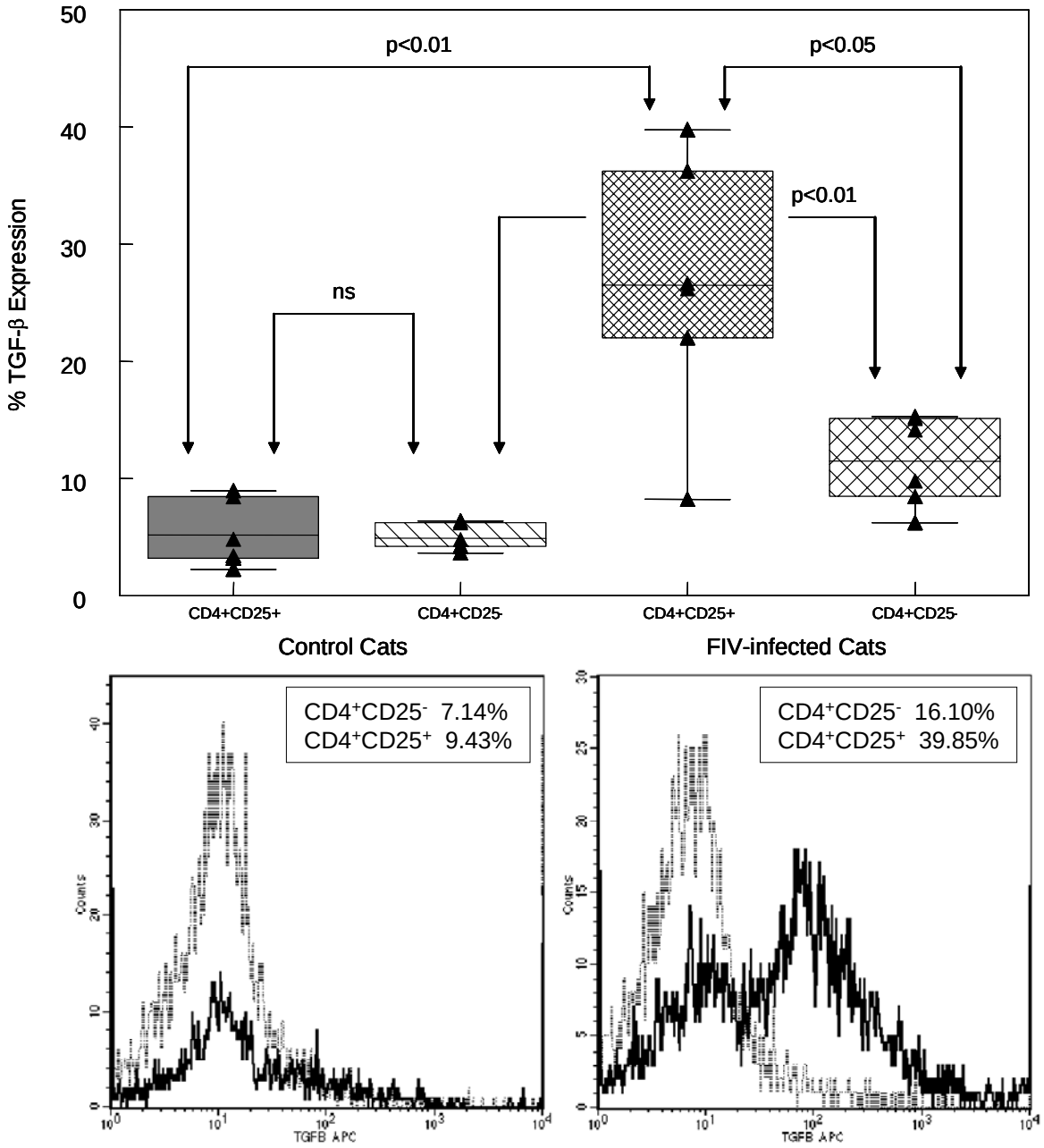

C
CD4+CD25-
CD4+CD25+
CD4 PERCP
CD25 FITC
D
8000
7000
6000
5000
4000
3000
2000
1000
0
Absolute CD4 T cell Number

**Figure 2.** Feline CD4+CD25+ T cells from FIV-infected display increased surface TGF-β. PBMC were obtained from the blood of FIV infected or SPF control cats. Cells were then stained with fluorescently labeled anti-CD4 PerCP, anti-CD25 FITC, and anti-TGF-β1 APC antibodies. Surface TGF-β1 expression on $CD4^+CD25^-$ and $CD4^+$ $CD25^+$ T cells was determined by flow cytometry. A slight, although not statistically significant, increase in the expression of TGF-β1 on $CD4^+CD25^-$ T cells was observed in PBMC of FIV-infected cats when compared to SPF control cats. However, a significant increase of surface TGF-β1 was found on $CD4^+CD25^+$ T cells in PBMC from FIV-infected cats compared to $CD4^+CD25^+$ T cells from SPF controls. Expression of TGF-β1 was similar on both $CD4^+CD25^-$ and $CD4^+$ $CD25^+$ T cells from control cats, while there was significantly higher expression of TGF-β1 on $CD4^+$ $CD25^+$ T cells from FIV-infected cats compared to $CD4^+CD25^-$ T cells from FIV-infected cats (top).
A representative histogram plot from n=6 for each group is shown (bottom). Surface TGF-β1 expression on $CD4^+CD25^-$ (dotted line) (Control 7.14%, MFI=43) (FIV 16.10%, MFI= 105),and $CD4^+$ $CD25^+$ (solid line) (Control 9.43%,MFI=873), (FIV 39.85%,MFI=848).


% TGF-β Expression
50
40
30
20
10
0
p<0.01
p<0.05
p<0.01
ns
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
Control Cats
FIV-infected Cats
CD4+CD25- 7.14%
CD4+CD25+ 9.43%
CD4+CD25- 16.10%
CD4+CD25+ 39.85%
Counts
TGFB APC

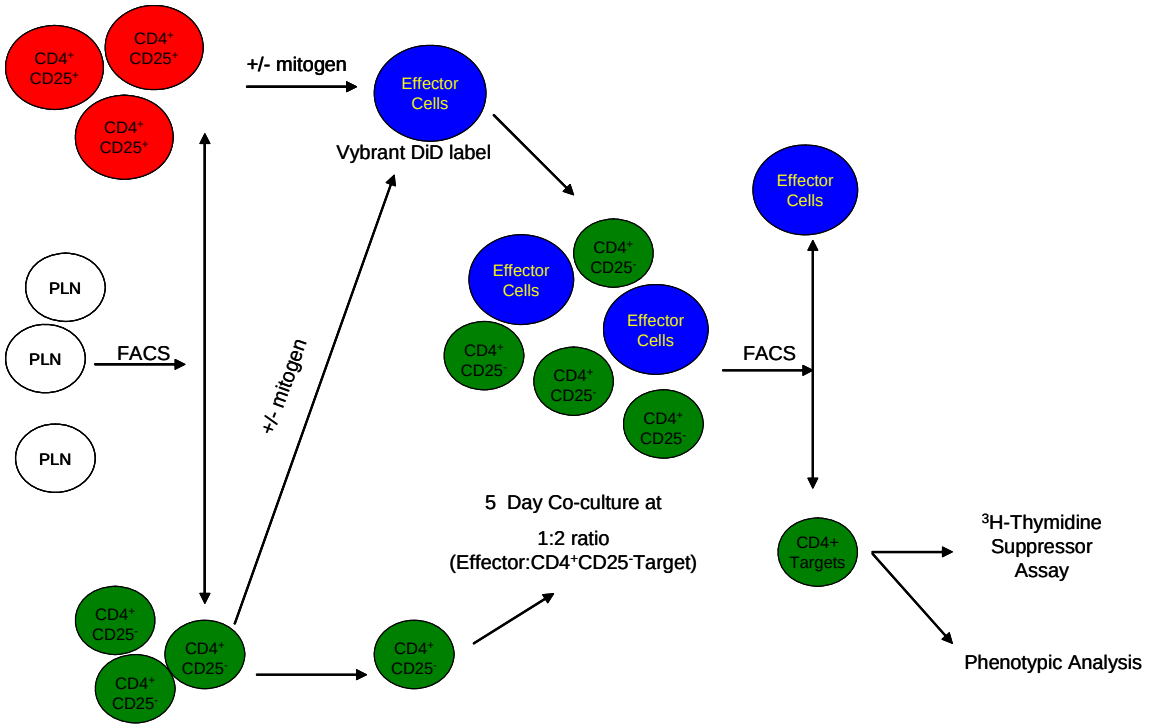



**Figure 3.** Schematic diagram of experimental design for $CD4^+$ $CD25^+$ Treg cell mediated conversion of feline $CD4^+CD25^-$ T cells to Treg-like cells.

Single cell suspensions were prepared from pooled PLN collected from FIV-infected or SPF control cats. Cells were stained with feline anti-CD25 FITC and anti-CD4 PerCP labeled monoclonal antibodies and $CD4^+$ $CD25^+$ and $CD4^+CD25^-$ T cell populations were purified by FACS. Effector cells for the co-culture experiments were generated from both purified populations by treatment with LPS/IL-2, IL-2 alone, or left untreated. Effector cells were then labeled with Molecular Probe Vybrant DiD membrane dye. Labeled effector cells were then added to autologous Con A-activated $CD4^+CD25^-$ T cell at a 1:2 ratio in medium supplemented with 10 U/mL IL-2. After 5 days, membrane negative cells were FACS purified from the co-culture and assayed for suppressor function and phenotype.

**Figure 4.** Activated feline $CD4^+CD25^+$ T cells induce regulatory function in $CD4^+CD25^-$ T cells.

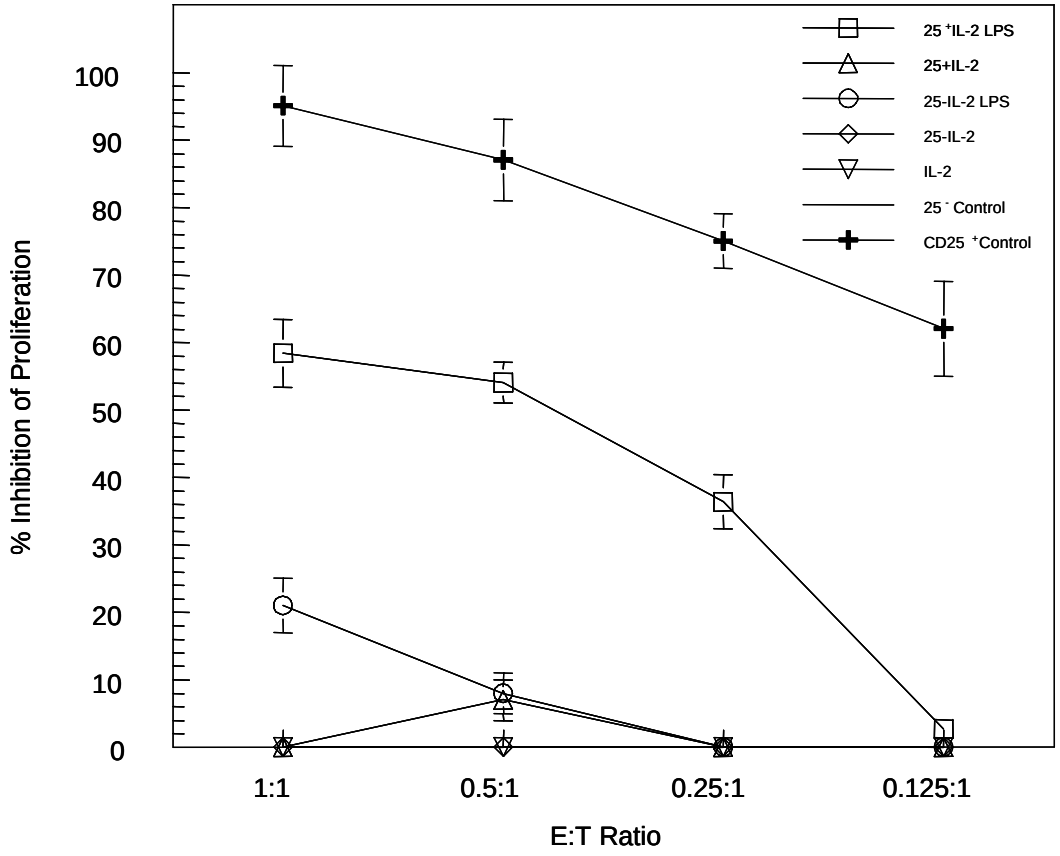
**Effector cell pretreatments**: $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were FACS purified from peripheral lymph nodes of control cats. Effector cells from control cats for co-culture experiments were generated as follows: $CD4^+CD25^+$ (□,△) and $CD4^+CD25^-$ (○,◇) T cells were pretreated with either LPS (10 ng/mL) and IL-2 (100U/mL) or IL-2 alone. After 4 days, effector cells were washed extensively and membrane labeled with Vybrant DiD.

**Target cell pretreatments**: For generation of target cells, $CD4^+CD25^-$ T cells from control cats were activated with Con A (5 μg/mL) and IL-2 (100 U/mL) for 4 hours immediately prior to co-culture and washed extensively.

**Co-culture experiments**: Membrane labeled effector cells from each pretreatment were plated at a 2:1 ratio with target cells, target cells were also cultured in media supplemented with IL-2 as a control (▽). After 5 days of co-culture in media supplemented with IL-2, effector and target cells were separated by FACS based on membrane labeling.

**Suppressor Assay**: FACS sorted membrane negative cells were used in a standard 72 hour $^3H$-Thymidine suppressor assay at the indicated ratios with $5x10^4$ $CD4^+CD25^-$ T cells enriched from PBMC that were activated with Con A for 4 hours and washed prior to the assay.

As shown above, co-culture of $CD4^+CD25^-$ T cells with $CD4^+CD25^+$ T cells freshly isolated from control cats were unable to induce suppressor function in the $CD4^+CD25^-$ population. However, after LPS/IL-2 pretreatment, there was significant induction of suppressor function. Activation of $CD4^+CD25^+$ T cells from control cats conferred the ability of this population to induce suppressor function in $CD4^+CD25^-$ T cells, and activation of the $CD4^+CD25^-$ T cells was also required. None of the other pretreatments were able to confer conversion potential that was significantly above that seen from control $CD4^+CD25^-$ T cells. Bars represent the mean ± standard deviation of three separate experiments.


25 +IL-2 LPS
25+IL-2
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25-IL-2
IL-2
25 - Control
CD25 +Control
% Inhibition of Proliferation
100
90
80
70
60
50
40
30
20
10
0
1:1
0.5:1
0.25:1
0.125:1
E:T Ratio

**Figure 5.** Feline CD4+CD25+ T cells from FIV-infected cats induce regulatory function in $CD4^+CD25^-$ T cells. **Effector cell pretreatments**: CD4+CD25+ and $CD4^+CD25^-$ T cells were FACS purified from peripheral lymph nodes. Effector cells from FIV-infected cats for co-culture experiments were generated as follows: CD4+CD25+ T cells (□) after FACS sorting were immediately membrane labeled with Vybrant DiD and used without pretreatment as we have previously demonstrated these cells are functionally activated. CD4+CD25- T cells were pretreated with either LPS (10 ng/mL) and IL-2 (100U/mL) (△) or IL-2 alone (○). After 4 days, CD4+CD25- effector cells were washed extensively and membrane labeled with Vybrant DiD.

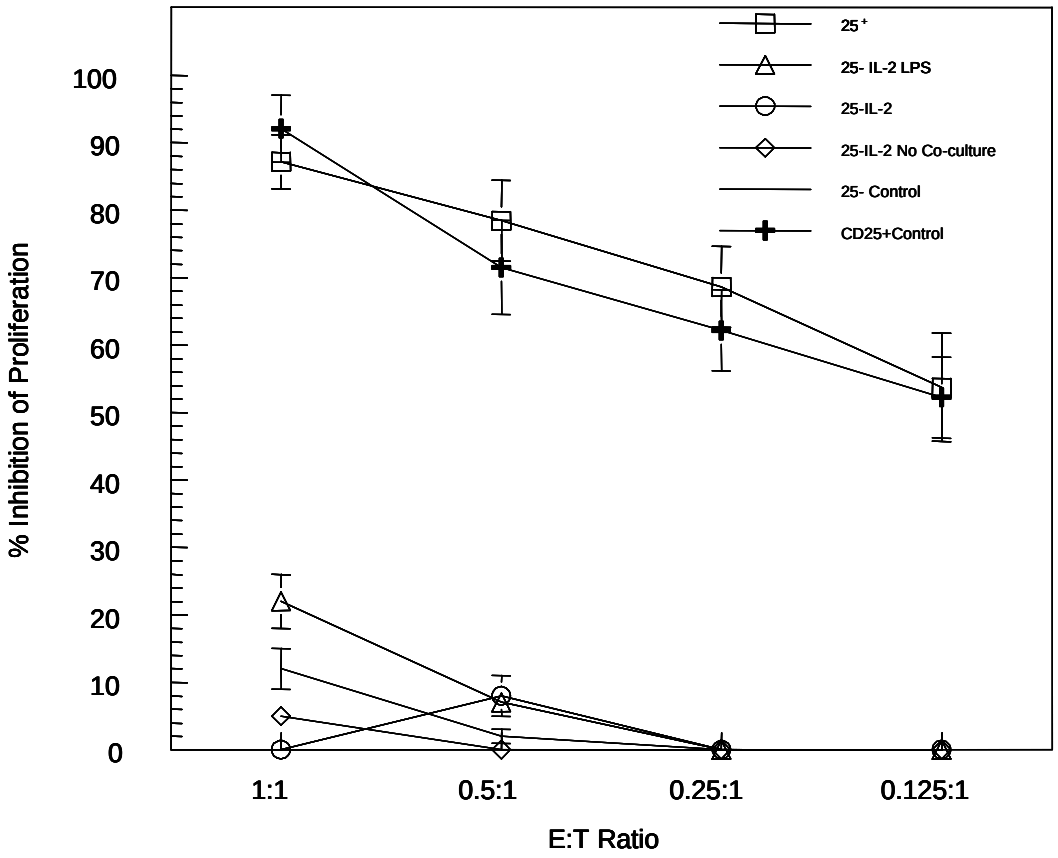
**Target cell pretreatments**: For generation of target cells, CD4+CD25- T cells from FIV-infected cats were used immediately or activated with Con A (5 μg/mL) and IL-2 (100 U/mL) for 4 hours immediately prior to co-culture and washed extensively.

**Co-culture experiments**: Membrane labeled effector cells from each pretreatment were plated at a 2:1 ratio with target cells, target cells were also cultured in media supplemented with IL-2 as a control (◇). After 5 days of co-culture in media supplemented with IL-2, effector and target cells were separated by FACS based on membrane labeling.

**Suppressor Assay**: FACS sorted membrane negative cells were used in a standard 72 hour $^3H$-Thymidine suppressor assay at the indicated ratios with $5x10^4$ CD4+CD25- T cells enriched from PBMC that were activated with Con A for 4 hours and washed prior to the assay.

As shown above, co-culture of $CD4^+CD25^-$ T cells with $CD4^+CD25^+$ T cells freshly isolated from FIV infected cats induced suppressor function in the $CD4^+CD25^-$ population equal to that seen from freshly isolated $CD4^+CD25^+$ T regulatory cells from FIV-infected cats. Activation of

$CD4^+CD25^-$ T cells was not required, as there was no difference in the induction of suppressor function when $CD4^+CD25^-$ T cells were *in vitro* activated. None of the other pretreatments were able to confer conversion potential that was significantly above that seen from control $CD4^+CD25^-$ T cells. Bars represent the mean ± standard deviation of three separate experiments.


25+
25- IL-2 LPS
25-IL-2
25-IL-2 No Co-culture
25- Control
CD25+Control
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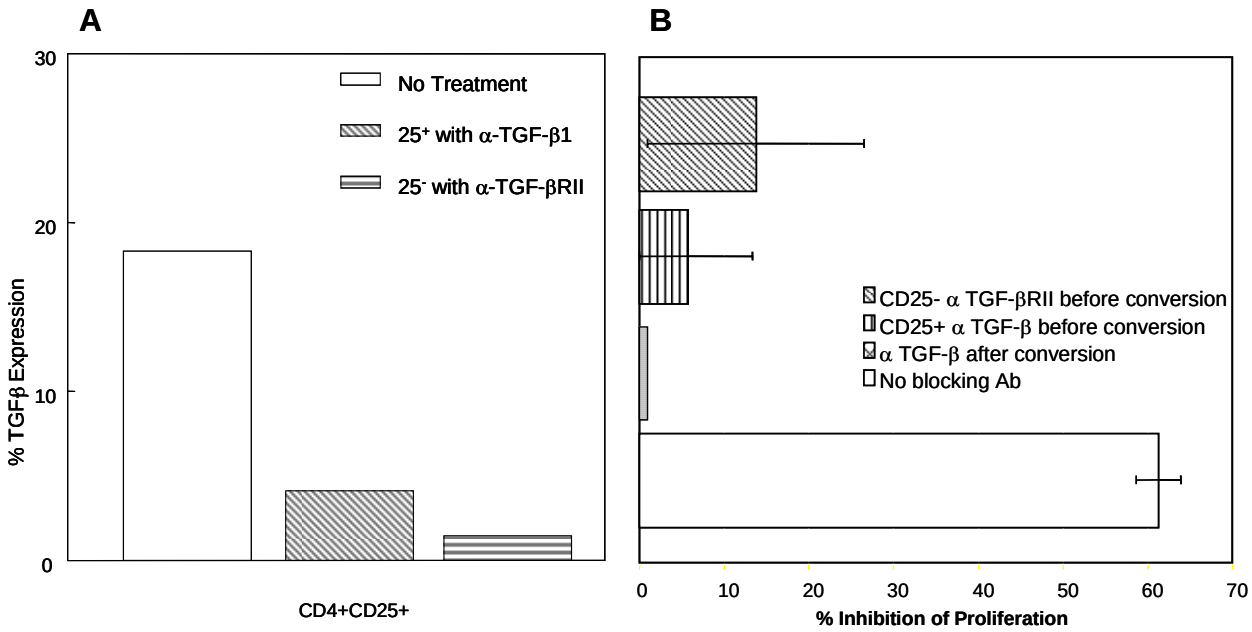
0.125:1
E:T Ratio

**Figure 6.** Feline $CD4^+CD25^+$ T cells mediate suppression and conversion of $CD4^+CD25^-$ T cells via TGF-β dependent mechanism. $CD4^+CD25^-$ T helper cells from PLN of FIV-infected cats were activated with Con A for 4 h washed and then treated or not with anti-TGF-βRII blocking antibody for 30 min prior to addition to the 5 day culture with membrane labeled autologous $CD4^+CD25^+$ Treg cells. Both up-regulation of surface TGF-β1 (a) and suppressor function (b) was abrogated by blocking TGF-βRII in the FACS purified membrane negative co-cultured $CD4^+CD25^-$ Th cells when compared to untreated cells. To further investigate the TGF-β/TGF-βRII interactions between $CD4^+CD25^+$ effector cells and $CD4^+CD25^-$ Th cells, membrane labeled $CD4^+CD25^+$ Treg cells from FIV-infected cats were treated with blocking anti-TGF-β1 antibody before addition to Con A-activated $CD4^+CD25^-$ T cells for co-culture. Surface TGF-β up-regulation (a) and suppressor function (b) was also significantly reduced in the $CD4^+CD25^-$ T cells by blockade of TGF-β1 on the surface of $CD4^+CD25^+$ Treg cells. Bars represent the mean ± standard deviation of three separate experiments. Finally, purified membrane negative converted Treg-like cells were treated with anti-TGF-β1 blocking antibody and then suppressor function was assayed by $^3H$ thymidine incorporation. Blocking up-regulated TGF-β1 on the surface of converted Treg-like cells completely abrogated their suppressor function over heterologous Con A-stimulated $CD4^+CD25^-$ T cells purified from PBMC. (c) Dot plot representation of data presented in (a). (d) Anti-TGF-β treatment of $CD4^+CD25^+$ or anti-TGF-βRII treatment of $CD4^+CD25^-$ T cells before co-culture abrogates up-regulation of FoxP3 mRNA. FACS sorted $CD4^+CD25^-$ and $CD4^+CD25^+$ were incubated for 30 min with or without

(Figure 6 continued)

the indicated antibodies before a 5 day co-culture. $CD4^+CD25^+$ T cells were membrane labeled with Vybrant DiD before co-culture to allow post culture FACS separation. RNA was extracted from FACS purified membrane negative cells. Shown is the agarose/ethidium bromide gel analysis of the RT-PCR products. Lane 1 $CD4^+CD25^-$ medium alone, Lane 2 $CD4^+CD25^+$ with anti-TGF-β, Lane 3 $CD4^+CD25^-$ with anti TGF-βRII, Lane 4 No treatment of either $CD4^+CD25^+$ or $CD4^+CD25^-$. FoxP3 is significantly up-regulated during co-culture only when no blocking antibodies were added.


A
B
30
20
10
0
% TGFβ Expression
No Treatment
25+ with α-TGF-β1
25- with α-TGF-βRII
CD4+CD25+
CD25- α TGF-βRII before conversion
CD25+ α TGF-β before conversion
α TGF-β after conversion
No blocking Ab
0
10
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30
40
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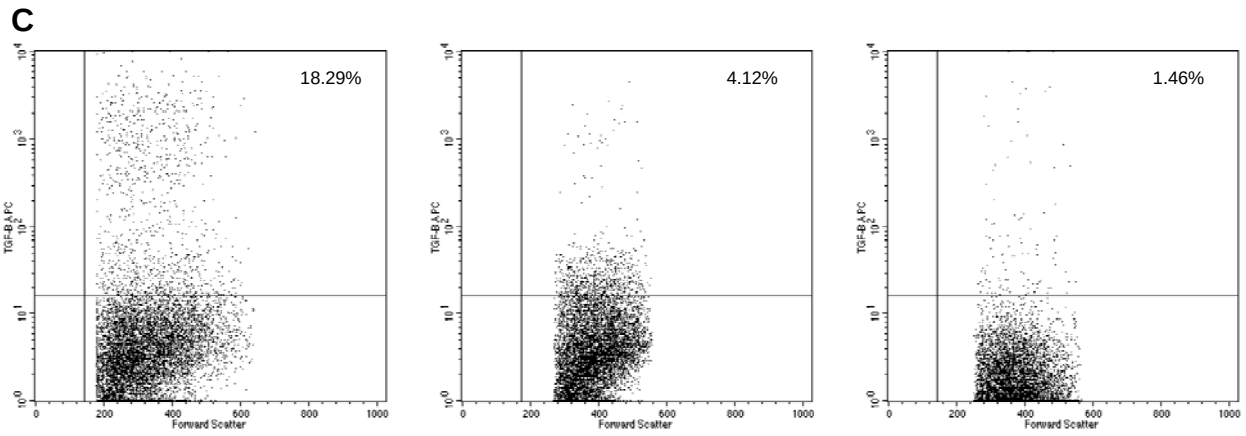
60
70
% Inhibition of Proliferation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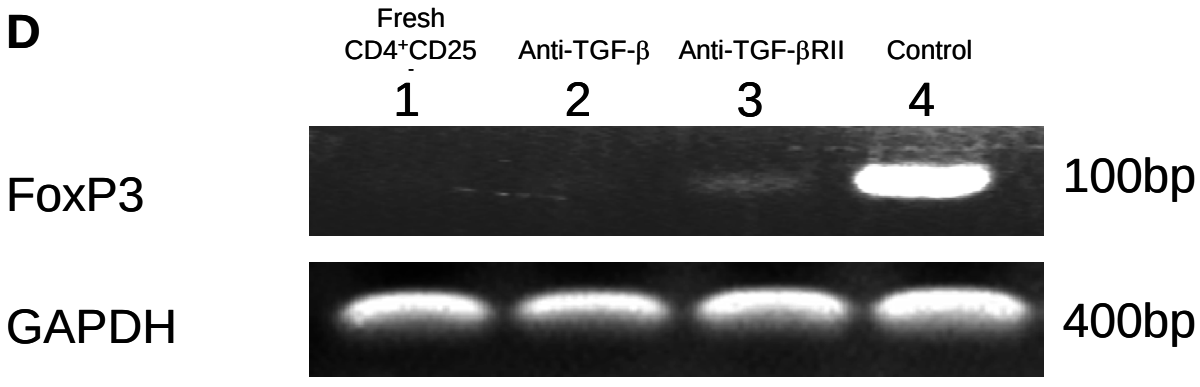
D
Fresh
CD4+CD25-
Anti-TGF-β
Anti-TGF-βRII
Control
1
2
3
4
FoxP3
100bp
GAPDH
400bp

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## SUMMARY

The preceding experiments have demonstrated the following with regard to further characterization of feline $CD4^+CD25^+$ Treg cells and the extra-thymic generation of Treg-like cells from $CD4^+CD25^-$ Th cells:

1. Feline FoxP3 is expressed significantly in $CD4^+CD25^+$ Treg cells and can be up-regulated $CD4^+CD25^-$ Th cells by TGF-β plus Con A
    a) FoxP3 primers designed from the published human sequence can be used to amplify a product from feline MYA-1 cell lines and $CD4^+CD25^+$ Treg cell
    b) Sequence analysis of the 385 bp product amplified by human FoxP3 primers revealed greater than 90% homology with both human and macaque FoxP3 published sequences
    c) Con A plus TGF-β1 induces FoxP3 expression in $CD4^+CD25^-$ T cells and correlates with up-regulation of both CD25 and TGF-β surface expression
    d) Con A induces expression of TGF-βRII but not TGF-β on $CD4^+CD25^-$ T cells
    e) Expression of CD25, TGF-β and FoxP3 in $CD4^+CD25^-$ T cells is induced by Con A plus TGF-β1and correlates with Treg-like suppressor function

f) $CD4^+CD25^-$ T cells isolated from both control and FIV-infected cats have equal ability to attain Treg-like function and phenotype after Con A and TGF-β1 treatment

g) Induction of regulatory phenotype and function in $CD4^+CD25^-$ T cells is dependent on both activation and TGF-β treatment

h) Blockade of TGF-βRII on Con A activated $CD4^+CD25^-$ T cells abrogates TGF-β1 mediated induction of both surface TGF-β and FoxP3 mRNA

i) Blockade of TGF-β on Con A plus TGF-β converted $CD4^+CD25^-$ T cells abrogates suppressor function

j) Induction of Treg-like function in $CD4^+CD25^-$ T cells correlates with induction both surface TGF-β and FoxP3 mRNA

2. $CD4^+CD25^+$ Treg cells induce Treg-like function in $CD4^+CD25^-$ Th cells

a) There is an increase of $CD4^+CD25^+$ T cells as a percentage of total $CD4^+$ T cells in PBMC of FIV-infected cats compared to age matched SPF control cats

b) The ratio of $CD4^+CD25^+$ Treg cells to $CD4^+CD25^-$ T cells is significantly increased in the PBMC of chronically FIV-infected cats and may be a predictor of disease progression

c) Surface TGF-β was significantly up-regulated on $CD4^+CD25^+$ Treg cells and to a lesser extent on $CD4^+CD25^-$ T cells from PBMC of

chronically FIV-infected cats, while expression was low on both $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells from PBMC of SPF control cats

d) $CD4^+CD25^+$ Treg cells from normal cats activated in vitro with IL-2 and LPS can induce Treg function in $CD4^+CD25^-$ T cells

e) $CD4^+CD25^-$ T cells or $CD4^+CD25^+$ Treg cells from normal cats not activated with LPS were unable to induce suppressor function in $CD4^+CD25^-$ T cells

f) $CD4^+CD25^+$ Treg cells from FIV-infected cats induce suppressor function in $CD4^+CD25^-$ T cells without in vitro activation

g) Blockade of TGF-β on Treg cells before co-culture with $CD4^+CD25^-$ T cells inhibits up-regulation of TGF-β on the $CD4^+CD25^-$ T cells

h) Blockade of TGF-βRII on $CD4^+CD25^-$ T cells before co-culture with Treg cells inhibits up-regulation of TGF-β on the $CD4^+CD25^-$ T cells

i) Blockade of TGF-β on Treg cells before co-culture with $CD4^+CD25^-$ T cells abrogates induction of suppressor function in the $CD4^+CD25^-$ T cells

j) Blockade of TGF-βRII on $CD4^+CD25^-$ T cells before co-culture with Treg cells abrogates induction of suppressor function in the $CD4^+CD25^-$ T cells

k) Blockade of TGF-β on Treg converted $CD4^+CD25^-$ T cells abrogates suppressor function

l) FoxP3 mRNA is up-regulated in $CD4^+CD25^-$ T cells from either normal or control cats when co-cultured with freshly isolated $CD4^+CD25^+$ Treg cells from FIV-infected cats but not control cats

## 6. CONCLUSION

Studies conducted on peripheral blood lymph node cells from HIV, FIV, and other retroviral infected individuals have suggested that $CD4^+CD25^+$ T cells are increased by chronic retroviral infection. Further investigation of these cells has shown they exhibit characteristic phenotype and function of T regulatory cells. Several possible mechanisms could explain this increase of Treg cells in infected subjects, including expansion of natural Treg cells, chronic antigen stimulation of $CD4^+CD25^-$ T cells, and direct cell mediated conversion of $CD4^+CD25^-$ T cells. Although it is well documented that TGF-β can induce Treg function in activated $CD4^+CD25^-$ T cells and reports suggest that $CD4^+CD25^+$ Treg cells also induce Treg function in activated $CD4^+CD25^-$ T cells, the mechanism of cell mediated conversion is poorly understood.

Studies pertaining to $CD4^+CD25^+$ Treg cells and the immune status of HIV-infected individuals are conflicting in their conclusions. An increase in Treg cells could favor the host by controlling HIV/FIV mediated hyper-activation antigen specific CD4 and CD8 CTL responses and limiting bystander cell destruction. Alternatively, increases in Treg cells could amplify retroviral induced immunosuppression by down-regulating HIV/FIV

and other pathogen specific T and B cell responses when stimulated with their cognate antigen.

To assess the effects of $CD4^+CD25^+$ Treg cells on $CD4^+CD25^-$ T cells we established a 5 day co-culture system using a membrane dye to label the Treg cells before co-culture with $CD4^+CD25^-$ T cells. Subsequently, we were able to FACS purify the original $CD4^+CD25^-$ T cells based on membrane staining, as activation by Con A induces CD25 expression in >90% of the population. This allowed us to further analyze the phenotype and function of the recovered cells. We were able to conclude that co-culture with in vitro activated or in vivo FIV activated Treg cells induced both surface TGF-β and FoxP3 mRNA in the recovered $CD4^+CD25^-$ T cells. Furthermore, these cells were able to suppress proliferation of freshly stimulated $CD4^+CD25^-$ T cells.

To investigate the mechanism of the induced Treg function and phenotype, we used blocking antibodies to TGF-β and TGF-βRII. This approach provided evidence that TGF-βRII on the surface of $CD4^+CD25^-$ T cells as well as TGF-β on the surface of Treg cells played critical roles in the development of Treg phenotype and function in $CD4^+CD25^-$ T cells subjected to the co-culture system. Further examination of the function of theses converted cells revealed a TGF-β dependent mechanism of suppression.

In conclusion, our results have important implications with respect to HIV/FIV infection pertaining to disruption of $CD4^+$ T cell homeostasis by providing evidence that there is at least a mechanism through TGF-β/TGF-βRII interaction that would allow for an increase in $CD4^+CD25^+$ Treg cell numbers during chronic lentiviral infection. Our studies have shown that chronically activated $CD4^+CD25^+$ Treg cells isolated from FIV-infected cats,

not only have the ability to suppress the proliferation of $CD4^+CD25^-$ T cells but also have the ability to induce suppressor function in these cells. The expanded population of Treg cells could be responsible for the decreases in CD4 Th numbers observed during HIV/FIV infection by depleting the Th pool or by increased potential for anergizing CD4 Th cells shortly after activation. We believe that the expanded population of Treg cells will have a negative effect on the status of FIV infection as $CD4^+CD25^+$ T cells are a reservoir for productive FIV infection. In addition, as activation of $CD4^+CD25^-$ T cells is a requirement for their conversion, these converted Treg cells are subject to repeated antigen specific activation and could therefore contribute to the profound immunodeficiency in response to HIV/FIV and other pathogenic antigens observed in HIV/FIV infected individuals.