ABSTRACT

EMANI, SIRISHA. MOLECULAR CHARACTERIZATION OF T REGULATORY CELLS IN FIV-INFECTION (Under the direction of Dr. Wayne Tompkins)

Naturally occurring $CD4^+CD25^+$ T regulatory cells (Treg) play important roles in maintaining immunologic self-tolerance in addition to controlling the magnitude of anti-microbial immune responses. However, the capacity of these $CD4^+CD25^+$ Treg cells to control immune responses both in vivo and in vitro is not well established. $CD4^+CD25^+$ Treg cell-mediated suppression can control autoimmune diseases; transplantation tolerance and graft verses host disease and, in contrast hinder tumor immunity and immunity to infectious agents. As Treg cells have been reported to be involved in several diseases, this study focused on molecular characteristics that enables them to maintain anergy and also resistance to programmed cell death along with the effect of FIV-infection on regulation of the above phenotypic characteristics. Our results show that feline $CD4^+CD25^+$ Treg cells are phenotypically and functionally anergic as indicated by elevated levels of the cyclin dependent kinase inhibitors, CdkI's, ($p21^{cip1}$,$p16^{ink4}$, and $p27^{kip1}$) , and resistance to mitogen-induced proliferation compared to their counter parts $CD4^+CD25^-$ T cells. Importantly, CdkI's are constitutively over-expressed only in FIV-infected cats. As expected Treg cells from FIV-infected cats that over-expressed CdkI's expressed low levels of the cyclins (mainly cyclins D) and phosphorylated retinoblastoma protein (pRb) that are responsible for cell cycle progression. We investigated the role of TGFβ signaling and found that TGFβ1 plus ConA stimulation was able to convert $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T cells with functional and phenotypic characteristics including upregulation of CdkI's and bcl-2. The differential expression of CdkI's and bcl-2 between the two $CD4^+$ T cell subsets may be linked to TGFβ-Smad pathway. Consistent with upregulation of CdkI's and bcl-2, we found that although natural and TGFβ1 converted $CD4^+CD25^+$ Treg cells are anergic, they are more resistant to activation induced cell death compared to $CD4^+CD25^-$ T cells functionally which correlated with increased bcl-2 to bax ratio in Treg cells. Thus, the molecular characterization of this unique population of Treg cells may be essential for understanding their role and function for developing effective therapeutics and vaccination especially against chronic infections such as Acquired Immune Deficiency Syndrome (AIDS).

MOLECULAR CHARACTERIZATION OF T REGULATORY CELLS IN FIV-INFECTION

BY

SIRISHA EMANI

A dissertation submitted to the Graduate Faculty of

North Carolina State University

In partial fulfillment of the

Requirements for the degree of

Doctor of Philosophy

Immunology

Raleigh, NC

2006

Approved by

_____

Dr. Wayne A Tompkins

Chair of Advisory Committee

_____ _____

Dr. Mary B Tompkins **Dr. Barbara Sherry**

_____

Dr. Frederick J Fuller

PERSONAL BIOGRAPHY

Sirisha Emani was born on August 12th, 1978, in Hyderabad , India. She received her primary education until high school from St. Ann's convent school, Hyderabad, India. She majored in Microbiology, Genetics, and Chemistry and obtained her bachelor's degree from Osmania University, Hyderabad, Andhra Pradesh, India. She started her research career as a junior scientist at Cirrus Pharmaceuticals, Research Triangle Park, North Carolina for a year from 1998-1999. She worked as a research analyst at Duke University Medical Center from 1999-2001. She then took admission as a graduate student in the PHP department, Immunology program, North Carolina State University, College of Veterinary Medicine to study the molecular characteristics of $CD4^{+}CD25^{+}$ T regulatory cells in relation to Feline Immunodeficiency Virus infection under the guidance of Dr. Wayne Tompkins. She received a doctorate degree in Immunology in spring 2006. Her future plans are to pursue post-doctoral training initially at Duke Medical Center for six months and then move to Boston, Massachusetts, for further educational advancement.

ACKNOWLEDGEMENTS

I would like to express gratefulness to my major advisor, Principle Investigator, and the director of the Immunology program, Dr. Wayne Tompkins for his constant enthusiasm, encouragement, and involvement in designing, modulating and helping me complete this research project. I would also like to thank Dr. Mary Tompkins for her support through out this research study and for allowing me to access the equipment, materials, and mainly the experimental animals required for the study. I would like to thank my committee members Dr. Frederick Fuller, Dr. Barbara Sherry, Dr. Mary Tompkins, and Dr. Wayne Tompkins for their valuable suggestions towards this work. I would also like to thank Dr. Thomas Siopes my external committee representative for his time taken to attend the important committee meetings. I would like to acknowledge the support provided by Deborah Anderson mainly with regard to experimental animal samples used in this study. I would like to thank Janet Dow for providing unlimited help with flow cytometry analysis and cell sorting facility. A special thanks to Linda Costine, our administrative coordinator for scheduling committee meetings, exams, and so on….Lastly I would like to appreciate the assistance given by the Immunology faculty members during the course of my study at North Carolina State University to help achieve my goal with flying colors.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1 : IMMUNE SUPPRESSOR FUNCTION OF $CD4^+CD25^+$ T regulatory CELLS IN FIV-INFECTED CATS IS ASSOCIATED WITH OVER-EXPRESSION OF CELL SURFACE TGF_1 AND TGF_RII AND UP-REGULATION OF CYCLIN DEPENDENT KINASE INHIBITORS.

CHAPTER 2 : TGFβ1/TGFβRII SIGNALING MAY PLAY A KEY ROLE IN THE MAINTENANCE OF ANERGIC STATE OF $CD4^+CD25^+$ T REGULATORY CELLS, YET ENABLING THEIR RESISTANCE TO ACTIVATION INDUCED APOPTOSIS VIA DIFFERENTIAL EXPRESSION OF SEVERAL G0/G1 CELL CYCLE PROTEINS.

LIST OF ABBREVIATIONS

AICD- Activation Induced Cell Death

Apaf-1- Apoptosis protease activating factor-1

APC- Allophycocyanin

CAD- Caspase Activated DNase

Cdk- Cyclin dependent kinase

CdkI- Cyclin dependent kinase Inhibitor

CREB-c AMP Response Element Binding Protein

Con-A- Concavalin-A

FITC- Fluorescein Isothyocynate

FIV- Feline Immunodeficiency Virus

GITR- Glucocorticoid-Induced TNF Receptor

rhIL-2- recombinant Interleukin-2

LPS- Lipopolysaccharide

NFκB- Nuclear Factor associated with Kappa light chain of B cells

PBMC- Peripheral Blood Mononuclear cells

PE- Phycoerythrin

PI- Propidium Iodide

TCR- T cell receptor

TGFβ – Transforming Growth Factor-beta

TNFα– Tumor Necrosis Factor-alpha

Treg – $CD4^{+}CD25^{+}$ T regulatory cells

1. INTRODUCTION

$CD4^+CD25^+$ T regulatory cells (Treg) are naturally anergic, arrested in the G0/G1 phase of the cell cycle. Similar to anergic $CD4^+$ Th cells, $CD4^+CD25^+$ Treg cells fail to produce IL-2 and proliferate in response to TCR engagement by antigens, but respond to polyclonal stimuli such as IL-2 (Powell, Ragheb et al. 1998; Lechler, Chai et al. 2001) . Naturally occurring $CD4^+CD25^+$ Treg cells, comprising 5-10% of the circulating $CD4^+$ T cell population, were originally defined as regulatory T cells that maintain peripheral self-tolerance (Maloy and Powrie 2001; Shevach 2002). More recent studies have demonstrated that Treg cells also play a major role in modulating the magnitude and duration of protective T cell immune responses to pathogens (Read, Malmstrom et al. 2000; Iwashiro, Messer et al. 2001; Belkaid, Piccirillo et al. 2002; Hori, Carvalho et al. 2002; Montagnoli, Bacci et al. 2002; Hisaeda, Maekawa et al. 2004; Belkaid and Rouse 2005). These pathogen-induced Treg cells, similar to natural Treg cells, are anergic and possess potent immunosuppressor function once activated by TCR engagement and/or certain nonspecific stimuli (Belkaid and Rouse 2005).

Treg cells are arrested in the early G0/G1 stage of the cell cycle, suggesting that anergic factors influence early events in the cell cycle. Progression through the G0/G1 to S phase of the cell cycle is regulated by several cyclins and cyclin-dependent kinases (Cdk) whose activities are in turn controlled by their inhibitors, CdkI's. Jackson SK reported that Th1 cell anergy could be linked to increased expression of the cyclin-dependent kinase inhibitors, $p21^{cip1}$ and $p27^{kip1}$. Current models propose that $p27^{kip1}$ inhibits cyclin-

dependent kinase-2 (cdk2) expression, thus maintaining the cell in a quiescent state(Coats, Whyte et al. 1999; Mohapatra, Agrawal et al. 2001; Mohapatra and Pledger 2001). Down-regulation of $p27^{kip1}$ is associated with entry of cells from G1-S phase and its up-regulation is associated with G_1 growth arrest. $p27^{kip1-/-}$ mice exhibit gigantism with disproportionately enlarged spleen, thymus, and lymph nodes (Fero, Rivkin et al. 1996; Kiyokawa, Kineman et al. 1996; Nakayama, Ishida et al. 1996), suggesting the importance of $p27^{kip1}$ in regulating the homeostatic proliferation of T cells in vivo. On the other hand, reports suggest that $p21^{cip1}$ may either act as an inhibitor of apoptosis (Suzuki, Tsutomi et al. 1999) or a promoter of apoptosis (Peschiaroli, Figliola et al. 2002). T cell development in mice deficient in $p21^{cip1}$ occurs normally. But, $p21^{cip1}$ knockout mice rapidly lose tolerance to nuclear antigens and develop an autoimmune-like disease reminiscent of systemic lupus in humans (Balomenos, Martin-Caballero et al. 2000), suggesting their role in regulating T cell proliferation and homeostasis.

T cell apoptosis also plays a critical role in maintaining immune homeostasis. It has been reported that human $CD4^+CD25^+$ Treg cells are prone to apoptosis which is associated with decreased levels of bcl-2 expression (Taams, Smith et al. 2001). Conversely, others have reported that mouse $CD4^+CD25^+$ Treg cells are resistant to apoptosis, as they do not undergo clonal deletion by viral superantigen *in-vivo* (Papiernik, do Carmo Leite-de-Moraes et al. 1997), and are resistant to Fas-induced apoptosis *in-vitro* (Banz, Pontoux et al. 2002). This urged us to determine if the reported resistance to activation induced cell death (AICD) of feline $CD4^+CD25^+$ Treg cells compared to their counterparts

$CD4^+CD25^-$ T cells in correlation with the expression of anti-apoptotic proteins and pro-apoptotic proteins involved in the mitochondrial apoptotic pathway.

Recently, it has been suggested that $CD4^+CD25^+$ Treg cell homeostasis can also be maintained in the presence of IL-2 and TGFβ1 (Horwitz, Zheng et al. 2004). The development of T regulatory cells (Fontenot, Gavin et al. 2003) and Treg cell suppressor activity (Chen, Jin et al. 2003), is known to be programmed by the transcription factor Foxp3, which is selectively regulated in peripheral immune compartments by TGFβ1, a major growth regulatory cytokine. TGFβ1 induces the expression of $p21^{cip1}$ and $p27^{kip1}$ in various cell types. Depending upon the cell type and the environment TGFβ1 exerts variable effects. Upon stimulation with TGFβ1 lymphocytes exhibit either a G_1 arrest or apoptosis. TGFβ1 is expressed in the G_1 phase of the cell cycle in growth-arrested cells (Howell, Sun et al. 1993) and binding of TGFβ1 to its receptor activates a wide range of pathways during cell cycle progression. TGFβ1 is constitutively expressed at elevated levels on $CD4^+CD25^+$ Treg cells, that are arrested in the G0/G1 phase of the cell cycle, compared to $CD4^+CD25^-$ T cells (Maggi, Cosmi et al. 2005). TGFβ1 by itself or along with other superfamily members, such as BMPs, and activins, can induce $p21^{cip1}$ transcription, an event that is thought to link cell growth inhibition, and/or apoptosis depending upon the target cell type (Zauberman, Oren et al. 1997; Ishisaki, Yamato et al. 1998; Jernvall, Aberg et al. 1998; Ghosh-Choudhury, Ghosh-Choudhury et al. 2000; Franzen and Heldin 2001). It has been a subject of debate whether TGFβ1-induced $p21^{cip1}$ expression is linked to inhibition of cell proliferation (Hu et al.,1998; Cheng et al., 2001). Transcriptional induction of $p21^{cip1}$ responsiveness to TGFβ1 could be mediated

either by SMAD pathway or by Ras/mitogen activated protein kinase (MAPK) pathway (Kivinen, Tsubari et al. 1999; Pardali, Kurisaki et al. 2000; Pouliot and Labrie 2002). The $p21^{cip1}$ promoter possesses binding site/s for transcription factors of the Sp1 family along with Smads (Moustakas, Pardali et al. 2002) which is targeted by both Smad- and Ras/MAPK-mediated pathways. TGFβ1 induces phosphorylation of cyclic AMP response element binding proteins (CREB) via MAPK pathway. IL-2Rα gene possesses binding sites for CREB/ATF, AP-1 and Smad3. It has also recently been reported that TGFβ1 along with anti-CD3 and anti-CD28 stimulation could induce IL-2Ralpha expression (CD25). Also the anti-apoptotic gene bcl-2 processes a CREB binding site on its promoter region (Wilson, Mochon et al. 1996). Therefore, TGFβ1 along with TCR stimulation could induce the expression of CD25, bcl-2, as well as the cell cycling protein inhibitors such as $p21^{cip1}$. Thus, the TGFβ1 pathway may be a useful mechanism to explain the phenomenon of cell growth inhibition, yet programmed cell survival in Treg cells.

2. BACKGROUND AND SIGNIFICANCE

A. Generation of $CD4^+CD25^+$ Tregulatory cells

Although the existence of a separate subset of T cells specifically involved in suppression of immune responses was proposed 30 yrs ago, it was only over the past 5-10 years that a distinct lineage of $CD4^+CD25^+$ T regulatory cell population or suppressor cells was identified. The function of the thymus, in addition to regulatory T cell differentiation and selection, is to maintain immunological self-tolerance by producing $CD4^+CD25^+$ Treg cells capable of preventing autoimmune diseases (Apostolou, Sarukhan et al. 2002). Clonal deletion, one of the major mechanisms for distinguishing self/non-self by the immune system, can establish tolerance to self-reactive B and T lymphocytes during their development in the thymus. However, the deletion mechanism is considered to be leaky, as it allows some of the self-reactive lymphocytes to escape clonal deletion and exit to the periphery. For the past few years it has become evident that Treg cells actively suppress self-reactive T cells in the periphery, avoiding the onset of harmful auto-immune diseases.

It has been demonstrated that the Treg cells acquire their regulatory activity through the thymic selection process and are released in the periphery as functionally committed $CD4^+CD25^+$ Treg cells (Apostolou, Sarukhan et al. 2002). Apart from their generation in the thymus from a defined lineage, recent studies have shown that these $CD4^+CD25^+$

Treg cells can also be activated in the periphery in response to self antigens or pathogen products (Thorstenson and Khoruts 2001; Zhang, Izikson et al. 2001; Hauet-Broere, Unger et al. 2003) or can be converted from normal $CD4^+CD25^-$ T cell subsets (Chen, Jin et al. 2003) via the TGFβ1 signaling pathway .

B. Phenotypic characteristics of Treg cells

$CD4^+CD25^+$ Treg cells constitute 5-10% of the peripheral $CD4^+$ T cells in mice, rats, cats, and humans (Maloy and Powrie 2001; Shevach 2002; Vahlenkamp, Bull et al. 2004). Several distinct subsets of T regulatory/suppressor cells had been described by the expression of surface markers and/or cytokine production profiles. The thymus-derived, naturally occurring $CD4^+CD25^+$ Treg cells, TGFβ1 producing Th3 cells (Fukaura, Kent et al. 1996) and IL-10 secreting Tr1 cells (McGuirk and Mills 2002) appear to be phenotypically different, yet functionally similar, as they suppress the proliferation and IL-2 production by stimulated T cells (Vahlenkamp, Tompkins et al. 2004). Although the CD25 (interleukin-2 receptor chain alpha) molecule is expressed on all conventional activated T cells, and not all T regulatory cells express CD25, to date CD25 is still used as a marker to characterize and isolate T regulatory cells. Several other cell surface markers and intracellular markers had been reported to be constitutively expressed by thymus-derived naturally occurring Treg cells, which slightly differ in murine and human species. Murine thymus-derived Treg cells constitutively co-express $CD25^+$, $CD122^+$ $CD69^+$, $CD44^+$, $CD45RB^{low}$, $GITR^+$, $CD103^+$(alphaE-integrin), $CD134^+$(OX-40), $CD54^+$(ICAM), whereas human thymus-derived Treg cells express $CD25^{high}$,$CD122^+$,

HLD-DR^{+},CD45RO^{+},CD95^{high},CD45RB^{low},CD38^{low}, CD62^{low},and GITR^{+} phenotype (Asano, Toda et al. 1996). Recent studies on human and murine Treg cells have used additional markers such as CD62L-selectin along with CD25 to differentiate activated T cells from Treg cells. Activated T cells down regulate CD62L-selectin (Mobley and Dailey 1992), whereas Treg cells constitutively express elevated levels of CD62L-selctin (McHugh, Whitters et al. 2002). The development of T regulatory cells (Fontenot, Gavin et al. 2003) and T cell suppressor activity (Curotto de Lafaille, Muriglan et al. 2001), is known to be programmed by the transcription factor Foxp3, which is selectively expressed in CD4^{+}CD25^{+} Treg cells. While Foxp3 is perhaps, the most selective marker for Treg cells, its usefulness for detection and isolation of Treg cells is somewhat limited by its intracellular location. The other markers associated with CD4^{+}CD25^{+} Treg cells are CTLA-4 and glucocorticoid –induced TNF receptor (GITR), that are induced by Foxp3 (Hori and Sakaguchi 2004). Recent data suggest that cell surface expressed TGFβ may be a useful marker for activated CD4^{+}CD25^{+} Treg cells (Chen and Wahl 2003).

C. Functional characteristics of Treg cells

Apart form the use of the above described phenotypic markers, Treg cells can be best identified using their functional properties. A classical feature of CD4^{+}CD25^{+} Treg cells is that they are anergic upon TCR mediated-stimulation in vitro, failing to produce IL-2 and undergo proliferation (Lechler, Chai et al. 2001). Some studies have reported that T cell anergy could be overcome by culturing Treg cells in the presence of IL-2 or by addition of anti-CD28 antibody that induces endogenous IL-2 production (Thornton and

Shevach 1998).

The other major functional characteristic of Treg cells is their ability to suppress proliferation and cytokine production by other activated T cells. T cell suppression by Treg cells is cell contact-dependent and occurs when Treg cells are activated via their T cell receptors. Several recent studies have also shown that TCR ligation is not always required for Treg cell immunosuppression of other T cells (Caramalho, Lopes-Carvalho et al. 2003). Treg cells express several Toll-like receptors (TLRs) including TLR-4, a receptor for LPS which can activate murine (Caramalho, Lopes-Carvalho et al. 2003) and feline Treg cells (Vahlenkamp, Bull et al. 2004). Also Vahlenkamp et al. (Vahlenkamp, Bull et al. 2004) reported that potent Treg cell suppressor function could be induced by ConA or rhIL-2 stimulation.

D. Activation of Treg cells in the peripheral immune compartment

$CD4^+CD25^+$ T regulatory cells possess immune suppressive activity in vitro, and can suppress the proliferation and cytokine production of other T cells, both $CD4^+$ and $CD8^+$, via an undefined cell contact-dependent mechanism (Takahashi, Kuniyasu et al. 1998; Thornton and Shevach 1998). On the other hand, $CD4^+CD25^+$ Treg cells stimulated with antigen presenting dendritic cells or IL-2 lose their anergic phenotype and can proliferate comparable to $CD4^+CD25^-$ T cells in-vivo and in-vitro while retaining the suppressor function (Yamazaki, Iyoda et al. 2003) Some authors reported that CTLA-4 is constitutively expressed on Treg cells and mediates T cell suppression via inhibition of

IL-2 expression (Krummel and Allison 1996; Read, Malmstrom et al. 2000). Others reported that CTLA-4 is not essential for suppression of other lymphocytes, as Treg-mediated suppression exists even in CTLA-4-/- mice. On the other hand, CTLA-4 engagement upregulates TGFβ and TGFβRII surface expression on Treg cells, which enhances their suppressor function (Chen and Wahl 2002). In contrast, glucocorticoid-induced TNF receptor (GITR), which is also predominantly expressed on $CD4^+CD25^+$ Treg cells, counterbalances their suppressor activity once it has been engaged by its ligand (McHugh, Whitters et al. 2002; Shimizu, Yamazaki et al. 2002).

It has also been reported in rodents that ligation of GITR with soluble GITR ligand (sGITR-L) can induce GITR-dependent NFκB activation, thereby up-regulating IL-2 and thus blocking in-vitro suppression mediated by Treg cells (Ji, Liao et al. 2004). Also, engagement of GITR on Treg cells with its ligand antagonizes TGFβ signal transduction by inhibiting phosporylation of Smad2/3, down strem signaling molecules of TGFβ-signaling pathway, to block suppression (Chen, Jin et al. 2003).

It has been well established that TGFβ1 is involved in down-regulation of T cell–mediated responses and in controlling autoimmunity (Letterio and Roberts 1998).TGFβ1 produced by Treg cells, bound to its receptor, expressed on other activated T cells could induce suppression of other T cells in a cell contact-dependent mechanism (Gorelik and Flavell 2000; Leveen, Larsson et al. 2002). In support of this, TGFβRII deficient mice exhibit uncontrolled T cell proliferation (Nakamura, Kitani et al. 2001; Zhang, Izikson et al. 2001). Furthermore, TLR-4 triggering of dendritic cells (DCs) has been reported to

induce IL-6, and IL-1 production, enhance Treg proliferation, and reduce the suppressive activity of $CD4^+CD25^+$ Tregs via GITR-dependent pathway (Pasare and Medzhitov 2003; Yamazaki, Iyoda et al. 2003). Th cell activation requires engagement of dendritic cells co-stimulatory molecules along with TLR-induced cytokines down-regulate Treg cell-mediated suppression during infection (Pasare and Medzhitov 2003). This could be one of the multiple mechanisms in which activation of cells of the innate immune system can override the suppressor activity of $CD4^+CD25^+$ Tregs, thus permitting adaptive immune responses to antimicrobial Ags to supersede the suppressive response to self Ags.

E. Involvement of T regulatory cells in auto-immune and infectious diseases

The $CD4^+CD25^+$ T regulatory cell population has a broad repertoire of receptors that recognize various self and non-self pathogen-associated antigens enabling Treg cells to regulate a broad range of antigen-specific responses. In the case of autoimmune diseases, it has been shown in several rodent models that depletion of $CD4^+CD25^+$ Treg cells leads to severe autoimmunity disorders, and passive transfer of these cells prevents autoimmune disease (Salomon, Lenschow et al. 2000; Stephens and Mason 2000). Treg cells have now been known to regulate a wide range of diseases that includes organ-specific autoimmune diseases (including gastritis, thyroiditis and insulin-dependent diabetes) (Salomon, Lenschow et al. 2000; Stephens and Mason 2000; Furtado, Olivares-Villagomez et al. 2001), inflammatory bowel disease (Read, Malmstrom et al. 2000), inflammatory lung disease (Hori, Carvalho et al. 2002), allograft rejection (Hara, Kingsley et al. 2001; Taylor, Noelle et al. 2001; Graca, Thompson et al. 2002), graft

versus host disease (Hoffmann, Ermann et al. 2002; Taylor, Lees et al. 2002), and allergy (Curotto de Lafaille, Muriglan et al. 2001).

In addition to regulating autoimmune and inflammatory diseases, Treg cells regulate sterilizing immunity to infectious organisms such as protozoa (Belkaid, Piccirillo et al. 2002), and fungi (Netea, Sutmuller et al. 2004), bacteria (Raghavan, Suri-Payer et al. 2004), and viruses (Aandahl, Michaelsson et al. 2004). It has also been shown that Treg cells have the ability to regulate innate immune natural killer T cells to prevent Helicobacter hepaticus-triggered colitis in T cell-deficient mice models (Maloy, Salaun et al. 2003). The other major role of Treg cells is the suppression of pathogen-induced inflammatory responses. These pathogen-specific Treg cells can prevent immuno-pathology induced by infections, but in contrast, can also increase the pathogen load and prolong persistence by suppressing protective immune responses. It has been recently suggested that the decreased pathogen induced responses lead to prolong persistence of infection which could serve as a mechanism for the maintenance of pathogen specific immunological memory. Depletion of Treg cells from mice infected with Leishmania major or Pneumocystis carini initiated an effective adaptive response but resulted in severe inflammation (Belkaid 2003). Similar immune responses were seen in $CD4^+CD25^+$ Treg cells depleted mice with malarial parasites but with hindered memory immune responses (Aseffa, Gumy et al. 2002; Belkaid, Piccirillo et al. 2002; Hori, Carvalho et al. 2002; Hisaeda, Maekawa et al. 2004). Recent studies also demonstrated that removal of Treg cells from the PBMC of HIV-infected individuals resulted in an effective antigen specific response as measured by cytokine production by antigen

stimulated $CD4^+$ and $CD8^+$ effector T cells (Aandahl, Michaelsson et al. 2004; Kinter, Hennessey et al. 2004). As Treg cells are known to be involved in several diseases, the phenotypic and genotypic characterization, and understanding their role and function may be useful for devising effective therapeutics and vaccination, especially against chronic infections such as AIDS.

F. Role of IL-2 in T regulatory cell function and homeostasis

According to the classical model of T cell activation, productive stimulation of T cells requires two major signals. The first is the engagement of MHC on APC with the TCR, and the second is the interaction of B7 on the APC with the T cell co-stimulatory receptor CD28 (Azuma, Yssel et al. 1993; Powell, Ragheb et al. 1998). In a normal cell, B7 receptors on APC initially engage the CD28 receptor on $CD4^+$ T cells transducing a signal that upregulates IL-2 production, an essential cytokine for transit out of the G0/G1 stage of the cell cycle, cell proliferation and the initiation of an immune response (Krummel and Allison 1995; Krummel and Allison 1996). Following APC activation T cells upregulate CTLA-4 on their surface and upon engagement of B7, transduces a signal for the down-regulation of IL-2 and termination of the immune response. If the T cells are stimulated only through the TCR, these T cells also fail to produce IL-2 enters G0/G1 arrested state of long-lasting, partial or total unresponsiveness termed anergy (Karandikar, Vanderlugt et al. 1996; Bluestone 1997; Scheipers and Reiser 1998; Walunas and Bluestone 1998). Anergic T cells possess increased expression of B7 and CTLA-4 molecules upon mitogen stimulation, yet fail to produce IL-2 and proliferate

upon re-stimulation with antigen-competent APC, but respond to polyclonal stimuli such as IL-2 (Chai and Lechler 1997; Powell, Ragheb et al. 1998; Lechler, Chai et al. 2001). The signaling pathway that is involved in IL-2 down-regulation in anergic cells is not well established.

IL-2 signaling is essential for Treg cell development in the thymus, homeostasis and function. On one hand, IL-2 potentially stimulates T cell growth and proliferation, and in the absence of IL-2, Treg cells cannot proliferate or survive in the thymus or in the periphery (Curotto de Lafaille, Lino et al. 2004; Bayer, Yu et al. 2005; Setoguchi, Hori et al. 2005). In contrast, IL-2 is necessary for the maintenance of homeostasis and tolerance to self (Malek and Bayer 2004; Nelson 2004). In the absence of IL-2 or its receptor, a lymphoproliferative auto-immune syndrome was reported in several rodent models (Sadlack, Merz et al. 1993; Suzuki, Kundig et al. 1995; Willerford, Chen et al. 1995) suggesting that IL-2/IL-2R is essential for Treg homeostasis and function.

It has been reported that $CD4^+CD25^+$ Treg cells from mice, humans, and cats constitutively express Foxp3 (Fantini, Becker et al. 2004). Foxp3, a member of fork-head transcription factors has been known to be important for the development and function of Treg cells. Foxp3 has the ability to inhibit IL-2, IL-4, and IFNγ production by direct interaction with the Rel family transcription factors, nuclear factor of activated T cells (NFAT) and NFκB, which are essential for target gene expression. Foxp3 may also directly bind to IL-2 promoter and block IL-2 transcription. Thus, while it appears that Treg cells are programmed for low levels of IL-2 production, some IL-2 is required for

their survival. How this balance is maintained is not known. However recent data showing a consensus binding element in the IL-2α gene for Smad4 may provide some insight. Kim et al. (Kim, Kim et al. 2005) identified a novel positive regulatory region in the IL-2α gene that mediates TGFβ1 induction of this gene via Smad3 binding. Thus, TGFβ1 signaling in Treg cells could constitutively sustain IL-2α up-regulation and the high affinity IL-2 receptor allowing the survival of Treg cells in the presence of low levels of IL-2.

G. Role of Cyclin dependent kinase Inhibitors (CdkI's) in Treg cell anergy

Anergic cells are blocked at the G0/G1 phase of the cell cycle and this blockade can be induced by cyclin dependent kinase inhibitors (CdkI's). Mitogen-dependent progression through the G1 phase and initiation of DNA synthesis (S phase) during cell cycle is regulated by several cyclin dependent kinases whose activities are in turn controlled by CdkI's. During G1/S phase progression, cyclin D (D1-D3 in most cells and only D2-D3 in lymphocytes) act in mid G1 phase followed by cyclin E and cyclin A in G1/S boundary. Cyclins A and B are involved during S and G2/M phases (Nurse 1994; Morgan 1995; Jackman and Pines 1997; Reed 1997). Cyclin dependent kinases (Cdk's) associate with cyclins and get phosphorylated upon activation. In response to mitogen signals, Cyclin D is induced and associates with Cdk4 and Cdk6 (Sherr 1993), whereas cyclin A associates with Cdk2. The activity of cyclin-Cdk complexes is repressed by CdkI's, which constrain entry into S phase (Hirama and Koeffler 1995; Reed 1997). CdkI's that govern these events have been divided into two groups based on their structure and Cdk

targets. The first class includes INK4 proteins (p16, p15, and p18) and consists of multiple ankyrin repeats, which bind to only cdk4 and cdk6 and are involved in the inhibition of the catalytic subunits of cdk4 and cdk6 (Serrano, Hannon et al. 1993; Guan, Jenkins et al. 1994; Hannon and Beach 1994). The second class of inhibitors includes $p21^{cip1}$, $p27^{kip1}$, p53and $p57^{kip2}$, which contain characteristic motifs within their amino terminal moieties that enable them to bind both cyclins and CDK subunits (Harper, Adami et al. 1993; Toyoshima and Hunter 1994; Lee, Reynisdottir et al. 1995). Action of these CdkI's can affect the activation of several cyclins and Cdk's that are involved in G1/S transition by inhibiting retinoblastoma protein (Rb) phosphorylation. In normal cells the complexes of D cyclins, Cdk4 and Cdk6 phosphorylates the retinoblastoma protein (pRb), causing the release of E2F transcription factors which activates transcription of cyclin E and cyclin A and other cell cycle related genes. Cyclin E binds Cdk2, promoting progression to the S phase (Sherr 1996; Funk and Galloway 1998). $p27^{kip1}$ and $p21^{cip1}$ are well studied CdkI's that regulate anergy. In resting T lymphocytes, $p27^{kip1}$ levels are high and $p21^{cip1}$ levels are low (Jackson, DeLoose et al. 2001). $p27^{kip1}$ is typically up-regulated via growth factor signaling, whereas $p21^{cip1}$ is induced via p53 in response to DNA damage (Philipp-Staheli, Payne et al. 2001; Wolfraim, Walz et al. 2004). As $p21^{cip1}$ is capable of binding with essentially all the cyclin-cyclin-dependent kinase complexes, it plays a major role in regulating T cell cycling and could be a principle factor in maintaining anergy. In support of this, $p21^{cip1}$ knockout mice rapidly lose tolerance to nuclear antigens and develop a lymphoproliferative autoimmune-like disease reminiscent of systemic lupus in humans (Balomenos, Martin-Caballero et al. 2000). To date, contradictory results have been reported by several groups regarding the

effect of $p27^{kip1}$ and $p21^{cip1}$ in cell cycle progression. Jackson S.K.(Jackson, DeLoose et al. 2001), stated that induction of anergy in Th1 cells is associated with increased levels of CdkI's ($p27^{kip1}$,and $p21^{cip1}$) in response to antigenic stimuli but not IL-2. Wolfraim et al. utilized $p21^{cip1-/-}$ and $p27^{kip1-/-}$ knockout mice to show that $p21^{cip1}$ and $p27^{kip1}$ act in synergy to maintain TGFβ induced G0/G1 cell cycle arrest. In contrast to the above studies, Funk J.O (Funk and Galloway 1998), reported that $p27^{kip1}$ and $p21^{cip1}$ are not required for induction and maintenance of T cell anergy.

TGFβ1 has also been shown to induce G0/G1 cell cycle-arrest via transcriptional activation of $p15^{ink4}$, $p21^{cipl}$ and $p27^{kip1}$, and post-transcriptional stabilization of $p21^{cip1}$ expression in a number of cells, including T and B lymphocytes (Kee, Rivera et al. 2001; Massague 2004; Wolfraim, Walz et al. 2004). Although there is little information on how TGFβ1 activates CdkI's expression, recent studies demonstrate that the $p21^{cipl}$ promoter contains consensus elements capable of binding SMAD4, a critical downstream element in the TGFβ1 signaling pathway (Yamato, Hashimoto et al. 2001). Cell surface expressed TGFβ1 has also been associated with activated $CD4^+CD25^+$ Treg cells, although it's role in Treg function is controversial (Maloy and Powrie 2001; Shevach 2002).

H. TGFβ-signaling and $CD4^+CD25^+$ Treg cell homeostasis and function

Historically CD4+CD25+ Treg cells have been defined as a thymus derived distinct $CD4^+$ cell lineage with unique anergic and immunosuppressive properties. Recently, it

has been demonstrated that $CD4^+CD25^+$ Treg cells can also be generated ex-vivo by culturing naïve peripheral $CD4^+CD25^-$ T cells in the presence of mitogen and TGFβ (Horwitz, Zheng et al. 2004). Stimulation of TCR, IL-2R and TGFβR can trigger Foxp3 expression in $CD4^+CD25^-$ T cell precursors and these Foxp3 expressing cells can act like conventional T regulatory cells (Fu, Zhang et al. 2004) . In contrast, Foxp3 expression and the suppressive function are prevented upon co-stimulation with CD28 in an IL-4 dependent manner (Fu, Zhang et al. 2004) .

TGFβ is a potent inhibitor of growth in a variety of cells including lymphoid cells (Zhang, Izikson et al. 2001). TGFβ1 is expressed in the G_1 phase of the cell cycle in growth-arrested cells (Chen, Jin et al. 2003) and binding of TGFβ1 to its receptor activates a wide range of pathways during cell-cycle progression. The inhibition of growth modulated by TGFβ could be via the suppression of the activity of cyclin-dependent kinases which leads to arrest of the cell cycle at G0/G1 phase (Alexandrow and Moses 1997). The inhibitory mechanism by which TGFβ1 modulates cyclin dependent kinases activity involves upregulation of Cyclin-dependent kinase inhibitors ($p21^{cip1}$, $p27^{kip1}$, and $p15^{ink}$) (Iavarone and Massague 1997).

TGFβ superfamily of cytokines contains more than forty proteins including TGFβ1, TGFβ2, and TGFβ3 (Shi, Wang et al. 1998). TGFβRI/II complex formation is induced by the binding of TGFβ ligands that in turn phosphorylates receptor-activated Smads 2/3 (R-Smads). R-Smads form complexes with Smad4 which allows their translocation into the nucleus where the complex interacts with Smad-binding elements (SBE) (Shi, Wang

et al. 1998). R-Smad complex promotes the transcription of several target genes depending upon the combinations of Smad complex interactions with co-activators and inhibitors of gene transcription (Shi and Massague 2003). As of this date there are seven TGFβI, four TGFβRII, and nine Smad 1-9 proteins involved in the TGFβ/ TGFβR signaling pathway.

TGFβ1 ligands bind specifically to a characteristic combination of TGFβRI and TGFβRII, which in turn activates a specific subgroup of R-Smad. Smad7 and Smad6 negatively regulate TGFβ signaling pathway (Shi and Massague 2003). Smad7 can inhibit TGFβ signaling either by preventing the interaction Smad2/3 with TGFβ1 receptor or by interacting with the ubiqutin ligases from the smurf family, which irreversibly terminates the signaling pathway (Bonni, Wang et al. 2001). Smad7 is induced via TGFβ-Smad2/3/4 complex along with AP1 and Sp1 transcription factors, providing an auto-regulatory loop for TGFβ1 / TGFβR signaling (Brodin, Ahgren et al. 2000). On the other hand, as mentioned earlier, Treg cells express Foxp3, and TGFβ1 induces Foxp3 expression which in turn down-regulates Smad7. Foxp3-mediated down-regulation of Smad7 induces a positive auto-regulatory loop of TGFβ-Smad signaling in naturally occurring and TGFβ1-induced Treg cells in human and murine models (Fantini, Becker et al. 2004). This may be one of the mechanisms by which Treg cells could maintain anergy by up-regulation of CdkI's via the positive loop of TGFβ- Smad signaling pathway.

As mentioned earlier, it has been reported that $CD4^+CD25^+$ Treg cells express elevated levels of activation molecules such as CD80, CD86, and CTLA-4 compared to their counterparts $CD4^+CD25^-$ T cells (Vahlenkamp, Bull et al. 2004). CTLA-4 in the presence of TCR ligation has been shown to produce TGFβ1 by purified naïve $CD4^+$ T cells or T cell clones, which may lead to immune suppression (Jonuleit, Schmitt et al. 2001). TGFβ1 is constitutively expressed at elevated levels on $CD4^+CD25^+$ Treg cells (Chen, Jin et al. 2003; Nakamura, Kitani et al. 2004), that are arrested in the G0/G1 phase of the cell cycle, compared to $CD4^+CD25^-$ T cells. TGFβ1 is also known to up-regulate and activate Smad2/Smad3/Smad4 transcription factors (ten Dijke and Hill 2004), which are in turn responsible for the expression of CdkI's that regulate cell cycle progression at the G1 phase of the cell cycle (Zhang and Lin 1997; Moustakas and Kardassis 1998; Ho, de Guise et al. 2004). However, TGFβ1-dependent expression of CdkI's in Treg cells is not yet determined. TGFβ1 by itself or along with other superfamily members, such as BMP's, and activins, could induce $p21^{cip1}$ transcription, an event that is thought to link cell growth inhibition, and/or apoptosis depending upon the target cell type (Zauberman, Oren et al. 1997; Ishisaki, Yamato et al. 1998; Jernvall, Aberg et al. 1998; Ghosh-Choudhury, Ghosh-Choudhury et al. 2000; Franzen and Heldin 2001). It is not clear if TGFβ1-induced $p21^{cip1}$ expression is linked to cell proliferation. Transcriptional induction of $p21^{cip1}$ responsiveness to TGFβ1 could be mediated either by the SMAD pathway or by the Ras/mitogen activated protein kinase (MAPK) pathway (Kivinen, Tsubari et al. 1999; Pardali, Kurisaki et al. 2000; Pouliot and Labrie 2002). $p21^{cip1}$ promoter pocessess binding site/s for transcription factors of the Sp1 family along with Smad's (Moustakas, Pardali et al. 2002) which is targeted by both Smad- and

Ras/MAPK-mediated pathways. Smad complex enhances the binding affinity of Sp1 transcription factor, by direct interaction with the $p21^{cip1}$ promoter DNA along with physical interaction with Sp1 (Pardali, Kurisaki et al. 2000). It has also been demonstrated that TGFβ1 activates Jun-family members that could also induce $p21^{cip1}$ expression by binding to the GC rich motifs on the proximal promoter, by physical interaction with Sp1 transcription factor. TGFβ1 induces phosphorylation of cyclic AMP response element binding proteins (CREB) via the MAPK pathway. IL-2R alpha gene possesses binding sites for CREB/ATF, AP1 and Smad3 transcription factors. TGFβ1 along with anti-CD3 and CD28 stimulation could induce IL-2Ralpha expression (CD25). On the other hand, anti-apoptotic gene bcl-2 can be activated transcriptionally by the transcription factor cAMP-response element binding protein (CREB) through a CRE site in the 5'-flanking region (Wilson, Mochon et al. 1996). Therefore, the TGFβ1 along with TCR stimulation in Treg cells could induce the expression of CD25, bcl-2, and also cell CdkI's such as $p21^{cip1}$. Thus, TGFβ1 pathway may be a useful mechanism to explain the phenomenon of cell growth inhibition yet programmed cell survival in Treg cells.

I. Regulation of apoptotic and anti-apoptotic proteins

The fate of the cell in terms of apoptosis vs. cell survival programming may also depend upon the stage in which IL-2 is down regulated. If IL-2 is deprived at G0/G1 to S phase transition of the cell cycle, which is solely dependent on IL-2, then this, may result in cell survival program (anergy) instead of apoptosis (Krummel and Allison 1996). In contrast, if the cell exited the G1 phase and is at the S or G2 phase of the cell cycle, which also

depends on IL-2, deprivation of IL-2 at this stage of the cell cycle may lead to apoptosis (Krummel and Allison 1996). As $CD4^+CD25^+$ Treg cells are arrested in the G0/G1 phase of the cell cycle, IL-2 may serve as a core component to understand the intracellular molecular mechanisms regulating anergic state and suppressor function of this unique $CD4^+$ T cell population.

The well known apoptotic pathways reported in mammalian cells are the death receptor (TNF-alphaR/TNF-alpha, Fas/FasL) pathway and the mitochondrial pathway (Bcl-family members). The death receptor pathway is activated when extracellular apoptotic ligands bind their receptors on the cells that receive extrinsic apoptotic signals. Upon receiving a death signal, intra-cellular death domains are activated which in turn activate pro-caspase 8 and/or caspase10, promoting an activation signal for the recruitment of caspase 3 (Boatright and Salvesen 2003). Caspase 3 activates caspase activating DNAse (CAD) by cleaving its inhibitor (ICAD) followed by ubiquitination of DNA (Yang, Liu et al. 1997). This death receptor pathway is important in maintaining the homeostasis of the immune system. On the other hand, the mitochondrial pathway is activated via the induction of intrinsic apoptotic signals. During this process mitochondria are permeabilized by pro-apoptotic proteins of the Bcl-family such as bax, and bid, releasing cytochrome C and other mitochondrial proteins (eg. proteases Apaf-1) (Gupta 2003). Cytochrome C binds to apoptotic protease-activating factor (Apaf-1) leading to the activation of caspase-9 and the intrinsic death pathway (Yang, Liu et al. 1997). Anti-apoptotic proteins such as Bcl-2 and Bcl-xL prevent pore formation, thus blocking apoptosis. Therefore, these pathways could be modulated by extracellular signaling cascades via TGFβ1 and intracellular

signaling cascades via MAP kinases.

The fate of the cell exposed to apoptotic stimulus is determined by the balance between the two groups of Bcl-family members. Expression of bcl-2 is known to be an important event in the regulation of cell survival (Adams and Cory 1998). The apparent contradictory responses of different cell types to TGFβ1 signaling may depend upon the relative expression of bcl-2 and bax, as the ratio of bcl-2 to bax in a cell appears to determine survival or apoptosis (Xiao, Oppenlander et al. 2001). A case in point is the different responses of the precursor $CD34^{+}CD38^{-}$ and the committed $CD34^{+}CD38^{+}$ cell to TGFβ1 signaling. The $CD34^{+}CD38^{-}$ precursor with relatively low levels of bcl-2 respond to TGFβ1 by undergoing apoptosis, whereas the committed $CD34^{+}CD38^{+}$ with higher levels of bcl-2 are not sensitive to TGFβ1-induced apoptosis (Xiao, Oppenlander et al. 2001).

TGFβ, an immune regulatory cytokine that plays a major role in homeostasis, has been reported to be involved in the inhibition of Fas or TNFαR mediated cell death (Schlapbach, Spanaus et al. 2000). Recent reports suggested that TGFβ1 induces the expression of Flice-inhibitory protein (FLIP) that could associate with Fas activated death domains, thus preventing the activation of the caspases mediated pathway (Irmler, Thome et al. 1997). FLIP is also known to be involved in the activation of transcription factors like NFκB and ERK inducing cell survival signals (Kataoka, Budd et al. 2000). TGFβ1 can also counteract the cytolytic effect caused by TNFα. TGFβ1 in combination with TNFα has been reported to reduce Fas expression, induce NFkB activation, and

upregulate anti-apoptotic protein bcl-XL in hepatic stellate cells (Saile, Matthes et al. 2001). On the other hand, in several cancer cell lines, TGFβ1 along with TNFα induced apoptosis via the down regulation of anti-apoptotic protein, bcl-2 (Schuster, Bender et al. 2003). TGFβ1 could induce bcl-2 expression via MAP kinase pathway. As mentioned earlier TGFβ1 induces the expression of CREB transcription factor, and bcl-2 promoter possesses binding sites for CREB (Wilson, Mochon et al. 1996). Thus the fate of the cell, programmed to die or survive, may depend upon TGFβ1 and TNFα- mediated regulation of different Bcl-family members.

J. Regulation of FIV replication in $CD4^+CD25^+$ Treg cells in Feline model

Feline Immunodeficiency Virus (FIV), similar to Human Immunodeficiency Virus (HIV), is a lentivirus that belongs to the Retroviridae family that cause's chronic and progressive acquired immunodeficiency syndrome in domestic cats. FIV has been classified into five clades (clades A-E) similar to HIV with at least nine clades (clades A-D, F-H, J and K). Similar to HIV infection, the immunopathogenesis of FIV infection in cats is characterized by $CD4^+$ T cell depletion, reduction in CD4/CD8 ratios, decreased responsiveness to T cell mitogens, and lymph node T cell apoptosis (Lawrence, Callanan et al. 1992; Guiot, Rigal et al. 1997; Tompkins, Bull et al. 2002). The anti-FIV cytotoxic activities, mediated by $CD8^+$ T cells that recognize whole virus, FIV gag and env proteins, are initiated during the early stages of infection and persist at the asymptomatic phase even with the loss of $CD4^+$ T cells similar to HIV-infection. Although the clinical stages of FIV and HIV infections are well understood, the mechanisms leading to the

reduction of $CD4^+$ T cell numbers and functions in these two viral infections are not effectively established. Therefore, FIV model in cats can serve as an efficient small animal AIDS model for humans. Thus, the FIV model may help in exploring the mechanisms involved in regulating FIV-replication by Treg cells, which may be comparable to HIV regulation by T regulatory cells in human subjects.

It has been reported that feline $CD4^+CD25^+$ Treg cells possess the major characteristics of murine and human Treg cells in that they are anergic to mitogenic stimulation, and relatively resistant to activation induced cell death (Vahlenkamp, Bull et al. 2004). FIV-infection results in chronic activation of Treg cells (Joshi, Vahlenkamp et al. 2004; Vahlenkamp, Bull et al. 2004). It has been reported that only activated Treg cells can non-specifically suppress the proliferation of other T cells. Treg cells from FIV-infected cats are endogenously activated and can suppress T cell proliferation without further activation in vitro (Vahlenkamp, Bull et al. 2004).

The ability of $CD4^+CD25^+$ Treg cells to be anergic, yet programmed to cell survival as opposed to apoptosis are unique features that may be essential long-term immunosurveillance and maintenance of peripheral self-tolerance, which may depend on altered CdkI's and anti-apoptotic proteins (bcl-2) expression. Because of this long-term apoptosis resistant phenotypic nature, $CD4^+CD25^+$ Treg cells can serve as a stable reservoir for productive FIV/HIV infection. It has been shown that FIV preferentially replicates in $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cells (Joshi, Vahlenkamp et al. 2004). Interestingly, the transcription factors like NFκB and AP1 involved in the

regulation of IL-2 gene expression are also known to regulate transcription of HIV/FIV genes (Joshi, Garg et al. 2005). HIV/FIV long terminal repeats also possess binding sites for several transcriptional factors like AP1, ATF, CEBP, and Sp1 (Joshi, Garg et al. 2005). The activation of the transcription factors such as AP1, CEBP, and ATF were demonstrated to correlate with FIV-transcription (Joshi, Garg et al. 2005). As mentioned earlier, TGFβ1 signaling has been known to be involved in regulating several of these transcriptional factors such as Sp1, ATF, and NFκB. Therefore, TGFβ1 signaling may be one of the mechanisms which enable potent suppressor Treg cells to maintain anergy, down-regulate IL-2, yet be programmed for survival, and also serve as excellent reservoirs for FIV/HIV replication.

K. Based on the above literature, the research was focused on the following specific aims.

Specific Aim 1 To determine if feline Treg cells are functionally anergic. If so, what are the phenotypic molecules (mainly the CdkI's) that could be responsible for the maintenance of anergic state in $CD4^+CD25^+$ Treg cells, compared to $CD4^+CD25^-$ T cells?

Specific Aim 2 To determine the activation status of Treg cells from FIV-infected cats compared to the control cats using phenotypic surface markers such as CD62L-selectin.

Specific Aim 3 To determine if Treg cells are more resistant to activation induced apoptosis compared to their counterparts $CD4^+CD25^-$ T cells. If so, how is the expression profile of pro-apoptotic and anti-apoptotic proteins in $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cells?

Specific Aim 4 To determine the surface expression profile of TGFβRII on the two purified $CD4^+$ T cell subsets.

Specific Aim 5 To determine if TGFβ converted feline $CD4^+CD25^-$ T cells are functionally and phenotypically anergic similar to that of naturally occurring $CD4^+CD25^+$ Treg cells.

L. LITERATURE CITED

1. Aandahl, E. M., J. Michaelsson, et al. (2004). "Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens." J Virol **78**(5): 2454-9.
2. Adams, J. M. and S. Cory (1998). "The Bcl-2 protein family: arbiters of cell survival." Science **281**(5381): 1322-6.
3. Alexandrow, M. G. and H. L. Moses (1997). "Kips off to Myc: implications for TGF beta signaling." J Cell Biochem **66**(4): 427-32.
4. Apostolou, I., A. Sarukhan, et al. (2002). "Origin of regulatory T cells with known specificity for antigen." Nat Immunol **3**(8): 756-63.
5. Asano, M., M. Toda, et al. (1996). "Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation." J Exp Med **184**(2): 387-96.
6. Aseffa, A., A. Gumy, et al. (2002). "The early IL-4 response to Leishmania major and the resulting Th2 cell maturation steering progressive disease in BALB/c mice are subject to the control of regulatory CD4+CD25+ T cells." J Immunol **169**(6): 3232-41.
7. Azuma, M., H. Yssel, et al. (1993). "Functional expression of B7/BB1 on activated T lymphocytes." J Exp Med **177**(3): 845-50.
8. Balomenos, D., J. Martin-Caballero, et al. (2000). "The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development." Nat Med **6**(2): 171-6.
9. Banz, A., C. Pontoux, et al. (2002). "Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells." J Immunol **169**(2): 750-7.

10. Bayer, A. L., A. Yu, et al. (2005). "Essential role for interleukin-2 for CD4(+)CD25(+) T regulatory cell development during the neonatal period." J Exp Med **201**(5): 769-77.
11. Belkaid, Y. (2003). "The role of CD4(+)CD25(+) regulatory T cells in Leishmania infection." Expert Opin Biol Ther **3**(6): 875-85.
12. Belkaid, Y., C. A. Piccirillo, et al. (2002). "CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity." Nature **420**(6915): 502-7.
13. Belkaid, Y. and B. T. Rouse (2005). "Natural regulatory T cells in infectious disease." Nat Immunol **6**(4): 353-60.
14. Bluestone, J. A. (1997). "Is CTLA-4 a master switch for peripheral T cell tolerance?" J Immunol **158**(5): 1989-93.
15. Boatright, K. M. and G. S. Salvesen (2003). "Mechanisms of caspase activation." Curr Opin Cell Biol **15**(6): 725-31.
16. Bonni, S., H. R. Wang, et al. (2001). "TGF-beta induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation." Nat Cell Biol **3**(6): 587-95.
17. Brodin, G., A. Ahgren, et al. (2000). "Efficient TGF-beta induction of the Smad7 gene requires cooperation between AP-1, Sp1, and Smad proteins on the mouse Smad7 promoter." J Biol Chem **275**(37): 29023-30.
18. Caramalho, I., T. Lopes-Carvalho, et al. (2003). "Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide." J Exp Med **197**(4): 403-11.
19. Chai, J. G. and R. I. Lechler (1997). "Immobilized anti-CD3 mAb induces anergy in murine naive and memory CD4+ T cells in vitro." Int Immunol **9**(7): 935-44.
20. Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-86.
21. Chen, W. and S. M. Wahl (2002). "TGF-beta: receptors, signaling pathways and autoimmunity." Curr Dir Autoimmun **5**: 62-91.

22. Chen, W. and S. M. Wahl (2003). "TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression." Cytokine Growth Factor Rev **14**(2): 85-9.
23. Coats, S., P. Whyte, et al. (1999). "A new pathway for mitogen-dependent cdk2 regulation uncovered in p27(Kip1)-deficient cells." Curr Biol **9**(4): 163-73.
24. Curotto de Lafaille, M. A., A. C. Lino, et al. (2004). "CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion." J Immunol **173**(12): 7259-68.
25. Curotto de Lafaille, M. A., S. Muriglan, et al. (2001). "Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes." J Exp Med **194**(9): 1349-59.
26. Fantini, M. C., C. Becker, et al. (2004). "Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7." J Immunol **172**(9): 5149-53.
27. Fero, M. L., M. Rivkin, et al. (1996). "A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice." Cell **85**(5): 733-44.
28. Fontenot, J. D., M. A. Gavin, et al. (2003). "Foxp3 programs the development and function of CD4+CD25+ regulatory T cells." Nat Immunol **4**(4): 330-6.
29. Franzen, A. and N. E. Heldin (2001). "BMP-7-induced cell cycle arrest of anaplastic thyroid carcinoma cells via p21(CIP1) and p27(KIP1)." Biochem Biophys Res Commun **285**(3): 773-81.
30. Fu, S., N. Zhang, et al. (2004). "TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors." Am J Transplant **4**(10): 1614-27.
31. Fukaura, H., S. C. Kent, et al. (1996). "Induction of circulating myelin basic protein and proteolipid protein-specific transforming growth factor-beta1-secreting Th3 T cells by oral administration of myelin in multiple sclerosis patients." J Clin Invest **98**(1): 70-7.
32. Funk, J. O. and D. A. Galloway (1998). "Inhibiting CDK inhibitors: new lessons from DNA tumor viruses." Trends Biochem Sci **23**(9): 337-41.

33. Furtado, G. C., D. Olivares-Villagomez, et al. (2001). "Regulatory T cells in spontaneous autoimmune encephalomyelitis." Immunol Rev **182**: 122-34.
34. Ghosh-Choudhury, N., G. Ghosh-Choudhury, et al. (2000). "Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells." Biochim Biophys Acta **1497**(2): 186-96.
35. Gorelik, L. and R. A. Flavell (2000). "Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease." Immunity **12**(2): 171-81.
36. Graca, L., S. Thompson, et al. (2002). "Both CD4(+)CD25(+) and CD4(+)CD25(-) regulatory cells mediate dominant transplantation tolerance." J Immunol **168**(11): 5558-65.
37. Guan, K. L., C. W. Jenkins, et al. (1994). "Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function." Genes Dev **8**(24): 2939-52.
38. Guiot, A. L., D. Rigal, et al. (1997). "Spontaneous programmed cell death (PCD) process of lymphocytes of FIV-infected cats: cellular targets and modulation." Vet Immunol Immunopathol **58**(2): 93-106.
39. Gupta, S. (2003). "Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review)." Int J Oncol **22**(1): 15-20.
40. Hannon, G. J. and D. Beach (1994). "p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest." Nature **371**(6494): 257-61.
41. Hara, M., C. I. Kingsley, et al. (2001). "IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo." J Immunol **166**(6): 3789-96.
42. Harper, J. W., G. R. Adami, et al. (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-16.
43. Hauet-Broere, F., W. W. Unger, et al. (2003). "Functional CD25- and CD25+ mucosal regulatory T cells are induced in gut-draining lymphoid tissue within 48 h after oral antigen application." Eur J Immunol **33**(10): 2801-10.
44. Hirama, T. and H. P. Koeffler (1995). "Role of the cyclin-dependent kinase inhibitors in the development of cancer." Blood **86**(3): 841-54.

45. Hisaeda, H., Y. Maekawa, et al. (2004). "Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells." Nat Med **10**(1): 29-30.
46. Ho, J., C. de Guise, et al. (2004). "Activin induces hepatocyte cell growth arrest through induction of the cyclin-dependent kinase inhibitor p15INK4B and Sp1." Cell Signal **16**(6): 693-701.
47. Hoffmann, P., J. Ermann, et al. (2002). "Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation." J Exp Med **196**(3): 389-99.
48. Hori, S., T. L. Carvalho, et al. (2002). "CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice." Eur J Immunol **32**(5): 1282-91.
49. Hori, S. and S. Sakaguchi (2004). "Foxp3: a critical regulator of the development and function of regulatory T cells." Microbes Infect **6**(8): 745-51.
50. Horwitz, D. A., S. G. Zheng, et al. (2004). "Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease." Semin Immunol **16**(2): 135-43.
51. Howell, G. M., L. Sun, et al. (1993). "The role of growth regulatory aberrations in progression of human colon carcinoma." Cancer Metastasis Rev **12**(3-4): 275-86.
52. Iavarone, A. and J. Massague (1997). "Repression of the CDK activator Cdc25A and cell-cycle arrest by cytokine TGF-beta in cells lacking the CDK inhibitor p15." Nature **387**(6631): 417-22.
53. Irmler, M., M. Thome, et al. (1997). "Inhibition of death receptor signals by cellular FLIP." Nature **388**(6638): 190-5.
54. Ishisaki, A., K. Yamato, et al. (1998). "Smad7 is an activin-inducible inhibitor of activin-induced growth arrest and apoptosis in mouse B cells." J Biol Chem **273**(38): 24293-6.
55. Iwashiro, M., R. J. Messer, et al. (2001). "Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection." Proc Natl Acad Sci U S A **98**(16): 9226-30.
56. Jackman, M. R. and J. N. Pines (1997). "Cyclins and the G2/M transition." Cancer Surv **29**: 47-73.

57. Jackson, S. K., A. DeLoose, et al. (2001). "Induction of anergy in Th1 cells associated with increased levels of cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1." J Immunol **166**(2): 952-8.
58. Jernvall, J., T. Aberg, et al. (1998). "The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot." Development **125**(2): 161-9.
59. Ji, H. B., G. Liao, et al. (2004). "Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression." J Immunol **172**(10): 5823-7.
60. Jonuleit, H., E. Schmitt, et al. (2001). "Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood." J Exp Med **193**(11): 1285-94.
61. Joshi, A., H. Garg, et al. (2005). "Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors." J Virol **79**(8): 4965-76.
62. Joshi, A., T. W. Vahlenkamp, et al. (2004). "Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation." Virology **321**(2): 307-22.
63. Karandikar, N. J., C. L. Vanderlugt, et al. (1996). "CTLA-4: a negative regulator of autoimmune disease." J Exp Med **184**(2): 783-8.
64. Kataoka, T., R. C. Budd, et al. (2000). "The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways." Curr Biol **10**(11): 640-8.
65. Kee, B. L., R. R. Rivera, et al. (2001). "Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta." Nat Immunol **2**(3): 242-7.
66. Kim, H. P., B. G. Kim, et al. (2005). "Smad-dependent cooperative regulation of interleukin 2 receptor alpha chain gene expression by T cell receptor and transforming growth factor-beta." J Biol Chem **280**(40): 34042-7.
67. Kinter, A. L., M. Hennessey, et al. (2004). "CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are

associated with favorable clinical markers of disease status." J Exp Med **200**(3): 331-43.

68. Kivinen, L., M. Tsubari, et al. (1999). "Ras induces p21Cip1/Waf1 cyclin kinase inhibitor transcriptionally through Sp1-binding sites." Oncogene **18**(46): 6252-61.
69. Kiyokawa, H., R. D. Kineman, et al. (1996). "Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1)." Cell **85**(5): 721-32.
70. Krummel, M. F. and J. P. Allison (1995). "CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation." J Exp Med **182**(2): 459-65.
71. Krummel, M. F. and J. P. Allison (1996). "CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells." J Exp Med **183**(6): 2533-40.
72. Lawrence, C. E., J. J. Callanan, et al. (1992). "Decreased mitogen responsiveness and elevated tumor necrosis factor production in cats shortly after feline immunodeficiency virus infection." Vet Immunol Immunopathol **35**(1-2): 51-9.
73. Lechler, R., J. G. Chai, et al. (2001). "T-cell anergy and peripheral T-cell tolerance." Philos Trans R Soc Lond B Biol Sci **356**(1409): 625-37.
74. Lee, M. H., I. Reynisdottir, et al. (1995). "Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution." Genes Dev **9**(6): 639-49.
75. Letterio, J. J. and A. B. Roberts (1998). "Regulation of immune responses by TGF-beta." Annu Rev Immunol **16**: 137-61.
76. Leveen, P., J. Larsson, et al. (2002). "Induced disruption of the transforming growth factor beta type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable." Blood **100**(2): 560-8.
77. Maggi, E., L. Cosmi, et al. (2005). "Thymic regulatory T cells." Autoimmun Rev **4**(8): 579-86.
78. Malek, T. R. and A. L. Bayer (2004). "Tolerance, not immunity, crucially depends on IL-2." Nat Rev Immunol **4**(9): 665-74.
79. Maloy, K. J. and F. Powrie (2001). "Regulatory T cells in the control of immune pathology." Nat Immunol **2**(9): 816-22.

80. Maloy, K. J., L. Salaun, et al. (2003). "CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms." J Exp Med **197**(1): 111-9.
81. Massague, J. (2004). "G1 cell-cycle control and cancer." Nature **432**(7015): 298-306.
82. McGuirk, P. and K. H. Mills (2002). "Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases." Trends Immunol **23**(9): 450-5.
83. McHugh, R. S., M. J. Whitters, et al. (2002). "CD4(+)CD25(+) immunoregulatory T cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor." Immunity **16**(2): 311-23.
84. Mobley, J. L. and M. O. Dailey (1992). "Regulation of adhesion molecule expression by CD8 T cells in vivo. I. Differential regulation of gp90MEL-14 (LECAM-1), Pgp-1, LFA-1, and VLA-4 alpha during the differentiation of cytotoxic T lymphocytes induced by allografts." J Immunol **148**(8): 2348-56.
85. Mohapatra, S., D. Agrawal, et al. (2001). "p27Kip1 regulates T cell proliferation." J Biol Chem **276**(24): 21976-83.
86. Mohapatra, S. and W. J. Pledger (2001). "Interdependence of cdk2 activation and interleukin-2Ralpha accumulation in T cells." J Biol Chem **276**(24): 21984-9.
87. Montagnoli, C., A. Bacci, et al. (2002). "B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to Candida albicans." J Immunol **169**(11): 6298-308.
88. Morgan, D. O. (1995). "Principles of CDK regulation." Nature **374**(6518): 131-4.
89. Moustakas, A. and D. Kardassis (1998). "Regulation of the human p21/WAF1/Cip1 promoter in hepatic cells by functional interactions between Sp1 and Smad family members." Proc Natl Acad Sci U S A **95**(12): 6733-8.
90. Moustakas, A., K. Pardali, et al. (2002). "Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation." Immunol Lett **82**(1-2): 85-91.
91. Nakamura, K., A. Kitani, et al. (2004). "TGF-beta 1 plays an important role in the mechanism of CD4+CD25+ regulatory T cell activity in both humans and mice." J Immunol **172**(2): 834-42.

92. Nakamura, K., A. Kitani, et al. (2001). "Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta." J Exp Med **194**(5): 629-44.
93. Nakayama, K., N. Ishida, et al. (1996). "Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors." Cell **85**(5): 707-20.
94. Nelson, B. H. (2004). "IL-2, regulatory T cells, and tolerance." J Immunol **172**(7): 3983-8.
95. Netea, M. G., R. Sutmuller, et al. (2004). "Toll-like receptor 2 suppresses immunity against Candida albicans through induction of IL-10 and regulatory T cells." J Immunol **172**(6): 3712-8.
96. Nurse, P. (1994). "Ordering S phase and M phase in the cell cycle." Cell **79**(4): 547-50.
97. Papiernik, M., M. do Carmo Leite-de-Moraes, et al. (1997). "T cell deletion induced by chronic infection with mouse mammary tumor virus spares a CD25-positive, IL-10-producing T cell population with infectious capacity." J Immunol **158**(10): 4642-53.
98. Pardali, K., A. Kurisaki, et al. (2000). "Role of Smad proteins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta." J Biol Chem **275**(38): 29244-56.
99. Pasare, C. and R. Medzhitov (2003). "Toll-like receptors: balancing host resistance with immune tolerance." Curr Opin Immunol **15**(6): 677-82.
100. Pasare, C. and R. Medzhitov (2003). "Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells." Science **299**(5609): 1033-6.
101. Peschiaroli, A., R. Figliola, et al. (2002). "MyoD induces apoptosis in the absence of RB function through a p21(WAF1)-dependent re-localization of cyclin/cdk complexes to the nucleus." Oncogene **21**(53): 8114-27.
102. Philipp-Staheli, J., S. R. Payne, et al. (2001). "p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer." Exp Cell Res **264**(1): 148-68.

103. Pouliot, F. and C. Labrie (2002). "Role of Smad1 and Smad4 proteins in the induction of p21WAF1,Cip1 during bone morphogenetic protein-induced growth arrest in human breast cancer cells." J Endocrinol **172**(1): 187-98.

104. Powell, J. D., J. A. Ragheb, et al. (1998). "Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy." Immunol Rev **165**: 287-300.

105. Raghavan, S., E. Suri-Payer, et al. (2004). "Antigen-specific in vitro suppression of murine Helicobacter pylori-reactive immunopathological T cells by CD4CD25 regulatory T cells." Scand J Immunol **60**(1-2): 82-8.

106. Read, S., V. Malmstrom, et al. (2000). "Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation." J Exp Med **192**(2): 295-302.

107. Reed, S. I. (1997). "Control of the G1/S transition." Cancer Surv **29**: 7-23.

108. Sadlack, B., H. Merz, et al. (1993). "Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene." Cell **75**(2): 253-61.

109. Saile, B., N. Matthes, et al. (2001). "The bcl, NFkappaB and p53/p21WAF1 systems are involved in spontaneous apoptosis and in the anti-apoptotic effect of TGF-beta or TNF-alpha on activated hepatic stellate cells." Eur J Cell Biol **80**(8): 554-61.

110. Salomon, B., D. J. Lenschow, et al. (2000). "B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes." Immunity **12**(4): 431-40.

111. Scheipers, P. and H. Reiser (1998). "Fas-independent death of activated CD4(+) T lymphocytes induced by CTLA-4 crosslinking." Proc Natl Acad Sci U S A **95**(17): 10083-8.

112. Schlapbach, R., K. S. Spanaus, et al. (2000). "TGF-beta induces the expression of the FLICE-inhibitory protein and inhibits Fas-mediated apoptosis of microglia." Eur J Immunol **30**(12): 3680-8.

113. Schuster, N., H. Bender, et al. (2003). "Transforming growth factor-beta and tumor necrosis factor-alpha cooperate to induce apoptosis in the oligodendroglial cell line OLI-neu." J Neurosci Res **73**(3): 324-33.

114. Serrano, M., G. J. Hannon, et al. (1993). "A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4." Nature **366**(6456): 704-7.

115. Setoguchi, R., S. Hori, et al. (2005). "Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization." J Exp Med **201**(5): 723-35.

116. Sherr, C. J. (1993). "Mammalian G1 cyclins." Cell **73**(6): 1059-65.

117. Sherr, C. J. (1996). "Cancer cell cycles." Science **274**(5293): 1672-7.

118. Shevach, E. M. (2002). "CD4+ CD25+ suppressor T cells: more questions than answers." Nat Rev Immunol **2**(6): 389-400.

119. Shi, Y. and J. Massague (2003). "Mechanisms of TGF-beta signaling from cell membrane to the nucleus." Cell **113**(6): 685-700.

120. Shi, Y., Y. F. Wang, et al. (1998). "Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling." Cell **94**(5): 585-94.

121. Shimizu, J., S. Yamazaki, et al. (2002). "Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance." Nat Immunol **3**(2): 135-42.

122. Stephens, L. A. and D. Mason (2000). "CD25 is a marker for CD4+ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both CD25+ and CD25- subpopulations." J Immunol **165**(6): 3105-10.

123. Suzuki, A., Y. Tsutomi, et al. (1999). "Mitochondrial regulation of cell death: mitochondria are essential for procaspase 3-p21 complex formation to resist Fas-mediated cell death." Mol Cell Biol **19**(5): 3842-7.

124. Suzuki, H., T. M. Kundig, et al. (1995). "Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta." Science **268**(5216): 1472-6.

125. Taams, L. S., J. Smith, et al. (2001). "Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population." Eur J Immunol **31**(4): 1122-31.

126. Takahashi, T., Y. Kuniyasu, et al. (1998). "Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state." Int Immunol **10**(12): 1969-80.

127. Taylor, P. A., C. J. Lees, et al. (2002). "The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality." Blood **99**(10): 3493-9.

128. Taylor, P. A., R. J. Noelle, et al. (2001). "CD4(+)CD25(+) immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade." J Exp Med **193**(11): 1311-8.

129. ten Dijke, P. and C. S. Hill (2004). "New insights into TGF-beta-Smad signalling." Trends Biochem Sci **29**(5): 265-73.

130. Thornton, A. M. and E. M. Shevach (1998). "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production." J Exp Med **188**(2): 287-96.

131. Thorstenson, K. M. and A. Khoruts (2001). "Generation of anergic and potentially immunoregulatory CD25+CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen." J Immunol **167**(1): 188-95.

132. Tompkins, M. B., M. E. Bull, et al. (2002). "Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis." J Infect Dis **185**(8): 1077-93.

133. Toyoshima, H. and T. Hunter (1994). "p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21." Cell **78**(1): 67-74.

134. Vahlenkamp, T. W., M. E. Bull, et al. (2004). "B7+CTLA4+ T cells engage in T-T cell interactions that mediate apoptosis: a model for lentivirus-induced T cell depletion." Vet Immunol Immunopathol **98**(3-4): 203-14.

135. Vahlenkamp, T. W., M. B. Tompkins, et al. (2004). "Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells." J Immunol **172**(8): 4752-61.

136. Walunas, T. L. and J. A. Bluestone (1998). "CTLA-4 regulates tolerance induction and T cell differentiation in vivo." J Immunol **160**(8): 3855-60.

137. Willerford, D. M., J. Chen, et al. (1995). "Interleukin-2 receptor alpha chain regulates the size and content of the peripheral lymphoid compartment." Immunity **3**(4): 521-30.

138. Wilson, B. E., E. Mochon, et al. (1996). "Induction of bcl-2 expression by phosphorylated CREB proteins during B-cell activation and rescue from apoptosis." Mol Cell Biol **16**(10): 5546-56.

139. Wolfraim, L. A., T. M. Walz, et al. (2004). "p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness." J Immunol **173**(5): 3093-102.

140. Xiao, M., B. K. Oppenlander, et al. (2001). "Transforming growth factor-beta(1) induces apoptosis in CD34(+)CD38(-/low) cells that express Bcl-2 at a low level." Exp Hematol **29**(9): 1098-108.

141. Yamato, K., S. Hashimoto, et al. (2001). "Activation of the p21(CIP1/WAF1) promoter by bone morphogenetic protein-2 in mouse B lineage cells." Oncogene **20**(32): 4383-92.

142. Yamazaki, S., T. Iyoda, et al. (2003). "Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells." J Exp Med **198**(2): 235-47.

143. Yang, J., X. Liu, et al. (1997). "Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked." Science **275**(5303): 1129-32.

144. Zauberman, A., M. Oren, et al. (1997). "Involvement of p21(WAF1/Cip1), CDK4 and Rb in activin A mediated signaling leading to hepatoma cell growth inhibition." Oncogene **15**(14): 1705-11.

145. Zhang, X., L. Izikson, et al. (2001). "Activation of CD25(+)CD4(+) regulatory T cells by oral antigen administration." J Immunol **167**(8): 4245-53.

146. Zhang, Y. and S. C. Lin (1997). "Molecular characterization of the cyclin-dependent kinase inhibitor p27 promoter." Biochim Biophys Acta **1353**(3): 307-17.

3. CHAPTER-1

IMMUNE SUPPRESSOR FUNCTION OF $CD4^+CD25^+$ T regulatory CELLS IN FIV-INFECTED CATS IS ASSOCIATED WITH OVER-EXPRESSION OF CELL SURFACE TGFβ1 AND TGFβRII AND UP-REGULATION OF CYCLIN DEPENDENT KINASE INHIBITORS

ABSTRACT

Cell cycling from G0/G1 to the S phase is regulated by a family of cyclin-dependent kinases (Cdk) and their inhibitors (CdkI's). $CD4^+CD25^+$ T regulatory cells (Treg) are arrested in the G0/G1 phase of the cell cycle and fail to produce IL-2 and proliferate in response to TCR engagement. The expression profile of the Cdk and CdkI genes in $CD4^+CD25^+$ Treg cells and the role they play in maintaining the anergic state has not been studied. We previously reported that $CD4^+CD25^+$ Treg cells from FIV-infected but not uninfected cats are activated in-vivo and possess potent immuno-suppressor function in the absence of additional in-vitro stimulation. In this study we assessed the expression profile of the CdkI's in Treg cells from FIV-infected and uninfected control cats. We demonstrated that the Cdk inhibitors $p2^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ are up-regulated in $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cells from FIV-infected cats. No significant difference in CdkI expression was detected in unstimulated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from uninfected cats. Upon stimulation with ConA or LPS alone, $p21^{cip1}$ expression was enhanced in $CD4^+CD25^+$ Treg cells but not in $CD4^+CD25^-$ T cells from uninfected cats, and the presence of rhIL-2 blocked this effect. Up-regulation of CdkI's in $CD4^+CD25^+$ Treg cells from FIV-infected cats is associated with increased expression of TGFβ1 and TGFβRII on the cell surface. We also demonstrated that feline $CD4^+CD25^+$ Treg cells from FIV-infected cats but not control cats are able to suppress the proliferation of activated $CD4^+CD25^-$ T cells. Taken together, these findings suggest that feline $CD4^+CD25^+$ Treg cells from FIV-infected cats, in contrast to $CD4^+CD25^+$ Treg cells from control cats are functionally and phenotypically activated in vivo, and that this activation may be regulated by the TGFβ1/TGFβR signaling pathway.

Keywords: $CD4^+CD25^+$ T regulatory cells; TGFβ1; Cdk; CdkI; FIV

INTRODUCTION

According to the classical model of T cell activation, productive stimulation of T cells requires two major signals. The first is the engagement of MHC-bound peptide on APC with the TCR and the second is the interaction of B7 on the APC with the T cell co-stimulatory receptor CD28 (Greenfield, Nguyen et al. 1998; Harris and Ronchese 1999). Under normal conditions, B7 receptors on APC initially engage the CD28 receptor on $CD4^+$ T cells transducing a signal that up-regulates IL-2 production, an essential cytokine for transit out of the G0/G1 stage of the cell cycle, induction of cell proliferation and the initiation of an immune response (Powell, Ragheb et al. 1998). If T cells are stimulated only through the TCR in the absence of co-stimulation, this partial activation leads to a state of long-lasting, partial or total unresponsiveness, termed anergy (Powell, Ragheb et al. 1998). In addition to $CD4^+$ T cells that can be rendered anergic by TCR engagement in the absence of B7-CD28 co-stimulation, a second population of $CD4^+$ T cells, the $CD4^+CD25^+$ Treg cells appear to be naturally anergic, arrested in the G0/G1 phase of the cell cycle. Similar to anergic $CD4^+$ T cells, $CD4^+CD25^+$ Treg cells fail to produce IL-2 and proliferate in response to TCR engagement by antigens, but respond to polyclonal stimuli such as IL-2 (Powell, Ragheb et al. 1998; Lechler, Chai et al. 2001). Naturally occurring $CD4^+CD25^+$ Treg cells, comprising 5-10% of the circulating $CD4^+$ T cell population, were originally defined as regulatory T cells that maintain peripheral self-

tolerance (Maloy and Powrie 2001; Shevach 2002). More recent studies have demonstrated that Treg cells also play a major role in modulating the magnitude and duration of protective T cell responses to pathogens (Read, Malmstrom et al. 2000; Iwashiro, Messer et al. 2001; Belkaid, Piccirillo et al. 2002; Hori, Carvalho et al. 2002; Montagnoli, Bacci et al. 2002; Hisaeda, Maekawa et al. 2004; Belkaid and Rouse 2005). These pathogen-induced Treg cells, similar to natural T cells, are anergic and possess potent immunosuppressor function once activated by TCR engagement and/or certain nonspecific stimuli (Belkaid and Rouse 2005). While anergic T cells, including pathogen-induced $CD4^+CD25^+$ Treg cells, are known to be arrested at the G0/G1 phase of the cell cycle, there is little information on what receptors or intracellular signaling proteins maintain IL-2 suppression and the anergic state of Treg cells.

Progression through the G0/G1 to S phase of the cell cycle is regulated by several cyclins and cyclin-dependent kinases (Cdk) whose activities are in turn controlled by their respective inhibitors (CdkI's). During G1/S phase progression, cyclin D (D1-D3 in most cells and only D2-D3 in lymphocytes) are activated in mid G1 phase followed by cyclin E and cyclin A in G1/S boundary (Morgan 1995; Reed 1997; Sherr and Roberts 1999). In response to mitogen signals, Cyclin D is induced and associates with Cdk4 and Cdk6 (Sherr and Roberts 1999), whereas cyclin A associates with Cdk2. The activity of cyclin-Cdk complexes is repressed by CdkI's, which constrain entry into S phase (Reed 1997) . The CdkI's that govern these events have been divided into two groups based on their structure and Cdk targets. The first class includes INK4 proteins (p16, p15, & p18) and consists of multiple ankyrin repeats, which bind to only Cdk4 and Cdk6 (Sherr and

Roberts 1999). The second class of inhibitors includes $p2^{cip1}$, $p27^{kip1}$, p53 and p57, which contain characteristic motifs within their amino terminal moieties that enable them to bind both cyclins and Cdk subunits (Sherr and Roberts 1999). As $p21^{cip1}$ is capable of binding with essentially all the cyclin-cyclin-dependent kinase complexes, it plays a major role in regulating T cell cycling and could be a principle factor in maintaining anergy. In support of this, $p21^{cip1}$ knockout mice rapidly lose tolerance to nuclear antigens and develop a lymphoproliferative autoimmune-like disease reminiscent of systemic lupus in humans (Balomenos, Martin-Caballero et al. 2000).

While numerous factors are involved in induction of cell-cycle arrest, cytokines, most notably TGFβ1 may play a major role in inducing cell growth arrest in immune cells by up-regulation of CdkI's (Dennler, Goumans et al. 2002; Gartel and Tyner 2002). TGFβ1 has been shown to induce G0/G1 cell cycle-arrest via transcriptional activation of $p15^{ink4}$, $p21^{cipl}$ and $p27^{kip1}$ expression in a number of cells including T and B lymphocytes (Kee, Rivera et al. 2001; Massague 2004; Wolfraim, Walz et al. 2004). Although there is little information on how TGFβ1 activates CdkI's expression, recent studies demonstrate that the $p21^{cipl}$ promoter contains sequences capable of binding SMAD4, a critical downstream element in the TGFβ1 signaling pathway (Yamato, Hashimoto et al. 2001). Cell surface expressed TGFβ1 has also been associated with activated $CD4^{+}CD25^{+}$ Treg cells, although it's role in Treg function is controversial (Maloy and Powrie 2001; Shevach 2002).

Recent studies have demonstrated that feline $CD4^+CD25^+$ Treg cells possess all the functional characteristics of Treg cells described in humans and rodents in that they are anergic, arrested at the G0/G1 phase of the cell cycle, and possess potent immunosuppressive activity when activated (Vahlenkamp, Tompkins et al. 2004). Moreover, we recently reported that $CD4^+CD25^+$ Treg cells from FIV-infected cats, in contrast to Treg cells from uninfected control cats, are activated in-vivo and fully armed for potent contact-dependent immunosuppressor function (Vahlenkamp, Tompkins et al. 2004). To determine what intracellular factors might regulate $CD4^+CD25^+$ Treg cell anergy and suppressive function and what affect FIV-infection of cats might have on these factors, this study focused on the expression profiles of the CdkI's in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets in FIV-infected and uninfected cats. We demonstrate that in-vivo activated, anergic feline $CD4^+CD25^+$ Treg cells from FIV-infected cats are functionally and phenotypically activated, as indicated by the enhanced expression of CdkI's and potent suppressor function. Moreover, Treg cells from FIV-infected cats over-express TGFβ1 and TGFβRII on their surface, suggesting that this signaling pathway in addition to up-regulating CdkI's and maintaining the anergic state of Treg cells, may play a role in the immunosuppressive function of these cells.

MATERIALS AND METHODS

Animals and Viral Infection

The cats used for this study were specific pathogen free (SPF) 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 NCSU1 isolate of FIV, a pathogenic clade A virus (English, Nelson et al. 1994), as described by Bucci et al (Bucci, English et al. 1998). The samples were collected from FIV^+ cats infected for more than 3yrs and from uninfected control cats, 4 to 6 years of age. FIV-infected cats were housed separately from uninfected control cats. FIV-infection was confirmed by immunoblot analysis and provirus detection by PCR using primers specific for the FIV-p24 encoding sequence.

Cell Collection

Whole blood was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) and PBMC isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as previously described (Tompkins, Ogilvie et al. 1987). LN cells were obtained from LN biopsies and single-cell suspensions were prepared by gently injecting sterile PBS into the tissue using 18G needle repeatedly,

until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion. Viability was always >90%.

$CD4^+$ T cell subset separation

To obtain single lymphocyte subsets, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells derived from PBMC and pooled LN cells were either enriched by biomagnetic bead depletion and selection or by fluorescent activated cell sorting (FACS) (MoFlo; DakoCytomation, Fort Collins, CO). Biomagnetic bead separation was performed using goat anti-mouse IgG-coated beads (Dynabeads M-450; Dynal, Great Neck, NY) as described by Bucci et al. (Bucci, English et al. 1998). PBMC and LN were depleted of B cells with anti-CD21 mAb (Sertec, Raleigh, NC)-coated beads, and $CD8^+$ cells were depleted with anti-CD8 (mAb 3.357 (Tompkins, Gebhard et al. 1990))-coated beads. The $CD4^+$ cell population was then enriched for $CD25^+$ cells by positive selection using anti-feline CD25 mAb (9F23; kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan)). The enriched $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were analyzed for purity by flow cytometric analysis and were greater than 90% pure. The purity of the FACS sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were greater than 95%. In both cases, viability of the purified $CD4^+$ T cell subsets was determined by trypan blue dye exclusion and was always >90%. All assays were based on viable cell numbers.

Flow cytometry analysis

At least 5 x 10^5 PBMC or purified cell populations were stained for surface expression of CD4 and CD25 markers using APC-conjugated anti-CD4 (mAb 30A (Tompkins, Gebhard et al. 1990)), FITC-conjugated anti-CD25 mAb (mAb 9F23), and PE-conjugated anti-CD62L selectin (BD-pharmagen). Monoclonal antibodies for TGFβ1 (mouse anti-human, mAb 240 clone 9016) and TGFβRII (mouse anti-human FAB2HIP clone 25508) were obtained from R and D. Systems. Data were acquired on a FACS caliber flow cytometer (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 Cell Quest software.

T cell stimulation assays

T cell subsets were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, and 1 mM HEPES buffer. For ConA stimulation, 1 x 10^5 viable cells/well were cultured in a round-bottom 96-well plate in the presence or absence of 5 μg/ml Con A and/or 100 U/ml recombinant human IL-2 (rhIL-2) (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD). All assays were run in triplicate. After 2 days of incubation, cells were pulsed with 1 μCi of [^{3}H]TdR/well and harvested 16hrs later using a Filtermake Harvester (Packard Bioscience, Meriden, CT). [^{3}H]TdR uptake as a measure of proliferation was determined in cpm using a Top Count NXT Microplate scintillation counter (Packard Bioscience).

Co-culture immunosuppression assay

Purified $CD4^+CD25^-$ T target cells (10^6 cells/ml) were stimulated for 3hrs with 5 μg/ml ConA, washed twice in RPMI 1640, and plated at 5×10^4 viable cells/well in 96-well plates. Varying numbers of freshly isolated autologous $CD4^+CD25^+$ Treg effector cells were added to the target cells to yield E:T ratios ranging from 0.125:1 to 1:1. The assays were run in triplicate. Effector and target cells were cocultured for 3 days, then pulsed with 1μCi of [^{3}H]TdR/well, and harvested 16hrs later as described above. Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ T target cells alone and calculated as follows: percent inhibition = (cpm of target cells alone - cpm of coculture) / cpm of target cells alone.

PCR Analysis

Total RNA was isolated from $1x10^6$-$2x10^6$ $CD4^+$ T cell subsets using RNA extraction kit (Qiagen) and cDNA was prepared using random oligo(dT) primers (reverse transcription system kit from Promega). $p21^{cip1}$ message expression was detected using feline specific $p21^{cip1}$ primer sequences: *5' GGAGCGATGGAACTTCGACTTTGT 3'* (forward) and *5' GCCGGCGTTTGGATGATAGAAAT 3'* (reverse). The expression level of GAPDH was determined to ensure equal amounts of RNA in the samples.

Immunobloting

Equal numbers of purified $CD4^+$ T cell subsets ($1x10^6$ – $2x10^6$ cells) were lysed using NP-40 lyses buffer for 16-24hrs at 4C. The concentrations of the proteins were

determined using a spectrophotometer (Biorad Smartspec 3000), 30-50μg of proteins were then resolved on 4-12% SDS-polyacrylamide gels (Novex Inc.). The separated proteins were transferred onto nitrocellulose and immunoblotted with specific mAbs to $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ (Santa Cruz biotechnology Inc) followed by HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc). Immunodetection was performed by ECL (Pierce) using HyperFilm ECL. To normalize the result, some blots were stripped with the Western Blot stripping buffer (Pierce) and reprobed with anti-actin mAb (Santa Cruz Biotechnology inc.). Specific antibody conjugated blots were analyzed for specific proteins using Lumicapture analyst camera detection system (Lumi- Imager).

Statistical Analysis

The Mann-Whitney U test was used to assess significance of difference between the medians in determining the CD62L-selectin expression, TGFβ , TGFβRII on the purified $CD4^+$ T cell subsets in FIV-infected and control cats. p-values < 0. 05 were considered significant.

RESULTS

Anergic phenotype and suppressor function of $CD4^+CD25^+$ Treg cells from FIV-infected and control cats

We reported previously that while $CD4^+CD25^+$ Treg cells from both FIV-infected and control cats were anergic to immune stimulation, only those from FIV-infected cats

displayed strong suppressor function in the absence of in vitro mitogenic stimulation (Vahlenkamp, Tompkins et al. 2004). To confirm that the $CD4^+CD25^+$ Treg cells from the cats used in this study display this pattern of anergy and suppressor function, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets were purified by flow cytometry sorting of PBMC from FIV-infected and control cats and assessed for anergy and suppressor function. **Figure 1** shows a typical response of the $CD4^+$ T cell subsets in these assays. **Figure 1A** shows the $CD4^+CD25^-$ T cells from FIV-infected and control cats proliferate efficiently in response to ConA stimulation, whereas $CD4^+CD25^+$ Treg cells are anergic to ConA stimulation but are responsive to ConA plus rhIL-2 stimulation. In the case of immune suppressor function, $CD4^+CD25^+$ Treg cells from FIV-infected cats demonstrate a strong dose-dependent anti-proliferative effect against ConA-stimulated $CD4^+CD25^-$ T cells, whereas $CD4^+CD25^+$ Treg cells from control cats suppressed the $CD4^+CD25^-$ T cell proliferative response only at the highest E:T ratio **(Figure 1B)** as has been previously reported for murine and feline Treg cells (Caramalho, Lopes-Carvalho et al. 2003; Vahlenkamp, Tompkins et al. 2004). Thus, the $CD4^+CD25^+$ Treg cells from FIV-infected cats and control cats to be used in these studies displayed the predicted functional characteristics, suggesting that they are functionally activated in FIV-infected cats.

CdkI genes are differentially expressed in PBMC from FIV-infected and control cats

Progression through G0/G1 to S phase of the cell cycle is regulated by cyclins and cyclin-dependent kinases (Cdk), whose activities in turn are regulated by their specific inhibitors, CdkI's. As Treg cells are anergic and arrested in the G0/G1 phase of the cell

cycle (Vahlenkamp, Tompkins et al. 2004), we analyzed the expression of some of the CdkI's in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells to determine their possible role in maintaining anergy. We initially examined the expression of the CdkI's, $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ in total PBMC isolated from FIV-infected and control cats. Whole cell lysates of unstimulated PBMC from control and FIV-infected cats were separated by PAGE, transferred onto nitrocellulose and immunoblotted with specific mAbs to the CdkI's $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$. As shown in **Figure 2,** enhanced expression of $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ proteins was observed in PBMC from FIV-infected cats compared to control cats.

$CD4^+CD25^+$ Treg cells from FIV-infected but not control cats preferentially over-express CdkI proteins and mRNA compared to $CD4^+CD25^-$ T cells

To determine whether $CD4^+CD25^+$ or $CD4^+CD25^-$ T cell subsets are contributing to the differential CdkI expression observed in the total PBMC population of FIV-infected cats and uninfected cats **(Figure 1),** highly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets were assessed for CdkI, cyclin, and retinoblastoma protein expression by immunoblotting. As $p21^{cip1}$ expression is reportedly transcriptionally regulated (Harr, Graves et al. 2005), we also examined $p21^{cip1}$ mRNA expression in the two $CD4^+$ T cell subsets from PBMC of FIV-infected and control cats. As shown in **Figure 3A**, there is a high level of constitutive $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ protein expression in $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cells from FIV-infected cats. In contrast, there is no discernable difference in the protein expression of CdkI's in $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from control cats (data shown for only $p21^{cip1}$, **Figure 3B**). $p21^{cip1}$

mRNA was also elevated in $CD4^+CD25^+$ Treg cells as compared to $CD4^+CD25^-$ T cells from FIV–infected cats but not control cats **(Figure 3C)**. Thus, $p21^{cip1}$, $p16^{ink4}$ and to lesser extent, $p27^{kip1}$ are over-expressed in unstimulated $CD4^+CD25^+$ Treg cells from FIV-infected cats. In contrast, cyclin D **(Figure 3D)**, and phosphorylated retinoblastoma protein (pRb) **(Figure 3D)** were expressed at elevated levels in $CD4^+CD25^-$ T cells compared to $CD4^+CD25^+$ Treg cells. This suggests that only $CD4^+CD25^+$ Treg cells but not $CD4^+CD25^-$ T cells are arrested in the G0/G1 phase of the cell cycle.

Mitogenic activation of $CD4^+CD25^+$ Treg cells from control cats induces $p21^{cip1}$ protein expression

As observed by others with murine Treg cells (Caramalho, Lopes-Carvalho et al. 2003), we reported that feline $CD4^+CD25^+$ Treg cells from FIV-infected cats could be induced to strong suppressor function by mitogen ± IL-2 or by IL-2 itself (Vahlenkamp, Tompkins et al. 2004). To assess the $p21^{cip1}$ response to mitogenic stimulation $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from FIV-infected and control cats were stimulated with ConA ± rhIL-2 and assayed for $p21^{cip1}$ protein by western blot analysis. ConA stimulation of $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells from FIV-infected cats had no effect on $p21^{cip1}$ expression, whereas $p21^{cip1}$ was markedly up-regulated in ConA-stimulated $CD4^+CD25^+$ Treg cells, but not in ConA-stimulated $CD4^+CD25^-$ T cells from control cats **(Figure 4A)**. ConA induction of $p21^{cip1}$ in $CD4^+CD25^+$ Treg cells from control cats was over-ridden by addition of rhIL2 **(Figure 1A)**. As we have reported previously that LPS stimulation of $CD4^+CD25^+$ Treg cells from control cats induced immunosuppressor function, we examined the effect of LPS on $p21^{cip1}$ expression. Stimulation with LPS

increased $p21^{cip1}$ expression in $CD4^+CD25^+$ Treg cells from control cats, which was over-ridden by the addition of rhIL-2 **(Figure 4B)** . LPS had little effect on either $CD4^+$ T cell subsets from FIV-infected cats **(Figure 4B)**. Thus, there is a correlation between previously reported mitogen-induced Treg immuno-suppressor function and induction of $p21^{cip1}$ expression. Interestingly, IL-2 down-regulates both the induced and basal levels of $p21^{cip1}$ expression in $CD4^+CD25^+$ Treg cells, which is consistent with its ability to over-ride anergy (Vahlenkamp, Tompkins et al. 2004).

TGFβ1 is up-regulated on the surface of $CD4^+CD25^+$ Treg cells from FIV-infected cats

$CD4^+CD25^+$ Treg cells from humans and mice secrete high levels of TGFβ1 and express TGFβ1 on their surface when appropriately stimulated (Nakamura, K, JEM, Annunziato 2001; Annku, F, JEM, 2002; Cosmi L, Blood, 2003). In view of our observation that CdkI's are up-regulated in Treg cells from FIV-infected cats and the fact that TGFβ1 upregulates expression of CdkI's and induces G0/G1 cell cycle arrest (Dannlers), we examined the expression of TGFβ1 on the surface of PBMC $CD4^+$ T cells from control and FIV-infected cats by flow cytometry. **Figure 5A** shows a significant increase in numbers of $CD4^+CD25^+$ Treg cells expressing cell surface TGFβ1 (39.9%) in FIV-infected cats as compared to TGFβ1 positive $CD4^+CD25^+$ Treg cells (10.4%) from control cats. There is no significant difference in expression of TGFβ1 on $CD4^+CD25^-$ T cells from FIV-infected (16.1%) and control cats (11.4%). Analysis of 6 asymptomatic FIV-infected cats and six age-matched control cats revealed a consistent pattern of increased numbers of $CD4^+CD25^+$ TGFβ1 Treg cells in the FIV-infected cats as

compared to controls **(Figure 5B)**. These data support the CdkI expression data and the immunosuppressor function data suggesting that $CD4^+CD25^+$ Treg cells from FIV-infected cats are activated in vivo.

TGFβRII is up-regulated on the cell surface of $CD4^+CD25^+$ Treg cells from FIV-infected cats.

There is considerable data on the expression of TGFβ1 on activated Treg cells. However there is no information on possible changes in TGFβ1 receptor expression. To assess TGFβRII expression on Treg cells, we performed 3-color flow cytometry on purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell subsets from FIV-infected and control cats. Similar to observations of increased TGFβ1 positive $CD4^+CD25^+$ Treg cells from FIV-infected cats compared to control cats **(Figure 5)**, TGFβRII was preferentially expressed on the surface of $CD4^+CD25^+$ Treg cells from FIV-infected (19%) compared to control (4%) cats **(Figure 6A)**. A very small fraction of $CD4^+CD25^-$ T cells from both FIV-infected and control cats expressed cell surface TGFβRII, although significantly more $CD4^+CD25^-$ T cells from FIV-infected cats expressed TGFβRII than those from control cats **(Figure 6A)**. Flow cytometric analysis of 6 asymptomatic FIV-infected cats and 6 age-matched controls confirmed that significantly more $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from FIV-infected cats expressed cell surface TGFβRII **(Figure 6B)** as compared to their counterparts from control cats. There was no significant difference in TGFβRII expression between $CD4^+CD25^{bright}$ and $CD4^+CD25^{dim}$ from FIV-infected cats **(Figure 6C)**. Whether these numbers of TGFβRII positive cells accurately reflect the actual numbers of TGFβRII positive T cells, particularly in the FIV-infected cats is not clear

from these data, as the high level of cell surface TGFβ1 expression could block antibody binding to the TGFβRII. However, 3- and 4-color flow cytometry analysis revealed that 80-90% of $CD4^+CD25^+TGF\beta1^+$ T cells also expressed TGFβRII (data not shown) suggesting that the TGFβRII and its ligand are co-expressed on Treg cells.

L-Selectin expression in $CD4^+CD25^+$ vs. $CD4^+CD25^-$ T cells from peripheral blood of FIV-infected and control cats

As shown in **Figures 2, 3, and 4**, we were able to consistently detect $p21^{cip1}$ in unstimulated $CD4^+CD25^-$ T cells from control cats. However, we were not able to detect this protein in $CD4^+CD25^-$ T cells from FIV-infected cats. As CdkI's generally accumulate in G0/G1 resting cells but are down regulated following activation (Balomenos, D, Imm Today, 2000), we speculated that the differential expression of $p21^{cip1}$ in $CD4^+CD25^-$ T cells between FIV-infected and control cats may be due to the activation state of the cells. Loss of L-selectin (CD62L) expression on lymphocytes is a useful phenotypic marker for T cell activation (Gebhart). Therefore, we analyzed the $CD4^+$ T cell subsets from FIV-infected and control cats for the expression of CD62L-selectin. **Figure 7A** shows a typical 3-color analysis for the expression of CD62L on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from a FIV-infected and control cats. Greater than 80% of the $CD4^+$ cells, whether or not they expressed CD25, were $CD62L^+$ in the control cat. In contrast, only 41% of the $CD4^+CD25^-$ T cells and 15% of the $CD4^+CD25^+$ Treg cells from the FIV-infected cat expressed CD62L. Analysis of data from a number of cats consistently demonstrate a difference in CD62L expression on $CD4^+$ subsets

between FIV-infected and normal cats **(Figure 7B),** indicating a large percent of the cells in both $CD4^+$ subsets are activated in FIV-infected cats. As reported in murine and human models $CD4^+CD25^{bright}$ cells express elevated levels of CD62L-selectin in FIV-infected cats but not control cats compared to $CD4^+CD25^{dim}$ T cells **(Figure 7C)**. The failure to detect $p21^{cip1}$ in $CD4^+CD25^-$ T cells from FIV-infected cats as opposed to $CD4^+CD25^-$ T cells from control cats may be the result of T cell activation in FIV-infected cats.

DISCUSSION

Treg cells are characterized by G0/G1 cell cycle arrest and are anergic to immune activation, as manifested by their inability to produce IL-2 and exit the G0/G1 stage of cell cycle in response to antigen-specific TCR engagement (Powell, Ragheb et al. 1998). Another major characteristic of $CD4^+CD25^+$ Treg cells described in human and rodent species is their ability, once activated through their TCR, to suppress proliferative responses of other antigen- or mitogen-stimulated $CD4^+$ or $CD8^+$ T cells (Thornton and Shevach 1998; Jackson, DeLoose et al. 2002). We recently described $CD4^+CD25^+$ Treg cells in the cat possessed all the major phenotypic and functional characteristic of Treg cells described in other species i.e. they are G0/G1 cell cycle arrested, IL-2 deficient and anergic to mitogenic stimulation, and when activated capable of suppressing IL-2 production and proliferation of activated T cells (Vahlenkamp, Tompkins et al. 2004).

The hallmark of naturally occurring Treg cells, including those in normal cats is that they require activation by antigen-TCR engagement or a potent mitogen before they can display immunosuppressive function (Thornton and Shevach 1998). In marked contrast to natural Treg cells, $CD4^+CD25^+$ Treg cells from cats chronically infected with the feline AIDS lentivirus (FIV) are activated in-vivo and displayed strong T cell immunosuppressive function in the absence of in-vitro stimulation (Vahlenkamp, Tompkins et al. 2004). In this study we confirmed that $CD4^+CD25^+$ Treg cells from FIV-infected cats, as opposed to normal cats, are functionally immunosuppressive and that this activation phenotype is associated with up-regulation of the Cdk inhibitors $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ and over-expression of TGFβ1 and TGFβRII on their surface.

Induction and maintenance of T cell anergy due to inappropriate TCR signaling has been associated with over-expression of the cyclin-dependent kinase inhibitors $p21^{cip1}$ and $p27^{kip1}$ (Jackson, DeLoose et al. 2002; Wolfraim, Walz et al. 2004). A direct relationship between $p27^{kip1}$ over-expression and immunological unresponsiveness was demonstrated in $p27^{kip1}$-transfected murine and human $CD4^+$ T cells (Boussiotis VA, 2000). As $CD4^+CD25^+$ Treg cells, unlike Th cells are naturally anergic, it was of interest to determine if the CdkI's were constitutively up-regulated in these cells or required an induction signal for expression.

In this study we demonstrated that the CdkI's, $p21^{cip1}$, $p16^{ink4}$, and $p27^{kip1}$ proteins are expressed at elevated levels in the total PBMC of FIV-infected cats compared to control cats. Upon further examination of $CD4^+$ T cell subsets, we observed an increase in

$p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ expression in the $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cells. Interestingly, up-regulation of CdkI's in $CD4^+CD25^+$ Treg cells was restricted to those cells from FIV-infected cats.

As these cats are FIV-infected and chronically antigenemic, we speculated that activation of CdkI's in $CD4^+CD25^+$ Treg cells may be due to immune activation. This is consistent with the observation that $CD4^+CD25^+$ Treg cells from FIV- infected cats but not control cats are functionally immunosuppressive and express enhanced levels of cell surface B7.1 and B7.2 in the absence of in vitro stimulation (Vahlenkamp, Bull et al. 2004; Vahlenkamp, Tompkins et al. 2004). We extend this observation in this study by demonstrating that $CD4^+CD25^+$ Treg cells from FIV-infected cats that displayed increased expression of CdkI's also possessed strong immunosuppressive function in the absence of in-vitro stimulation. Others have demonstrated in-vivo activation of immunosuppressive Treg cells in a number of chronic infections, such as HIV, lieshmania and malaria (Belkaid 2003; Hisaeda, Maekawa et al. 2004; Tsunemi, Iwasaki et al. 2005); Belkaid and Rouse, Nat Imm, 2005), but have not examined the CdkI expression profile. While we have not identified the $CD4^+CD25^+$ Treg cell activation factors in chronically infected cats, a number of studies have demonstrated that naïve or natural Treg cells could be induced to suppressor activity by antigenic or mitogenic stimulation (51). We also reported that $CD4^+CD25^+$ Treg cells from naïve cats could be induced to strong suppressor function by LPS and IL-2 (Vahlenkamp, Tompkins et al. 2004). In this study, we also demonstrated that $p21^{cip1}$ expression in $CD4^+CD25^+$ Treg cells from control cats was markedly enhanced by ConA or LPS stimulation. As

expected, these mitogen did not induce $p21^{cip1}$ expression in $CD4^+CD25^-$ T cells from either FIV-infected or control cats. Consistent with the observation that exogenous IL-2 is able to override the anergic state of feline $CD4^+CD25^+$ Treg cells in vitro (Vahlenkamp, Tompkins et al. 2004), we observed that rhIL-2 reduced the mitogen-induced, as well as basal levels of $p21^{cip1}$ expression in T cells.

Over-expression of CdkI's in $CD4^+CD25^+$ Treg cells from FIV-infected cats could result from up-regulation of the TGFβ1 signaling pathway. In many cell types, TGFβ1 causes a G0/G1 cell-cycle arrest by inhibiting Cdk activity via activation of $p15^{ink4b}$ and $p21^{cip1}$ expression (Dennler S). A number of studies have demonstrated that TGFβ1 is up-regulated on the surface of $CD4^+CD25^+$ Treg cells following treatment with appropriate activation stimuli (Nakumura N, Annumziato F, Cosmi; (Hoffmann, Ermann et al. 2002), while others have demonstrated that treatment of $CD4^+CD25^-$ T cells with TCR stimuli in the presence of TGFβ1 converted them to phenotypic and functional Treg cells (Chen W, JEM 198; 2003; Fantini MC, JI, 2004). As the TGFβ1 signaling pathway also is capable of up-regulating $p21^{cip1}$ and $p27^{kip1}$ expression and inducing G0/G1 cell cycle arrest, we examined Treg cell from FIV-infected cats for cell surface TGFβ1 expression. Our data clearly demonstrates that the numbers of $CD4^+CD25^+$ Treg cells expressing TGFβ1 on their surface is increased in FIV-infected cats compared to control cats. Importantly, we were also able to demonstrate that TGFβRII is also up-regulated on the surface of $CD4^+CD25^+$ Treg cells from FIV-infected cats, but not control cats. To the best of our knowledge this is the first report of up-regulation of TGFβRII on the plasma membrane of activated $CD4^+CD25^+$ Treg cells. These data provide a direct link between

TGFβ1/TGFβRII expression, activation of CdkI's and Treg suppressor function. As the biological half-life of TGFβ1-family ligands is short and they usually act in an autocrine manner, it is possible that phenotypic and functional activation of Treg cells in FIV-infected cats is the result of autocrine TGFβ1/TGFβR signaling. This is consistent with the observation that activated Treg cells secrete high levels of biological active TGFβ1 (Nakumar, Maloy and Powers). This is also supported by 3- and 4-color flow cytometry analysis revealing that TGFβ1 and TGFβRII were co-expressed on the subset of $CD4^+CD25^+$ Treg cells. Preliminary data analyzing downstream elements of the TGFβ1/TGFβR signaling pathway shows that SMAD2 is phosphorylated and Rb protein in hypophosphorlated in Treg cells from FIV-infected cats suggesting that the pathway is functional (S. Emani, preliminary data). Whether the TGFβ1/TGFβR signaling pathway and up-regulation of CdkI's play a role in the immunosuppressor function of Treg cells remains to be determined. However, it is interesting that TGFβ1 stimulation of $CD4^+CD25^-$ T cells in the presence of TCR engagement converts them to $CD25^+TGF\beta1^+Foxp3^+$ T cells with potent inmmunosuppressive properties (Chen, Fantini). We have also demonstrated that feline $CD4^+CD25^-$ T cells could be converted to $CD4^+CD25^+TGF\beta1^+Foxp3^+$ immunosuppressive Treg cells by stimulation with ConA plus TGFβ1 (C. Petty unpublished data). While TGFβ1 signaling may regulate Treg suppressor activity, it is unlikely to directly relate to up-regulation of CdkI's as we have observed that IL-2 treatment of Treg cells reduces both the basal level and induced $p21^{cip1}$ expression, yet has been shown to substantially increase both feline (Vahlenkamp, Tompkins et al. 2004) and murine (Fu, Zhang et al. 2004) Treg suppressor function.

Whether cell surface expressed TGFβ1 plays a direct role in mediating T cell suppression was not evaluated in this study. The role of TGFβ1 immune suppression by rodent and human Treg cells is controversial. While Nakamura et al. reported that both cell bound and soluble TGFβ1 could mediate T cell immune suppression other have reported that soluble TGFβ1 does not mediate suppression (Takaski T, Int Imm 1998, Thornton AM, JEM 1998). Additional studies are in progress to determine the possible role of TGFβ1 in mediating feline $CD4^+CD25^+$ Treg immunosuppressor function.

An additional observation of interest from this study is that, while we could consistently measure $p21^{cip1}$ in unstimulated $CD4^+CD25^-$ T cells from control cats, it was unusual to detect this protein in $CD4^+CD25^-$ T cells from FIV-infected cats. This could be due to the activation state of the $CD4^+$ T cells from FIV-infected cats with long-term asymptomatic infection. It is well established that resting or naïve Th cells constitutively express $p27^{kip1}$ protein which is rapidly degraded following activation (Kwon TK, 1997; Appleman LJ, JI, 2000). Analysis of CD62L-selectin expression on $CD4^+CD25^-$ T cells from naïve cats used in this study indicated that the majority were phenotypically naïve as indicated by high expression of cell surface CD62L-selectin. In contrast, the majority of $CD4^+CD25^-$ T cells from FIV– infected cats that did not express $p21^{cip1}$ also did not express cell surface CD62L-selectin, indicating that they are activated. A similar state of chronic T cell immune hyperactivation has been described previously in FIV infected cats (Vahlenkamp, Tompkins et al. 2004) and in HIV–infected patients with established infections (Eggena, Barugahare et al. 2005). Whether and how this state of T cell immune hyperactivation would affect $p21^{cip1}$ expression remains to be determined.

Although, this study did not address the factors responsible for activating Treg cells in FIV–infected cats, there are a number of possibilities. While natural autoreactive Treg cells are thought to be activated by TCR engagement by their cognate antigen (Maloy and Powrie), pathogen-induced Treg cells can be activated by a number of mechanisms including antigen-specific TCR engagement as has been suggested for a number of pathogens (Price, Schaumburg et al. 2005). $CD4^+CD25^+$ Treg cells also express a number of TLR's and could be activated by pathogen associated molecular pathogens (PAMPS) such as LPS, as has been reported for murine and feline Treg (Caramalho, Lopes-Carvalho et al. 2003; Vahlenkamp, Tompkins et al. 2004). The cytokine microenvironment is another possibility, as IL-2 has been shown to be a potent inducer of Treg immunosuppressor function (Caramalho, Lopes-Carvalho et al. 2003; Vahlenkamp, Tompkins et al. 2004) and a number of cytokine including TGFβ1, IFNγ, and IL-6 have been reported to up-regulate $p21^{cip1}$ expression in other cell types (Gartel and Tyner 2002). Direct interactions between FIV and Treg cells is another possibility, as FIV has been shown to preferentially establish a productive infection in $CD4^+CD25^+$ Treg cells, although the infection does not appear to be cytolytic (Joshi, Garg et al. 2005). HIV has similarly been shown to preferentially establish productive infection in $CD4^+CD25^+$ Treg cells as opposed to $CD4^+CD25^-$ T cells (Borvak, Chou et al. 1995). Studies are currently in progress to explore the possible mechanisms of $CD4^+CD25^+$ Treg cells activation in FIV-infected cats.

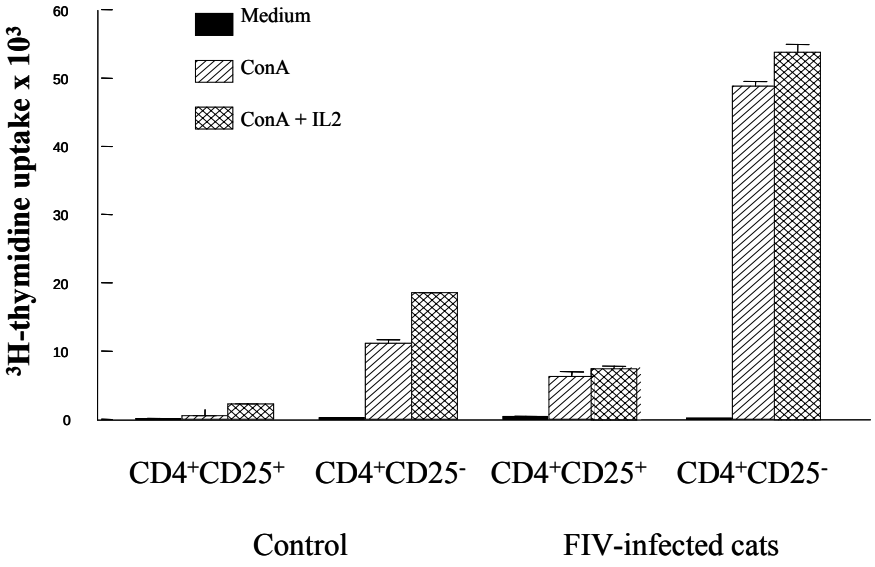
In conclusion, while $CD4^+CD25^+$ Treg cells from both FIV-infected and naïve cats are functionally anergic, as manifested by their inability to produce IL-2 and proliferate upon mitogenic stimulation, only those from FIV-infected cats are activated for strong suppressor function in-vivo. Functional activation of Treg cells from FIV-infected cats correlates with up-regulation of TGFβ1 and TGFβRII on their surface and over expression of the CdkI's, $p21^{cip1}$, $p27^{kip1}$ and $p15^{ink4}$. As TGFβ1/TGFβR signaling is known to induce G0/G1 cell-cycle arrest by up-regulation of CdkI's, it can be speculated that the other phenotypic and functional responses observed in Treg cells from FIV-infected cats derive from the TGFβ1/TGFβR signaling pathway. Further, molecular characterization of $CD4^+CD25^+$ Treg cells from FIV chronically infected cats may be helpful in providing experimental basis for understanding the immunopathogenic potential of activated Treg cells and developing immunotherapeutic strategies in treatment for chronic diseases such as HIV/FIV infection.

ACKNOWLEDGEMENTS

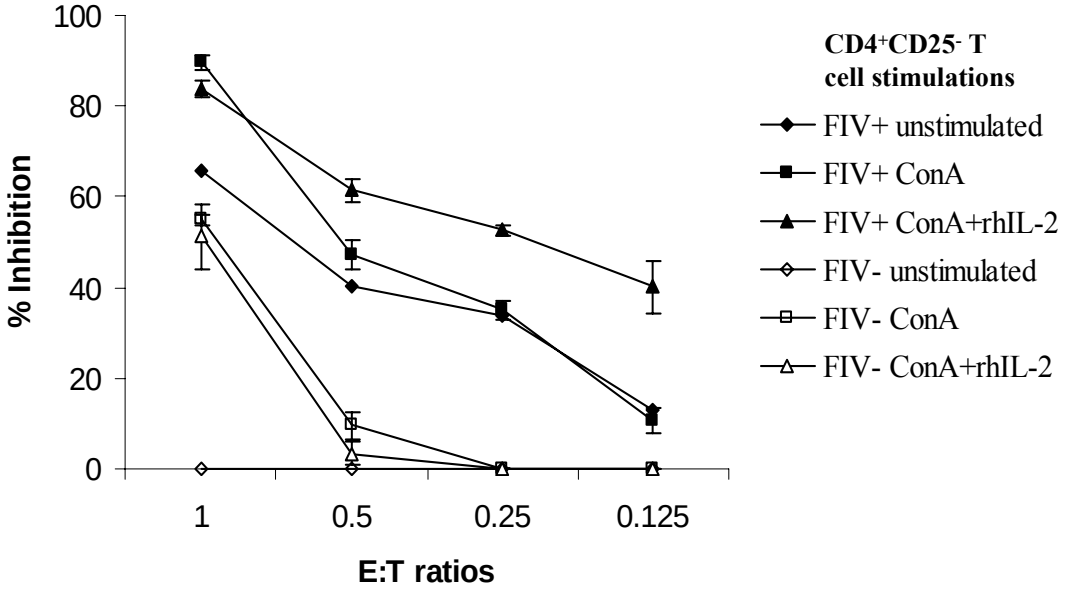
We thank Janet Dow and Debra Anderson for their technical assistance. We thank Dr. Koishi Ohno for providing the anti-CD25 antibody. We also thank NIH AIDS Research and Reference reagent program for providing rHuIL-2. The present study was supported by National Institute of Health grants AI38177, AI 43858, and AI058691.

Figure1. Suppressor activity and proliferative response of purified CD4$^+$ T cell subsets from PBMC of FIV-infected cats to Con A and/or rhIL-2 stimulation. **(A)** Mo-flow purified CD4$^+$ T cell subsets (2 x 10^5) were incubated with or without ConA (5 μg/ml) and/or rhIL-2 (100 U/ml) for 48hrs, pulsed with [^{3}H]TdR, and harvested 16hrs later. Bars represent the mean ± SEM of triplicates. **(B)** Suppressor activity of freshly isolated CD4$^+$CD25$^+$ Treg cells on autologus CD4$^+$CD25$^-$ T cells from FIV-infected cats and control cats. FACS-purified CD4$^+$CD25$^-$ T target cells were either unstimulated or stimulated with ConA (5μg/ml) +/- rhIL-2 (100 U/ml) for 3hrs, then cocultured for 3 days with autologus CD4$^+$CD25$^+$ Treg cells and were pulsed with [^{3}H]TdR 16hrs prior to the harvest. A constant number of CD4$^+$CD25$^-$ T target cells (5 x 10^4/well) were cultured with varying numbers of CD4$^+$CD25$^+$ Treg effector cells. Percent inhibition of proliferation was calculated based on proliferation of CD4$^+$CD25$^-$ T target cells alone ((cpm of target cells alone − cpm of coculture) / cpm of target cells alone). Bars represent mean +/- SEM of triplicate wells for each condition.

A


Medium
ConA
ConA + IL2
^{3}H-thymidine uptake x 10^3
60
50
40
30
20
10
0
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
Control
FIV-infected cats

B


% Inhibition
100
80
60
40
20
0
1
0.5
0.25
0.125
E:T ratios
CD4+CD25- T cell stimulations
FIV+ unstimulated
FIV+ ConA
FIV+ ConA+rhIL-2
FIV- unstimulated
FIV- ConA
FIV- ConA+rhIL-2

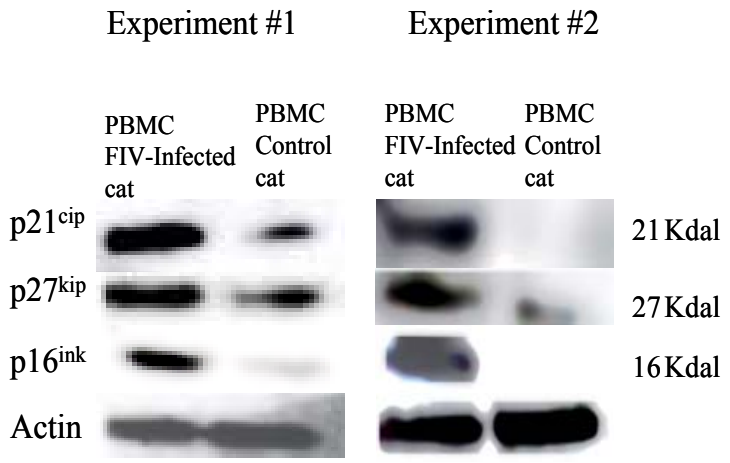


Figure 2. PBMC from FIV-infected cats express elevated levels of CdkI ($p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$) proteins compared to control cats. Fresh PBMC ($2x10^6$ cells) isolated from FIV-infected and control cats were lysed using NP-40 lysis buffer and separated on SDS-PAGE followed by immunoblotting with specific CdkI primary antibodies and HRP-conjugated goat anti-mouse IgG1 secondary antibody and detected by chemiluminescence. The blots were stripped using stripping buffer and reprobed with actin primary antibody and HRP-conjugated goat anti-mouse IgG1 secondary antibody followed by chemilumeniscence detection. These data are representative of gels from two separate experiments, each with an FIV-infected and control cats.

Figure 3. CdkI mRNA and protein expression in the purified CD4$^+$ T cell subsets from FIV-infected and control cats. Equal number of purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells ($2*10^6$) were lysed with NP-40 lysis buffer to obtain total cellular protein for the detection of $p21^{cip1}$, $p27^{kip1}$, $p16^{ink4}$, cyclin D, pRb expression. Total cell lysates from two individual infected cats **(A,D)** and one control cat **(B)** were separated by SDS-PAGE and analyzed by immunoblot using specific primary antibodies against CdkI's ($p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$), followed by HRP-conjugated goat anti-mouse IgG1 secondary antibody and detected by chemiluminescence. The blots were stripped using stripping buffer and reprobed with actin primary antibody and HRP-conjugated goat anti-mouse IgG1 secondary antibody followed by chemilumeniscence detection. **(C)** mRNA expression of $p21^{cip1}$ and GAPDH3 in FIV-infected and control cats.

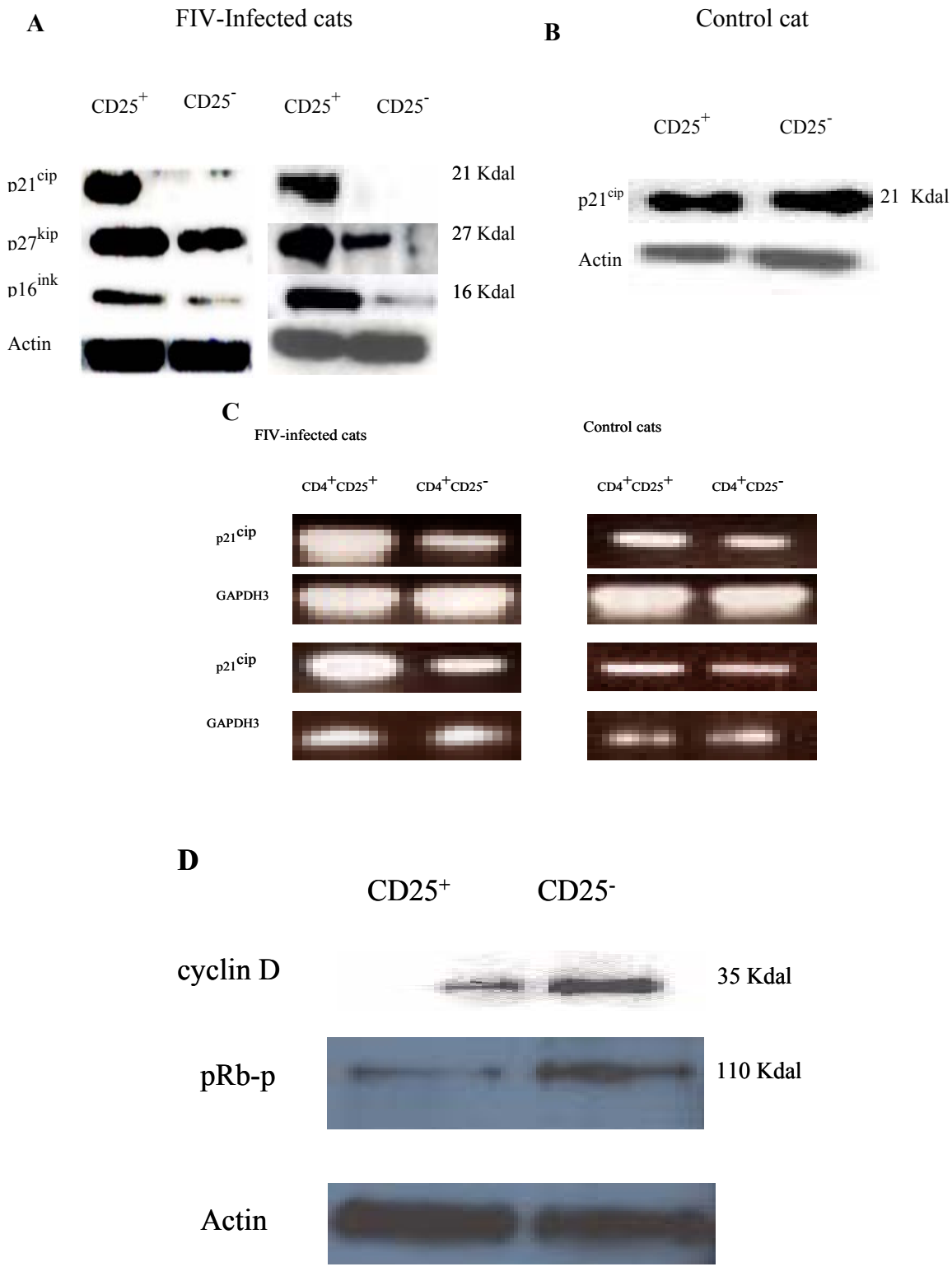
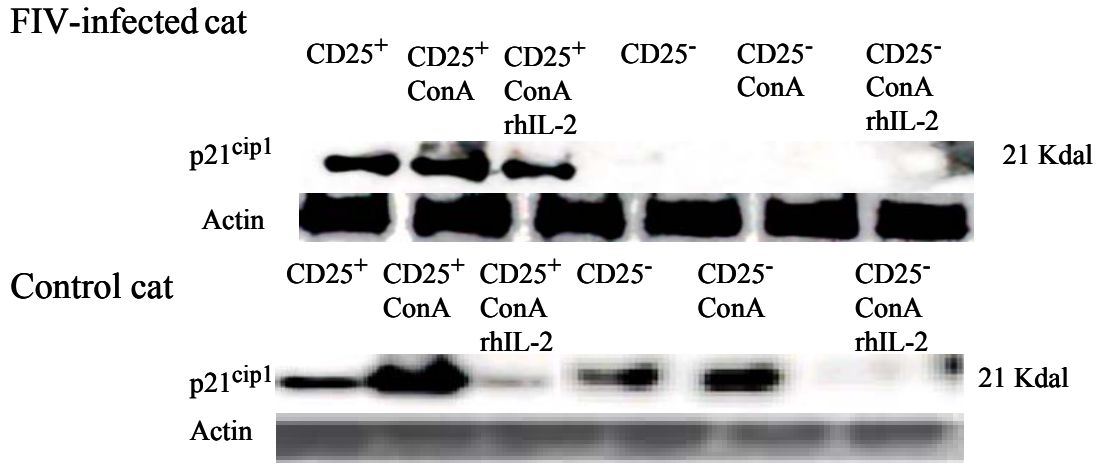

A
FIV-Infected cats
CD25+
CD25-
CD25+
CD25-
p21cip
p27kip
p16ink
Actin
21 Kdal
27 Kdal
16 Kdal
B
Control cat
CD25+
CD25-
p21cip
Actin
21 Kdal
C
FIV-infected cats
Control cats
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
p21cip
GAPDH3
p21cip
GAPDH3
D
CD25+
CD25-
cyclin D
35 Kdal
pRb-p
110 Kdal
Actin

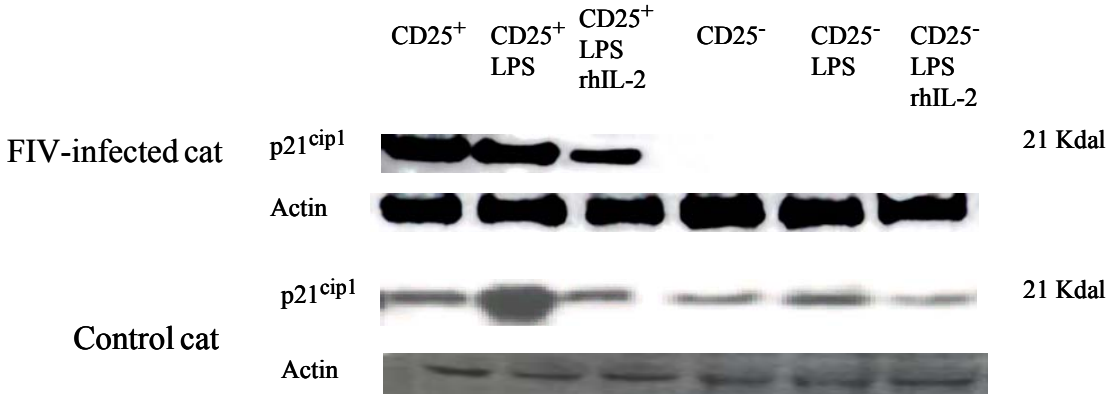
Figure 4. CdkI protein expression in ConA or LPS stimulated CD4$^+$ T cell subsets.

Bead purified CD4$^+$ T cell subsets were cultured with or without 5μg/ml ConA **(A)** or LPS 10ug/ml **(B)** with or without 100U/ml rhIL-2 from FIV infected cats and uninfected cats cultured for 2 days. Equal numbers ($2*10^6$) of cultured cells were lysed using NP-40 lysis buffer and separated on SDS-PAGE followed by immunoblotting with specific CdkI primary antibodies and HRP-conjugated goat anti-mouse IgG1 secondary antibody and detected by chemiluminescence. The blots were stripped using stripping buffer and re-probed with anti-actin primary antibody and HRP-conjugated goat anti-mouse IgG1 secondary antibody followed by chemilumeniscence detection.

A


FIV-infected cat
CD25+
CD25+ ConA
CD25+ ConA rhIL-2
CD25-
CD25- ConA
CD25- ConA rhIL-2
p21cip1
21 Kdal
Actin
Control cat
CD25+
CD25+ ConA
CD25+ ConA rhIL-2
CD25-
CD25- ConA
CD25- ConA rhIL-2
p21cip1
21 Kdal
Actin

B


CD25+
CD25+ LPS
CD25+ LPS rhIL-2
CD25-
CD25- LPS
CD25- LPS rhIL-2
FIV-infected cat
p21cip1
21 Kdal
Actin
p21cip1
21 Kdal
Control cat
Actin

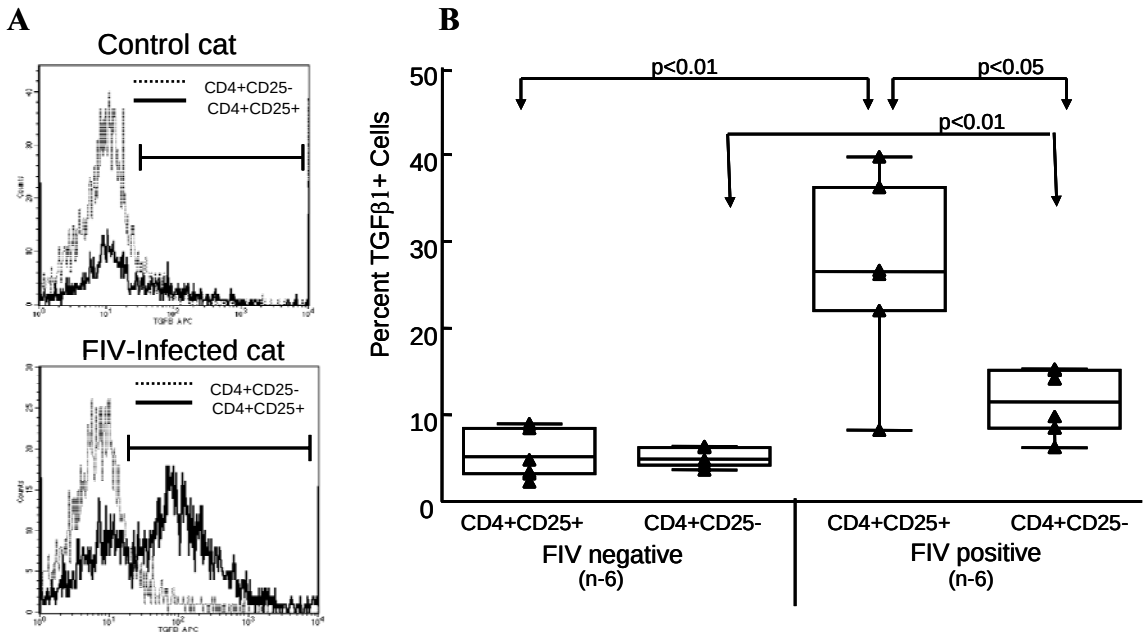
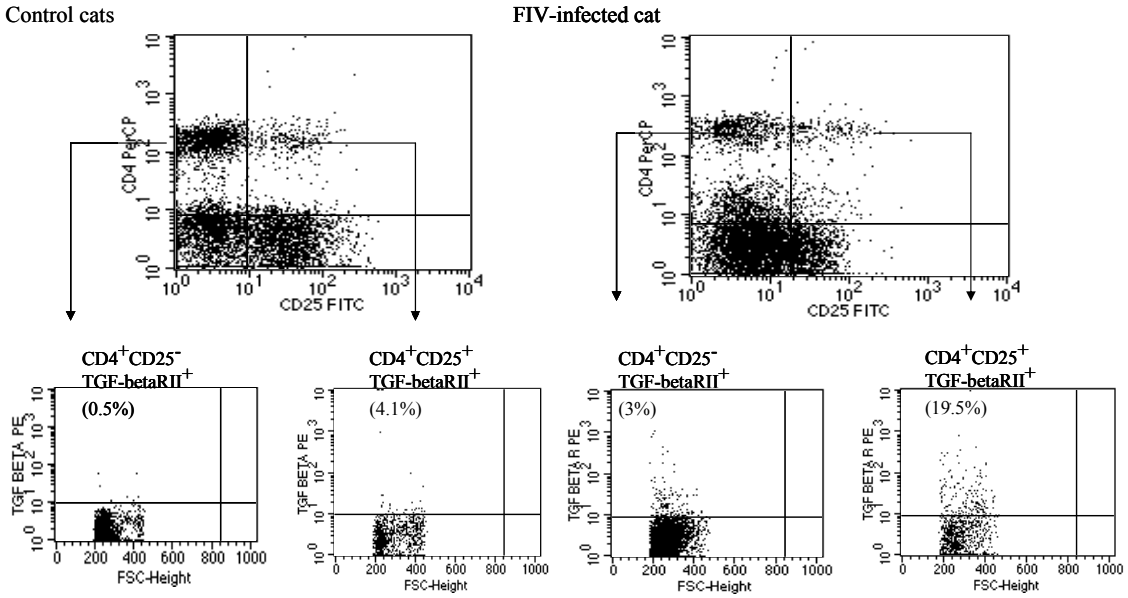


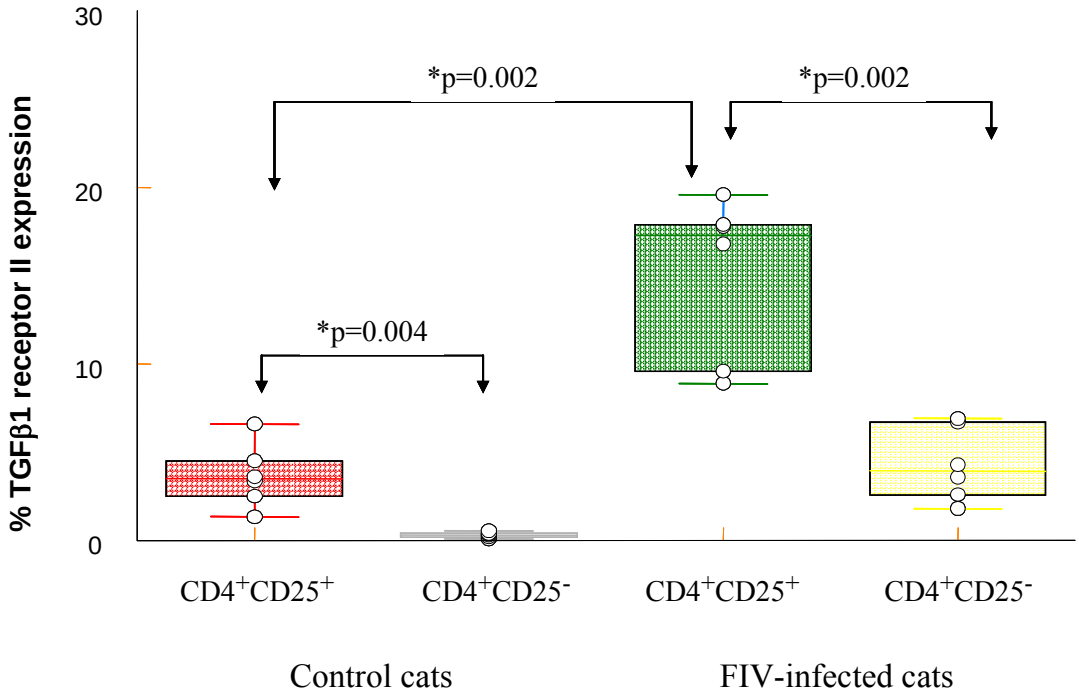
Figure 5. $CD4^+CD25^+$ T cells from FIV-infected cats express surface TGFβ1.

PBMC from FIV-infected and control cats were stained with anti-CD4-PE, anti-CD25-FITC, and anti-TGFβ1-APC. Data were acquired on a FACS Caliber flow cytometer. Lymphocytes were gated based on forward and side scatter and 20,000 gated events acquired for analysis using Cell Quest software. **(A)** Representative flow cytometry histogram of surface TGFβ1 expression on $CD4^+CD25^-$ (dotted line) and $CD4^+CD25^+$ (solid line) T cells from an FIV-infected and control cats. **(B)** Cumulative data for TGFβRII expression on $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from FIV-infected (n=6) and control cats (n=6) cats. The middle line in the box whisker plots indicated by an arrow represents the median of the sample. The middle line in the box whisker plots indicated by an arrow represents the median of the sample. p-values were calculated by Mann-Whitney U test.

A


Control cats
FIV-infected cat
CD4 PerCP
CD25 FITC
CD4+CD25- TGF-betaRII+ (0.5%)
CD4+CD25+ TGF-betaRII+ (4.1%)
CD4+CD25- TGF-betaRII+ (3%)
CD4+CD25+ TGF-betaRII+ (19.5%)
TGF BETA R PE
FSC-Height

B


% TGFβ1 receptor II expression
30
20
10
0
*p=0.002
*p=0.002
*p=0.004
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
Control cats
FIV-infected cats

C

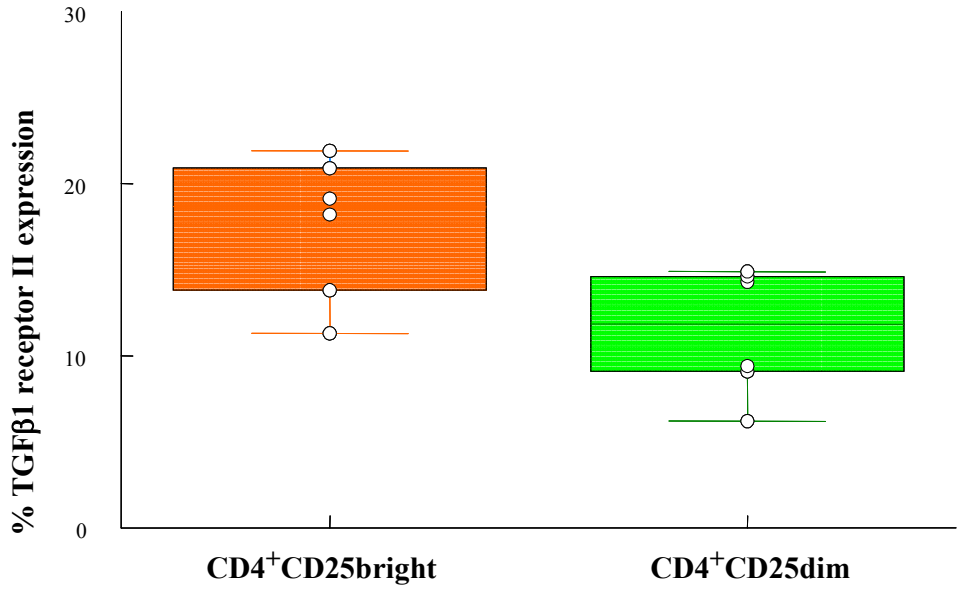
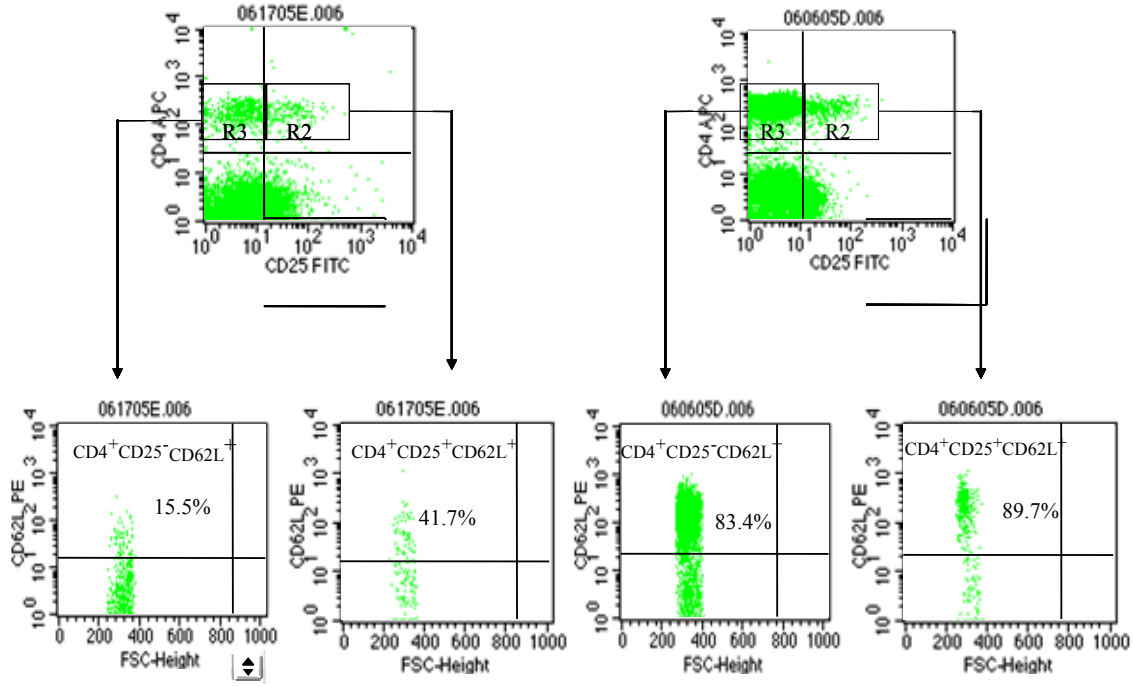
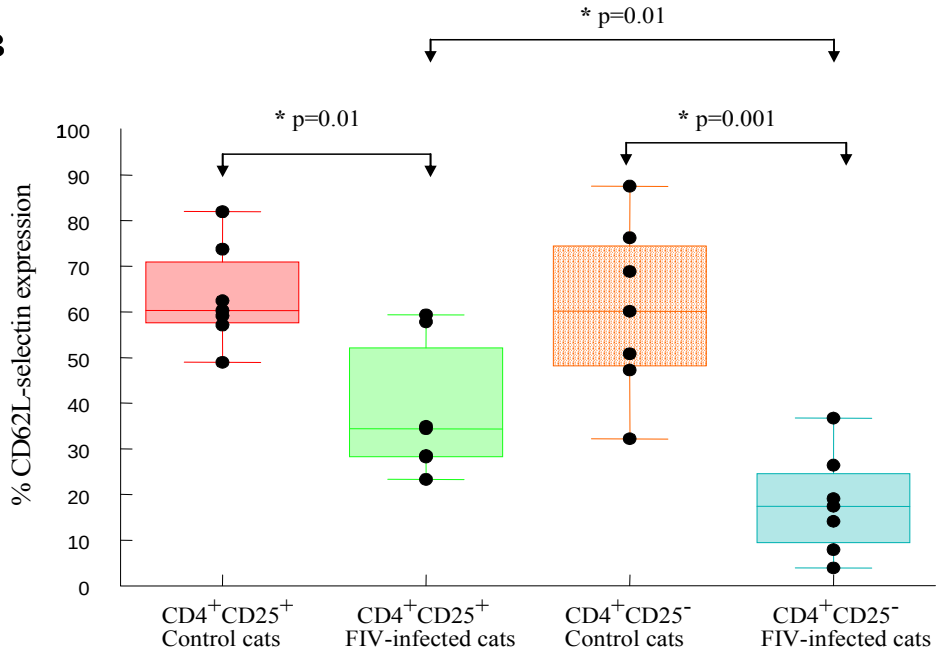


Figure 6. CD4$^+$ T cell subsets express TGFβRII at varying levels in FIV-infected and control cats. **(A)** Representative dot plots for TGFβRII expression in the two CD4$^+$ T cell subsets from FIV-infected and control cats. Mononuclear cells from freshly drawn cat blood were stained with anti-CD4 APC, anti- TGFβRII PE, and anti-CD25 FITC. The cells in this analysis were gated on lymphocytes via their forward and side scatter properties. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were analyzed for surface TGFβRII expression in FIV-infected and control cats. **(B)** Cumulative data for TGFβRII expression on CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from FIV-infected (n=6) and control cats (n=6) cats. **(C)** CD4$^+$CD25^{bright} vs CD4$^+$CD25^{dim} from FIV-infected (n=6) and control cats (n=6) cats. The middle line in the box whisker plots indicated by an arrow represents the median of the sample. The middle line in the box whisker plots indicated by an arrow represents the median of the sample. p-values were calculated by Mann-Whitney U test.

A


FIV-Infected cat
Control cat
061705E.006
060605D.006
CD4 APC
CD25 FITC
R3
R2
CD62L PE
FSC-Height
CD4+CD25-CD62L+
15.5%
CD4+CD25+CD62L+
41.7%
83.4%
89.7%

B


* p=0.01
* p=0.01
* p=0.001
% CD62L-selectin expression
CD4+CD25+ Control cats
CD4+CD25+ FIV-infected cats
CD4+CD25- Control cats
CD4+CD25- FIV-infected cats

C

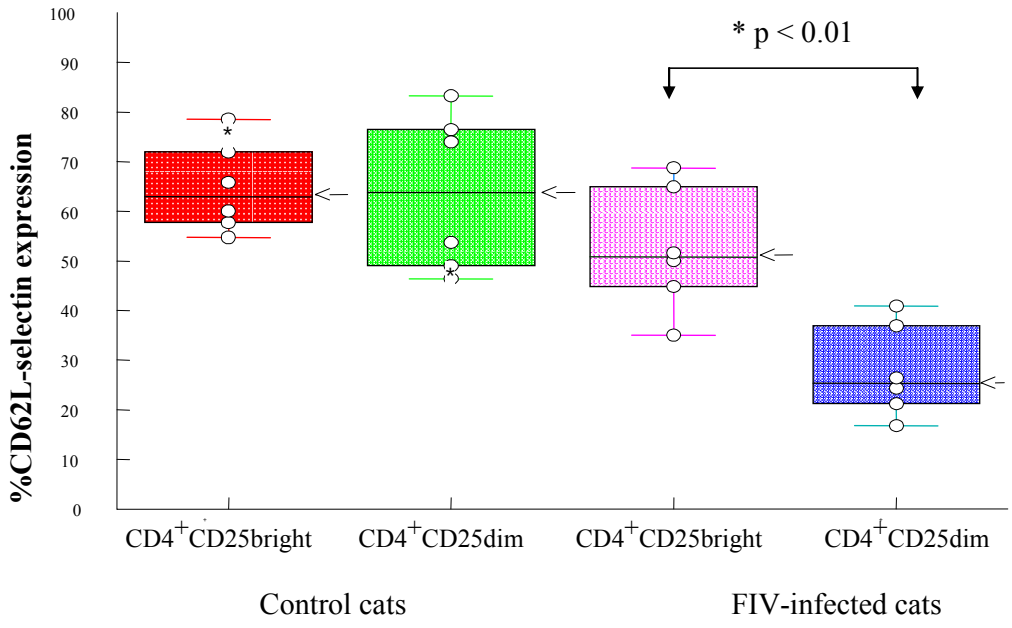


Figure 7. CD4$^+$ T cell subsets express CD62L-selectin at varying levels in FIV-infected and control cats. (**A**) Representative dot plots for CD62L-selectin expression in the two CD4$^+$ T cell subsets from FIV-infected and control cats. Mononuclear cells from freshly drawn cat blood were stained with anti-CD4 APC, anti-CD62L PE, and anti-CD25 FITC. The cells in this analysis were gated on lymphocytes via their forward and side scatter properties. CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells were analyzed for surface CD62L-selectin expression in FIV-infected and control cats. (**B**) Cumulative data for CD62L-selectin expression on CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. **(C)** CD4$^+$CD25^{bright} vs CD4$^+$CD25^{dim} from FIV-infected (n=7) and control cats (n=7) cats. The middle line in the box whisker plots indicated by an arrow represents the median of the sample. p-values < 0.01 as calculated by Mann-Whitney U test were considered significant.

REFERENCES

Balomenos, D., J. Martin-Caballero, et al. (2000). "The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development." Nat Med **6**(2): 171-6.

Belkaid, Y. (2003). "The role of CD4(+)CD25(+) regulatory T cells in Leishmania infection." Expert Opin Biol Ther **3**(6): 875-85.

Belkaid, Y., C. A. Piccirillo, et al. (2002). "CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity." Nature **420**(6915): 502-7.

Belkaid, Y. and B. T. Rouse (2005). "Natural regulatory T cells in infectious disease." Nat Immunol **6**(4): 353-60.

Borvak, J., C. S. Chou, et al. (1995). "Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection." J Immunol **155**(6): 3196-204.

Bucci, J., R. English, et al. (1998). "Mucosally transmitted feline immunodeficiency virus induces a CD8+ antiviral response that correlates with reduction of cell-associated virus." J Infect Dis **177**: 18-25.

Caramalho, I., T. Lopes-Carvalho, et al. (2003). "Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide." J Exp Med **197**(4): 403-11.

Dennler, S., M. J. Goumans, et al. (2002). "Transforming growth factor beta signal transduction." J Leukoc Biol **71**(5): 731-40.

Eggena, M. P., B. Barugahare, et al. (2005). "Depletion of regulatory T cells in HIV infection is associated with immune activation." J Immunol **174**(7): 4407-14.

English, R. V., P. Nelson, et al. (1994). "Development of clinical disease in cats experimentally infected with feline immunodeficiency virus." J Infec Dis **170**: 543-52.

Fu, S., N. Zhang, et al. (2004). "TGF-beta induces Foxp3 + T-regulatory cells from CD4 + CD25 - precursors." Am J Transplant **4**(10): 1614-27.

Gartel, A. L. and A. L. Tyner (2002). "The role of the cyclin-dependent kinase inhibitor p21 in apoptosis." Mol Cancer Ther **1**(8): 639-49.

Greenfield, E., K. Nguyen, et al. (1998). "CD28/B7 costimulation: a review." Crit Rev Immunol **18**: 389-418.

Harr, M. W., T. G. Graves, et al. (2005). "Variation in transcriptional regulation of cyclin dependent kinase inhibitor p21waf1/cip1 among human bronchogenic carcinomas." Mol Cancer **4**: 23.

Harris, N. and F. Ronchese (1999). "The role of B7 costimulation in T-cell immunity." Immunol Cell Biol **77**: 304-11.

Hisaeda, H., Y. Maekawa, et al. (2004). "Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells." Nat Med **10**(1): 29-30.

Hoffmann, P., J. Ermann, et al. (2002). "Donor-type CD4(+)CD25(+) regulatory T cells suppress lethal acute graft-versus-host disease after allogeneic bone marrow transplantation." J Exp Med **196**(3): 389-99.

Hori, S., T. L. Carvalho, et al. (2002). "CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice." Eur J Immunol **32**(5): 1282-91.

Iwashiro, M., R. J. Messer, et al. (2001). "Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection." Proc Natl Acad Sci U S A **98**(16): 9226-30.

Jackson, S. K., A. DeLoose, et al. (2002). "The ability of antigen, but not interleukin-2, to promote n-butyrate-induced T helper 1 cell anergy is associated with increased expression and altered association patterns of cyclin-dependent kinase inhibitors." Immunology **106**(4): 486-95.

Joshi, A., H. Garg, et al. (2005). "Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors." J Virol **79**(8): 4965-76.

Kee, B. L., R. R. Rivera, et al. (2001). "Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta." Nat Immunol **2**(3): 242-7.

Lechler, R., J. G. Chai, et al. (2001). "The contributions of T-cell anergy to peripheral T-cell tolerance." Immunology **103**(3): 262-9.

Maloy, K. J. and F. Powrie (2001). "Regulatory T cells in the control of immune pathology." Nat Immunol **2**(9): 816-22.

Massague, J. (2004). "G1 cell-cycle control and cancer." Nature **432**(7015): 298-306.

Montagnoli, C., A. Bacci, et al. (2002). "B7/CD28-dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to Candida albicans." J Immunol **169**(11): 6298-308.

Morgan, D. O. (1995). "Principles of CDK regulation." Nature **374**(6518): 131-4.

Powell, J. D., J. A. Ragheb, et al. (1998). "Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy." Immunol Rev **165**: 287-300.

Price, J. D., J. Schaumburg, et al. (2005). "Induction of a regulatory phenotype in human CD4+ T cells by streptococcal M protein." J Immunol **175**(2): 677-84.

Read, S., V. Malmstrom, et al. (2000). "Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation." J Exp Med **192**(2): 295-302.

Reed, S. I. (1997). "Control of the G1/S transition." Cancer Surv **29**: 7-23.

Sherr, C. J. and J. M. Roberts (1999). "CDK inhibitors: positive and negative regulators of G1-phase progression." Genes Dev **13**(12): 1501-12.

Shevach, E. M. (2002). "CD4+ CD25+ suppressor T cells: more questions than answers." Nat Rev Immunol **2**(6): 389-400.

Thornton, A. M. and E. M. Shevach (1998). "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production." J Exp Med **188**(2): 287-96.

Tompkins, M. B., D. H. Gebhard, et al. (1990). "Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat." Vet. Immunol. Immunopathol. **26**: 305-317.

Tompkins, M. B., G. K. Ogilvie, et al. (1987). "Induction of IL-2 and lymphokine activated killer cells in the cat." Vet Immunol Immunopathol **16**(1-2): 1-10.

Tsunemi, S., T. Iwasaki, et al. (2005). "Relationship of CD4+CD25+ regulatory T cells to immune status in HIV-infected patients." Aids **19**(9): 879-86.

Vahlenkamp, T., M. Tompkins, et al. (2004). "Feline immunodeficiency virus (FIV) infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory (Treg) cells." J Immunol **172**: 4752-61.
Vahlenkamp, T. W., M. E. Bull, et al. (2004). "B7+CTLA4+ T cells engage in T-T cell interactions that mediate apoptosis: a model for lentivirus-induced T cell depletion." Vet Immunol Immunopathol **98**(3-4): 203-14.
Vahlenkamp, T. W., M. B. Tompkins, et al. (2004). "Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells." J Immunol **172**(8): 4752-61.
Wolfraim, L. A., T. M. Walz, et al. (2004). "p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness." J Immunol **173**(5): 3093-102.
Yamato, K., S. Hashimoto, et al. (2001). "Activation of the p21(CIP1/WAF1) promoter by bone morphogenetic protein-2 in mouse B lineage cells." Oncogene **20**(32): 4383-92.

4. CHAPTER-2

TGFβ1/TGFβRII SIGNALING MAY PLAY A KEY ROLE IN THE MAINTENANCE OF ANERGIC STATE OF $CD4^+CD25^+$ T REGULATORY CELLS, YET ENABLING THEIR RESISTANCE TO ACTIVATION INDUCED APOPTOSIS VIA DIFFERENTIAL EXPRESSION OF SEVERAL G0/G1 CELL CYCLE PROTEINS

ABSTRACT

$CD4^+CD25^+$ T regulatory (Treg) cells that are known to be anergic, arrested in the G0/G1 phase of the cell cycle, yet programmed for cell survival. However, the molecular mechanism(s) involved in G0/G1 cell cycle arrest and cell survival signaling has not been defined. We previously reported that $CD4^+CD25^+$ Treg cells from chronically FIV-infected cats as opposed to those from control cats are functionally activated in vivo and express high levels of TGFβ1 on their surface and elevated levels of intercellular cyclin-dependent kinase inhibitors (CdkI's). To learn more of the potential role of cell surface expressed TGFβ1 in regulating the phenotypic and functional characteristics of Treg cells, we examine the role of exogenous TGFβ1 in conversion of $CD4^+CD25^-$ T cells to a Treg phenotype. Herein, we report that TGFβ1 in combination with mitogen could convert feline $CD4^+CD25^-$ T cells from peripheral blood to Treg-like cells in vitro. We also demonstrate that, similar to naturally occurring $CD4^+CD25^+$ Treg cells, TGFβ1 in vitro-converted $CD4^+CD25^-$ T cells are anergic and express elevated levels of CdkI, $p21^{cip1}$ and anti-apoptotic protein bcl-2 but not pro-apoptotic protein bax, compared to unstimulated controls. Both natural and TGFβ1 converted $CD4^+CD25^+$ Treg cells express high levels of the anti-apoptotic protein bcl-2 and are more resistant to activation-induced cell death than their counterparts $CD4^+CD25^-$ T cells.

Keywords: $CD4^+CD25^+$ T regulatory cells; TGFβ1; CdkI; apoptosis; anergy; bcl-2

INTRODUCTION

Naturally occurring $CD4^+CD25^+$ Treg cells, comprising 5-10% of the circulating $CD4^+$ T cell population, were originally defined as regulatory T cells that maintain peripheral self-tolerance (Maloy and Powrie 2001; Shevach 2002). More recent studies have demonstrated that Treg cells also play a major role in modulating the magnitude and duration of protective T cell responses to pathogens (Iwashiro, Messer et al. 2001; Montagnoli, Bacci et al. 2001; Belkaid, Piccirillo et al. 2002; Hori, Carvalho et al. 2002; Hisaeda, Maekawa et al. 2004; Belkaid and Rouse 2005). These pathogen-induced Treg cells, similar to natural Treg cells, are anergic, yet programmed for cell survival, and possess potent immunosuppressor function once activated by TCR engagement and/or certain nonspecific stimuli (Belkaid and Rouse 2005). While anergic T cells, including pathogen-induced $CD4^+CD25^+$ Treg cells, are known to be arrested at the G0/G1 phase of the cell cycle, and thought to be relatively long-lived in the circulation, there is little information on what receptors or intracellular signaling proteins maintain their unique functional state and homeostasis.

We recently demonstrated that feline $CD4^+CD25^+$ Treg cells possess all the functional characteristics of Treg cells described in humans and rodents in that they are anergic, arrested at the G0/G1 phase of the cell cycle, resistant to activation-induced cell death and possess potent immunosuppressive activity when activated (Joshi, Garg et al. 2005). Moreover, we recently reported that $CD4^+CD25^+$ Treg cells from chronically FIV-infected cats, in contrast to Treg cells from uninfected control cats, are activated in vivo

and fully armed for potent contact dependent immunosuppressor function (Vahlenkamp, Tompkins et al. 2004). Despite the activation status of $CD4^+CD25^+$ Treg cells in FIV-infected cats, which may result from the fact that they support a chronic productive FIV infection (Joshi, Vahlenkamp et al. 2004), their numbers remain relatively constant, or may actually increase throughout the course of the disease (Joshi, Vahlenkamp et al. 2004) .

The mechanism(s) regulating the survival and homeostasis of the Treg cell pool in the periphery is poorly understood. However, studies indicate that maintenance of peripheral Treg homeostasis is independent of the thymus. 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 TGFβ1 in the periphery and are not thymus dependent. Dieckmann, et al. (Dieckmann, Plottner et al. 2005) demonstrated that activated $CD4^+CD25^+$ Treg cells are capable of inducing a Treg phenotype from activated Th cells in a contact-dependent manner, suggesting that Treg cells may be recruited from the $CD4^+CD25^-$ T cell pool. In support of this, Chen et al. (Chen, Jin et al. 2003) reported that TGFβ1 is capable of converting TCR-stimulated $CD4^+CD25^-$ T cells into Foxp3 positive $CD4^+CD25^+$ Treg cells with potent immunosuppressive function. However, it is possible that the origin of the converted Treg cells in the periphery is not the $CD4^+$ Th pool, but rather a small population of $CD4^+CD25^-Foxp3^+$ cells that have been suggested to be a reservoir of precursor Treg cells (Romagnoli, Tellier et al. 2005). Whatever the source of cells maintaining peripheral Treg homeostasis, data suggest that

Treg cells, once activated, may recruit additional Treg cells from $CD4^+CD25^-$ T cell pool in a TGFβ1-dependent manner.

TGFβ1 is the prototypic member of a large family of structurally related pleiotropic-secreted cytokines that play a pivotal role in the control of differentiation, proliferation and the state of activation of many different cell types, including immune cells (Dennler, Goumans et al. 2002). Because of its dual role in pro-apoptotic and anti-apoptotic signaling, TGFβ1 is fundamental in the maintenance of homeostasis by regulating the balance between cell growth and apoptosis. Evidence from TGFβ1 knockout mice show that TGFβ1 plays a pivotal role in the maintenance of immune cell homeostasis. TGFβ1 null mice that are born, die shortly after weaning as a result of multifocal inflammatory disease with lymphocyte infiltration with multiple organs and autoimmune manifestations (Kulkarni, Huh et al. 1993; Christ, McCartney-Francis et al. 1994; Shull and Doetschman 1994; Yaswen, Kulkarni et al. 1996). TGFβ1 is though to maintain peripheral Treg homeostasis by its ability to induce cell-cycle arrest as well as promote resistance to AICD. In many cell types, TGFβ1 causes a G0/G1 cell-cycle arrest by inhibiting cyclin-dependent kinase (Cdk) activity via activation of cyclin-dependent kinase inhibitors (CdkI's) (Wolfraim, Walz et al. 2004). We recently reported that CdkI's $p21^{cip1}$, $p27^{kip1}$, and $p15^{ink4}$ are upregulated in G0/G1 arrested $CD4^+CD25^+$ Treg cells from FIV-infected cats. Upregulation of CdkI's in $CD4^+CD25^+$ Treg cells correlates with increased cell surface expression of TGFβ1 and TGFβRII (chapter 1). Others have reported increased expression of $p21^{cip1}$ and $p27^{kip1}$ in T cells anergized by TCR engagement in the absence of co-stimulation (Jackson, DeLoose et al. 2002). Wolfraim, et al. (Wolfraim, Walz et al.

2004) reported that TGFβ1 induced G0/G1 cell-cycle arrest of T cells is markedly diminished in $p21^{cip1}$ and $p27^{kip1}$ double knockout mice when T cells are activated under optimal co-stimulation conditions.

TGFβ1 also plays an important role in homeostasis of the immune system via its ability to regulate apoptosis (Sanchez-Capelo 2005). TGFβ1 inhibits or decreases the sensitivity of Fas/FasL and TNFα-induced apoptosis of activated T cells (Wahl, Orenstein et al. 2000). TGFβ1 has also been shown to suppress cytotoxic T cell-induced apoptosis by a contact-dependent mechanism between $CD4^+CD25^+$ Treg cells and cytotoxic T cells (Chen and Wahl 2003). Although the signaling pathways involved in TGFβ1-induced cell survival are not completely understood, recent data suggest that TGFβ1 may inhibit apoptotic signaling by upregulation of the NFκB cell survival signal (Saile, Matthes et al. 2001). Others have reported that TGFβ1 suppresses apoptosis in hepatic stellate cells by activating NFκB and by upregulating bcl-xl, the anti-apoptotic protein of the mitochondrial Fas-mediated pathway (Saile, Matthes et al. 2001). Similarly, TGFβ1 protects cultural embryonic rat neurons from apoptosis by increasing bcl-2 expression (Prehn, Bindokas et al. 1994). In contrast, in oligodendroglial precursor cells, TGFβ1 induces apoptosis which is associated with down-regulation of bcl-xl with no effect on bcl-2 (Schuster, Bender et al. 2003). While it has been reported that human $CD4^+CD25^+$ Treg cells are prone to apoptosis in association with decreased levels of bcl-2 expression (Taams, Smith et al. 2001), mouse $CD4^+CD25^+$ Treg cells are resistant to apoptosis, as they do not undergo clonal deletion when treated with superantigen in vivo (Papiernik, de Moraes et al. 1998), and are resistant to Fas-induced apoptosis in vitro (Banz, Pontoux et

al. 2002). Similar to murine Treg cells, Joshi, et al. (Joshi, Garg et al. 2005) reported that feline $CD4^+CD25^+$ Treg cells were relatively resistant to AICD compared to $CD4^+CD25^-$ T cells.

The apparent contradictory responses of different cell types to TGFβ1 signaling may depend upon the relative expression of bcl-2 and bax, as the ratio of bcl-2 to bax in a cell appears to determine survival or apoptosis (Xiao, Oppenlander et al. 2001). A case in point is the different responses of the precursor $CD34^+CD38^-$ and the committed $CD34^+CD38^+$ cell to TGFβ1 signaling. The $CD34^+CD38^-$ precursor with relatively low levels of bcl-2 respond to TGFβ by undergoing apoptosis, whereas the committed $CD34^+CD38^+$ with higher levels of bcl-2 are not sensitive to TGFβ1-induced apoptosis (Xiao, Oppenlander et al. 2001). Interestingly, bax expression is not changed in either cell in response to TGFβ1 signaling supporting the speculation that the bcl-2/bax ratio determines susceptibility or resistance to apoptosis.

As $CD4^+CD25^+$ are naturally anergic to antigen stimulation, we do not know whether the above discussed cellular responses to TGFβ1 signaling apply. To address some of these questions, this study focused on determining the mechanism(s) regulating cell growth arrest and apoptosis of $CD4^+CD25^+$ Treg cells from FIV-infected and control cats. We also explored the effects of TGFβ1 on expression of cell cycle genes and apoptotic genes in Treg cells. We observed that although Treg cells express elevated levels of CdkI's, they are resistant to apoptosis, which correlates with increased levels of anti-apoptotic proteins (bcl-2) and decreased levels of bax compared to $CD4^+CD25^-$ T cells. We also

report that TGFβ1 plus ConA treatment could convert feline $CD4^+CD25^-$ T cells into immune suppressor cells which possess phenotypic properties of naturally occurring $CD4^+CD25^+$ Treg cells, in that they express cell surface TGFβ1, and elevated levels of $p21^{cip1}$, and anti-apoptotic proteins (bcl-2).

MATERIALS AND METHODS

Animals and viral infection

The cats used for this study were specific pathogen free (SPF) 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 NCSU1 isolate of FIV, a pathogenic clade A virus (English, Nelson et al. 1994), as described by Bucci et al (Bucci, English et al. 1998). The samples were collected from FIV^+ cats infected for more than 3yrs and from uninfected control cats, 4 to 6 years of age. FIV-infected cats were housed separately from uninfected control cats. FIV-infection was confirmed by immunoblot analysis and provirus detection by PCR using primers specific for the FIV-p24 encoding sequence.

Cell collection

Whole blood was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) and PBMC isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as previously described (Tompkins,

Ogilvie et al. 1987). LN cells were obtained from LN biopsies and single-cell suspensions were prepared by gently injecting sterile PBS into the tissue using 18G needle repeatedly, until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion. Viability was always >90%.

$CD4^+$ T cell subset separation

To obtain single lymphocyte subsets, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells derived from PBMC and pooled LN cells were either enriched by biomagnetic bead depletion and selection or by fluorescent activated cell sorting (FACS) (MoFlo; DakoCytomation, Fort Collins, CO). Biomagnetic bead separation was performed using goat anti-mouse IgG-coated beads (Dynabeads M-450; Dynal, Great Neck, NY) as described by Bucci et al. (Bucci, English et al. 1998). PBMC and LN were depleted of B cells with anti-CD21 mAb (Serotec, Raleigh, NC)-coated beads, and $CD8^+$ cells were depleted with anti-CD8 (mAb 3.357 (Tompkins, Gebhard et al. 1990))-coated beads. The $CD4^+$ cell population was then enriched for $CD25^+$ cells by positive selection using anti-feline CD25 mAb (9F23; kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan)). The enriched $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were analyzed for purity by flow cytometric analysis and were greater than 90% pure. The purity of the FACS sorted $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were greater than 95%. In both cases, viability of the purified $CD4^+$ subsets was determined by trypan blue dye exclusion and was always >90%. All assays were based on viable cell numbers.

Flow cytometry analysis

At least 5 x 10^5 PBMC or purified cell populations were stained for surface expression of CD4 and CD25 markers using APC-conjugated anti-CD4 (mAb 30A (Tompkins, Gebhard et al. 1990)), FITC-conjugated anti-CD25 mAb (mAb 9F23), and PE-conjugated anti-CD62L selectin (BD-pharmagen). Monoclonal antibodies for TGFβ1 (mouse anti-human, mAb 240 clone 9016) were obtained from R&D Systems. Data were acquired on a FACS Caliber flow cytometer (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 Cell Quest software.

Propidium Iodide staining

Purified $CD4^+$ T cell subsets from PBMC were cultured with or without ConA (5μg/ml) or $CD4^+CD25^-$ T cells stimulated with rhIL-2 (100U/ml) or without ConA (5μg/ml) +/- TGFβ1 (10ng/ml) for 24hrs or 48hrs. Propidium Iodide was added to the cultured cells ($1*10^5$) for 10min incubated on ice followed by a wash in PBS. The cells were then assayed for viability immediately by flow cytometry analysis. For TGFβ1 plus ConA stimulated $CD4^+CD25^-$ T cells, only TGFβ1 positive cells stained positive for PI were determined.

T cell stimulation assays

T cell subsets were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-

glutamine, and 1 mM HEPES buffer. For ConA/TGFβ1 stimulation, 1 x 10^5 viable cells/well were cultured in a round-bottom 96-well plate in the presence or absence of 5 μg/ml Con-A or 100 U/ml recombinant human IL-2 (rhIL-2) (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD) or 10ng/ml TGFβ1 (R&D Biosciences) . All assays were run in triplicate. For anergy assays $CD4^+CD25^-$ T cells were stimulated for 2 days cells, and for suppressor assays, after 4-5 days of incubation stimulated cells were cocultured for 2 days with autologous ConA stimulated (24hrs) $CD4^+CD25^-$ T cells and were pulsed with 1 μCi of [^{3}H]TdR/well and harvested 16hrs later using a Filtermake Harvester (Packard Bioscience, Meriden, CT). [^{3}H]TdR uptake as a measure of proliferation was determined in counts per minute (cpm) using a Top Count NXT Microplate scintillation counter (Packard Bioscience).

Immunobloting

Equal numbers of Ficoll separated PBMC or FACS purified $CD4^+$ T cell subsets ($1x10^6$ – $2x10^6$ cells) stimulated for 4-5 days with rhIL-2, TGFβ1, ConA or ConA plus TGFβ1 were lysed using NP-40 lyses buffer for 16-24hrs at 4C. The concentrations of the proteins were determined using a spectrophotometer (Biorad Smartspec 3000), 30-50μg of proteins were then resolved on 4-12% SDS-polyacrylamide gels (Novex Inc.). The separated proteins were transferred onto nitrocellulose and immunoblotted with specific mAbs to $p21^{cip1}$, bcl-2, and bax (Santa Cruz biotechnology Inc) followed by HRP-conjugated secondary antibodies (Santa Cruz Biotechnology Inc). Immunodetection was performed by ECL (Pierce) using HyperFilm ECL. To normalize the result, some blots were stripped with the Western Blot stripping buffer (Pierce) and reprobed with anti-actin

mAb (Santa Cruz Biotechnology Inc.). Specific antibody conjugated blots were analyzed for specific proteins using Lumicapture analyst camera detection system (Lumi- Imager).

Statistical Analysis

The student t-test was used to assess significance of difference between different $CD4^+CD25^-$ T cell stimulations compared to unstimulated controls for detection of activation induced apoptosis in FIV-infected and control cats.

RESULTS

TGFβ1 along with mitogen stimulation can induce anergy in feline $CD4^+CD25^-$ T cells

It has also been recently demonstrated in rodent and human models that apart from naturally occurring $CD4^+CD25^+$ Treg cells generated in the thymus from a defined lineage, $CD4^+CD25^+$ Treg like anergic/suppressor cells may also be induced in the periphery by the action of several immunosuppressive molecules or cytokines like TGFβ1. Also that TGFβ1 along with TCR stimulation can convert naïve $CD4^+CD25^-$ T cells into $CD4^+CD25^+$ anergic/suppressor cells in vitro. To test this hypothesis in a feline model, flow cytometry purified $CD4^+CD25^-$ T cells were stimulated with rhIL-2, or TGFβ1, or TGFβ1 +/- ConA for 2 days and ^3H thymidine was added in the last 16hrs to determine anergy. As observed in **figure1,** TGFβ1 along with ConA stimulation of $CD4^+CD25^-$ T cells exhibited significantly reduced TCR-triggered proliferation

compared to $CD4^+CD25^-$ T cells stimulated with rhIL-2 or ConA or TGFβ1 alone. Thus, TGFβ1 plus TCR stimulated $CD4^+CD25^-$ T cells are functionally anergic similar to that of naturally occurring feline $CD4^+CD25^+$ Treg cells (chapter 1).

TGFβ1 along with ConA stimulation induces the expression of cyclin dependent kinase inhibitor ($p21^{cip1}$) protein.

We recently reported that feline $CD4^+CD25^+$ Treg cells are phenotypically anergic as indicated by the elevated levels of expression of cyclin dependent kinase inhibitor proteins ($p21^{cip1}$, $p27^{kip1}$, and $p16^{ink}$) (chapter 1). To determine whether TGFβ1 converted $CD4^+CD25^-$ T cells from control cats **(figure 2A)** and FIV-infected cats **(figure 2B)** are phenotypically anergic, bead purified $CD4^+CD25^-$ T cells were stimulated with or without rhIL-2, or ConA, or TGFβ1 +/- ConA for 4-5 days. Equal numbers of viable cells were lysed and total cellular proteins were separated on SDS-page gels and analyzed by immunobloting procedures. There was no effect on $p21^{cip1}$ expression in $CD4^+CD25^-$ T cells stimulated with rhIL-2 or ConA or TGFβ1 alone. In contrast, TGFβ1 along with ConA stimulation of $CD4^+CD25^-$ T cells induced elevated levels of $p21^{cip1}$ protein compared to unstimulated controls in FIV-infected and control cats **(figure 2).**

Feline $CD4^+CD25^-$ T cells stimulated with TGFβ1 along with ConA are potent suppressors similar to naturally occurring $CD4^+CD25^+$ Treg cells

To further reconfirm whether the TGFβ–anergized feline $CD4^+CD25^-$ T cells were functionally active and able to suppress responder Th cell proliferation (unpublished data), freshly isolated $CD4^+CD25^-$ naive T cells from FIV-infected cats and control cats were cultured with ConA+/- rhIL-2 with or without TGFβ1 for 4-5 days. These stimulated $CD4^+CD25^-$ T cells were cocultured for 48hrs with autologous $CD4^+CD25^-$ T cells cultured with ConA for 24hrs. ^{3}H-thymidine was added in the last 16hrs before harvest. Percent inhibition of $CD4^+CD25^-$ T cell proliferation was calculated. $CD4^+CD25^-$ T cells stimulated with either rhIL-2 or ConA or TGFβ1 alone from both FIV-infected cats **(figure 3A)** and control cats **(figure 3B)** had any suppressive action. In contrast, TGFβ1 and ConA converted $CD4^+CD25^-$ T cells from both control cts and FIV-infected cats were able to inhibit ConA stimulated $CD4^+CD25^-$ T cell proliferation similar to that of naturally occurring $CD4^+CD25^+$ Treg cells from FIV-infected cats.

TGFβ1 converted $CD4^+CD25^-$ Treg like cells similar to naturally occurring $CD4^+CD25^+$ Treg cells are more resistant to activation induced apoptosis compared to rhIL-2 stimulated $CD4^+CD25^-$ T cells.

Our laboratory previously demonstrated that feline $CD4^+CD25^+$ Treg cells are anergic functionally and phenotypically as indicated by elevated levels of cyclin dependent kinase inhibitors expression compared to $CD4^+CD25^-$ T cells (chapter 1). We also reported that FIV preferentially replicates in $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cell counterparts (Joshi, Vahlenkamp et al. 2004). To determine the potential of the purified $CD4^+$ T cell subsets for the resistance to activation induced

apoptosis, $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells from FIV-infected and control cats were stimulated with rhIL-2 or TGFβ1, or ConA +/- TGFβ1 for 24hrs or 48hrs. Cell viability was determined using flow cytometry by propidium iodide staining procedures. We demonstrated that Con-A stimulated $CD4^+CD25^+$ Treg cells from either FIV-infected or control cats are significantly more resistant to activation induced apoptosis at 24hrs and even more at 48hrs than $CD4^+CD25^-$ T cells in-vitro **(figure 4A).** Similar to naturally occurring Treg cells, only TGFβ1 along with mitogen stimulation of $CD4^+CD25^-$ T cells induced an anti-apoptotic phenotype compared to the rhIL-2 stimulated controls **(figure 4B).** As expected $CD4^+CD25^-$ T cells were able to proliferate significantly in response to ConA compared to rhIL-2 stimulated controls **(figure 4B)**.

Anti-apoptotic but not pro-apoptotic proteins are differentially expressed in the TGFβ1 converted $CD4^+CD25^-$ T cell subsets

To further analyze the molecular bases for the $CD4^+CD25^+$ Treg cells to maintain anergic phenotype yet be resistant to apoptosis compared to $CD4^+CD25^-$ T cells, expression of anti-apoptotic protein (bcl-2) and pro-apoptotic protein (bax) was determined in the two $CD4^+$ T cell subsets **(figure 5A).** Bead separated $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ T cells isolated from the PBMC of FIV-infected and control cats or $CD4^+CD25^-$ T cells stimulated for 4 days with rhIL-2, TGFβ1, or ConA+/- TGFβ1 were lysed to obtain protein. Total cell proteins were separated on SDS-PAGE gels and further analyzed by western blotting using specific antibodies such as bcl-2 and bax. As shown in **figure 5A** anti-apoptotic protein bcl-2 was expressed at elevated levels in $CD4^+CD25^+$ Treg cells compared to $CD4^+CD25^-$ T cells, and in contrast pro-apoptotic protein bax was

expressed at increased levels in $CD4^+CD25^-$ T cells from both FIV-infected and control cats. Stimulation of $CD4^+CD25^-$ T cells only with TGFβ1 plus ConA induced the expression of anti-apoptotic protein bcl-2, but had no effect on pro-apoptotic protein bax expression **(figure 5B).**

DISCUSSION

Treg cells are characterized by G0/G1 cell cycle arrest and are anergic to immune activation, as manifested by their inability to produce IL-2 and exit the G0/G1 stage of cell cycle in response to antigen-specific TCR engagement (Powell, Ragheb et al. 1998). Another major characteristic of $CD4^+CD25^+$ Treg cells described in human and rodent species is their ability, once activated through their TCR, to suppress proliferative responses of other antigen- or mitogen-stimulated $CD4^+$ or $CD8^+$ T cells (Thornton and Shevach 1998; Jackson, DeLoose et al. 2001). We recently described $CD4^+CD25^+$ Treg cells in the cat possessed all the major characteristics of phenotypic anergy as indicated by the expression of genes responsible for exiting G0/G1 phase of the cell cycle such as cyclin D, retinoblastoma protein and cyclin dependent kinase inhibitors (chapter 1). We also demonstrated the functional characteristics of Treg cells in cats, similar to those described in other species in that they are G0/G1 cell cycle arrested, IL-2 deficient and anergic to mitogenic stimulation, and when activated capable of suppressing IL-2 production and proliferation of activated T cells (Vahlenkamp, Tompkins et al. 2004).

We recently reported that $CD4^+CD25^+$ Treg cells from chronically FIV-infected cats, in contrast to Treg cells from uninfected control cats, are activated in vivo and fully armed for potent contact dependent immunosuppressor function (Vahlenkamp, Tompkins et al.

2004). In conjunction with functional anergy, in vivo activated $CD4^+CD25^+$ Treg cells from FIV-infected cats expressed elevated levels of CdkI's such as $p21^{cip1}$ compared to $CD4^+CD25^-$ T cells. No significant difference between the two $CD4^+$ T cell subsets was observed in control cats (chapter 1). We also reported that activated Treg cells from FIV-infected cats express elevated levels of TGFβ1 and its receptor on their cell surface compared to their counterparts $CD4^+CD25^-$ T cells. We also observed the colocalization of TGFβ1 with its receptor on the cell surface (Tompkins lab unpublished data).

$CD4^+CD25^+$ regulatory T cells (Treg) play an important role in the control of immune responses and the maintenance of immune homeostasis. Although Treg generally inhibit T cell proliferation, the precise regulatory mechanisms of Treg remain unknown, despite reports suggesting possible roles for TGFβ or CTLA-4. In this study, we have provided evidence that feline Treg like cells can be induced/converted from peripheral $CD4^+CD25^-$ naive responder T cells through costimulation of TCR and TGFβ signaling. Several important conclusions can be drawn from the current work. First, TGFβ1 treatment in the presence of TCR stimulation converts naive $CD4^+CD25^-$ responder feline T cells to anergized $CD4^+CD25^+$ Treg like cells that inhibits their proliferation in vitro compared to ConA stimulated $CD4^+CD25^-$ T cells.

The hallmark of naturally occurring Treg cells, including those in normal cats is that they require activation by antigen-TCR engagement or a potent mitogen before they can display immunosuppressive function (Vahlenkamp, Tompkins et al. 2004). TGFβ1 bound to its receptor on Treg cells could induce suppression of other T cells in a cell-contact

dependent manner. We reconfirmed that TGFβ1–converted anergic feline $CD4^+CD25^-$ T cells are potent suppressors similar to professional Treg cells. Functionally, when cocultured with ConA stimulated $CD4^+CD25^-$ T cells, TGFβ1-anergized T cells inhibit proliferation and cytokine production of the target T cells similar to naturally occurring $CD4^+CD25^+$ Treg cells.

Induction and maintenance of anergy in feline Treg cells has been associated with over-expression of the cyclin-dependent kinase inhibitors, CdkI's, $p21^{cip1}$, $p21^{kip1}$, and $p16^{ink}$ (chapter 1). TGFβ1 causes a G0/G1 cell-cycle arrest by inhibiting cyclin-dependent kinase (Cdk) activity via activation of cyclin-dependent kinase inhibitors (CdkI's) (Kee, Rivera et al. 2001; Massague 2004; Wolfraim, Walz et al. 2004). We recently reported that CdkI's $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ are upregulated in G0/G1 arrested $CD4^+CD25^+$ Treg cells from FIV-infected cats. Upregulation of CdkI's in $CD4^+CD25^+$ Treg cells correlates with increased cell surface expression of TGFβ1 and TGFβRII (chapter 1). In this study we have demonstrated that TGFβ1–converted anergic feline $CD4^+CD25^-$ T cells similar to the professional Treg cells, over-express CdkI, $p21^{cip1}$. Thus, TGFβ1 stimulation along with TCR-engagement can induce phenotypic and functional anergy in $CD4^+CD25^-$ T cells in vitro.

TGFβ1 suppresses apoptosis in cells by activating NFκB and by upregulating Bcl-family members such as bcl-X and bcl-2. $CD4^+CD25^+$ Treg cells exert regulatory effects through a cell-cell contact-dependent manner, and the frequency of Treg cells is crucial to the prevention of inflammation and autoimmunity, as well as induction of tumor antigen

tolerance. It has been shown previously that $CD4^+CD25^+$ Treg cells are potent targets for FIV-infection (Joshi, Vahlenkamp et al. 2004). The activation status of $CD4^+CD25^+$ Treg cells in FIV-infected cats may result from the fact that they support a chronic productive FIV-infection (Joshi, Vahlenkamp et al. 2004). Furthermore, subsets of immune cells are known to respond differently to activation induced apoptosis. These observations prompted us to explore the sensitivity of $CD4^+CD25^{+/-}$ T cells to activation induced cell death.

A homeostatic phenomenon tends to reduce the peripheral pool of proliferating lymphocytes by inducing apoptosis of activated T cells upon TCR re-engagement. TGFβ1 is important in the maintenance of homeostasis by regulating the balance between cell growth and apoptosis. TGFβ1 is also though to maintain peripheral Treg homeostasis by its ability to induce cell-cycle arrest as well as promote resistance to AICD. We have shown here that TGFβ1 induced Treg like cells similar to naturally occurring Treg cells are more resistant to activation induced apoptosis compared to ConA stimulated $CD4^+CD25^-$ T cells. This different susceptibility to cell death might be explained by the balance between pro- and anti-apoptotic factors such as FLIP (Irmler, Thome et al. 1997; Kataoka, Budd et al. 2000), and members of the Bcl-2 family such as bcl-2, and bax (Xiao, Oppenlander et al. 2001) of the mitochondrial Fas-mediated pathway (Saile, Matthes et al. 1999). In this study, we demonstrated that TGFβ1 along with TCR stimulation of $CD4^+CD25^-$ T cells similar to professional Treg cells can induce the expression of anti-apoptotic proteins like bcl-2. In contrast to Treg cells which express low levels of pro-apoptotic proteins, bax. TGFβ1 converted Treg like cells had no effect on bax expression. The expression of elevated levels of bcl-2 correlated with the anti-

apoptotic potential of activated $CD4^+CD25^+$ Treg cells. Therefore, more than the levels of expression of anti- and pro-apoptotic proteins, the ratio between the two may play a critical role in determining the fate of the cell, to die or live, upon activation.

Based on the above observations, we proposed a model that could explain the effects of TGFβ1 signaling in the maintenance of homeostasis by regulating proteins that are essential for the balance between cell growth and apoptosis in Treg cells **(Figure 6)**. TGFβ1 is expressed in the G_1 phase of the cell cycle in growth-arrested cells (Chen, Jin et al. 2003) and binding of TGFβ1 to its receptor activates a wide range of pathways during cell-cycle progression. TGFβRI/II complex formation is induced by the binding of TGFβ ligands that in turn phosphorylates receptor-activated Smads 2/3 (R-Smads). R-Smads form complexes with Smad4 which allows their translocation into the nucleus where the complex interacts with Smad-binding elements (SBE) (Shi, Wang et al. 1998). R-Smad complex promotes the transcription of several target genes depending upon the combinations of Smad complex interactions with co-activators and inhibitors of gene transcription (Shi and Massague 2003) including $p21^{cip1}$, and Foxp3. Foxp3 inturn down regulates the Smad7. Foxp3-mediated down-regulation of Smad7 induces a positive auto-regulatory loop of TGFβ-Smad signaling in naturally occurring and TGFβ1 induced Treg cells in human and murine models (Fantini, Becker et al. 2004).On the other hand Foxp3 inhibits IL-2 transcription. TGFβ1 signaling can also activate MAP-Kinase pathway that induces expression of several transcriptional factors such as ATF, Sp1, and c-Jun. $p21^{cip1}$ promoter possesses binding site/s for transcription factors of the Sp1 family along with Smad's (Moustakas, Pardali et al. 2002) which is targeted by both Smad- and

Ras/MAPK-mediated pathways. ATF transcription factor induces phosphorylation of cyclic AMP response element binding proteins (CREB) which inturn targets induction of several genes including bcl-2. Thus, TGFβ1 pathway could be one of the mechanisms by which Treg cells can maintain anergy, yet be programmed for cell survival.

In conclusion, TGFβ1 stimulation along with TCR- engagement can induce functional and phenotypic anergy in converted Treg like cells, as manifested by their inability to proliferate upon mitogenic stimulation and expression of CdkI, $p21^{cip1}$ respectively. TGFβ1 converted Treg like cells similar to naturally occurring Treg cells are more resistant to activation induced apoptosis compared to mitogen stimulated $CD4^{+}CD25^{-}$ T cells, which correlates with the expression of anti apoptotic proteins (bcl-2) and pro-apoptotic proteins (bax). Therefore, TGFβ/TGFβR signaling may be one of the mechanisms by which Treg cells could induce G0/G1 cell cycle arrest by up-regulation of CdkI's, yet be resistant to apoptosis by maintaining anti-apoptotic and pro-apoptotic protein ratios for survival.

ACKNOWLEDGEMENTS

We thank Janet Dow and Debra Anderson for their technical assistance. We thank Dr. Koishi Ohno for providing the anti-CD25 antibody. We also thank NIH AIDS Research and Reference reagent program for providing rHuIL-2. The present study was supported by National Institute of Health grants AI38177, AI 43858, and AI058691.

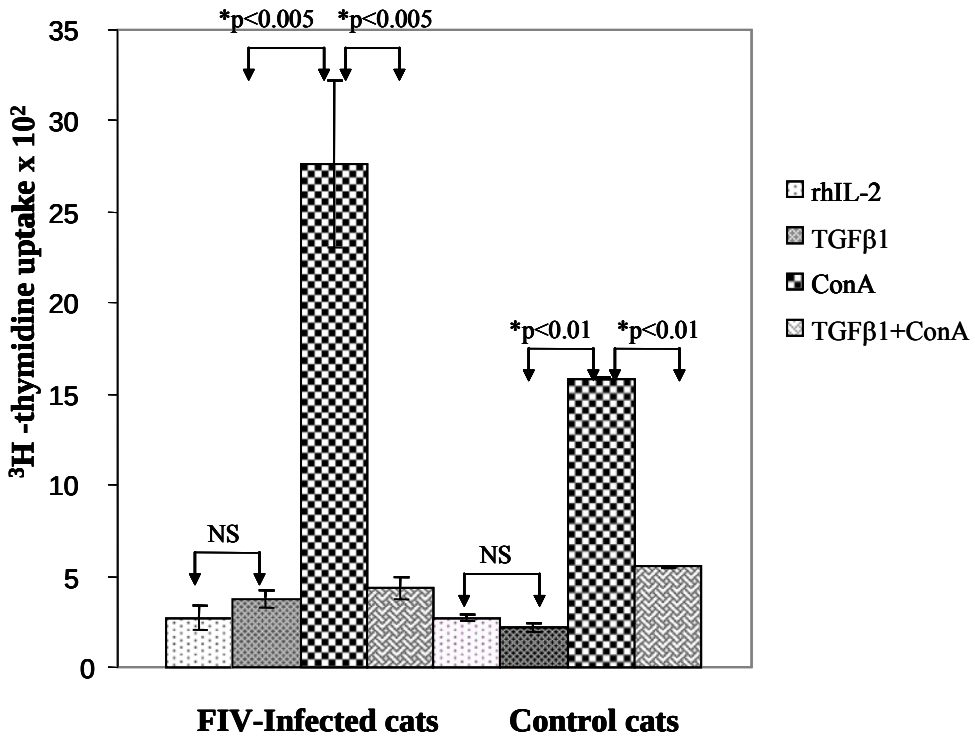


Figure1. TGFβ1 along with TCR triggering converts feline CD4$^+$CD25$^-$ T cells to anergic CD4$^+$CD25$^+$ Treg like cells in-vitro. Freshly isolated bead purified CD4$^+$CD25$^-$ T cells from FIV-infected cats and control cats were stimulated with or without rhIL-2, or ConA, or TGFβ1 +/- ConA for 2 days. ^{3}H-thymidine was added in the last 16hr period to determine cell proliferation. p-values were calculated using student t- test. *p-value < 0.01 was considered significant.

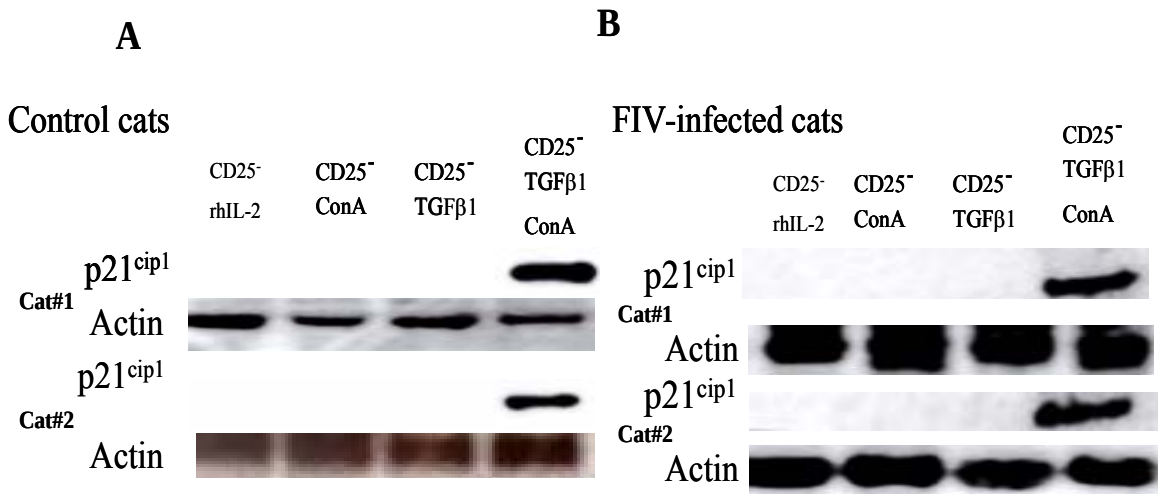
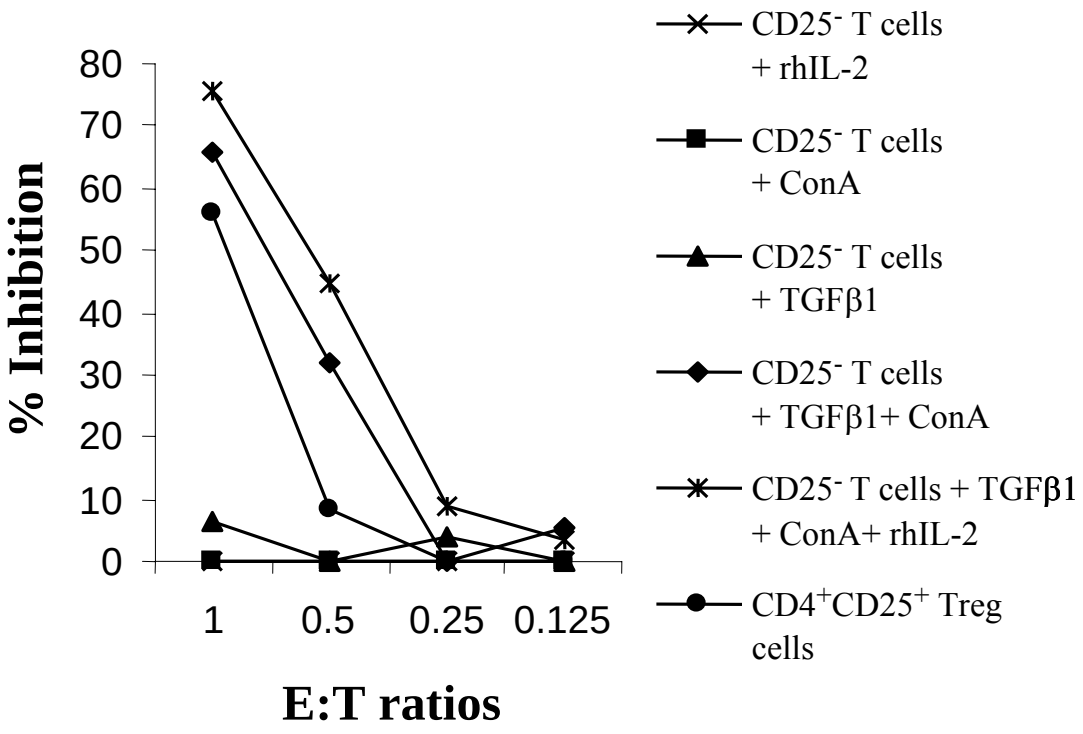


Figure 2. Induction of protein expression of cyclin dependent kinase inhibitor ($p21^{cip1}$) by TGFβ1. Equal number of purified $CD4^{+}CD25^{-}$ T cells ($1*10^{6}$ - $2*10^{6}$) from control cats **(A)** and FIV-infected cats **(B)** stimulated with or without TGFβ1 (10ng/ml) +/- ConA (5μg/ml) and /or rhIL-2 (100U/ml) for 4-5 days were lysed with NP-40 lysis buffer to obtain total cellular protein for the detection of $p21^{cip1}$. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblot using specific primary antibodies against CdkI ($p21^{cip1}$), 21Kdal protein, followed by HRP-conjugated goat anti-mouse IgG1 secondary antibody and detected by chemiluminescence. The blots were stripped using stripping buffer and reprobed with actin primary antibody and HRP-conjugated goat anti-mouse IgG1 secondary antibody followed by chemilumeniscence detection.

Figure 3. Suppressor activity of TGFβ1 converted CD4$^+$CD25$^-$ T cells on autologous CD4$^+$CD25$^-$ T cells from FIV-infected cats and control cats. FACS-purified CD4$^+$CD25$^-$ T target cells from control cats **(A)** and FIV-infected cats **(B)** were either stimulated with rhIL-2 (100U/ml) or ConA (5μg/ml) or TGFβ1 (10ng/ml) +/- ConA for 4-5 days. These stimulated CD4$^+$CD25$^-$ T cells or freshly isolated CD4$^+$CD25$^+$ Treg cells from FIV-infected cats were cocultured for 2 days with autologous CD4$^+$CD25$^-$ T cells cultured for 24hrs in the presence of ConA and were pulsed with [^{3}H]TdR 16hrs prior to the harvest. A constant number of CD4$^+$CD25$^-$ T target cells (5 x 10^4/well) were cultured with varying numbers of CD4$^+$CD25$^+$ Treg cells or the above stimulated CD4$^+$CD25$^-$ T effector cells. Percent inhibition of proliferation was calculated based on proliferation of CD4$^+$CD25$^-$ T target cells alone ((cpm of target cells alone − cpm of coculture) / cpm of target cells alone). Bars represent mean +/- SEM of triplicate wells for each condition.

A


% Inhibition
80
70
60
50
40
30
20
10
0
1
0.5
0.25
0.125
E:T ratios
CD25- T cells + rhIL-2
CD25- T cells + ConA
CD25- T cells + TGFβ1
CD25- T cells + TGFβ1+ ConA
CD25- T cells + TGFβ1 + ConA+ rhIL-2
CD4+CD25+ Treg cells

B

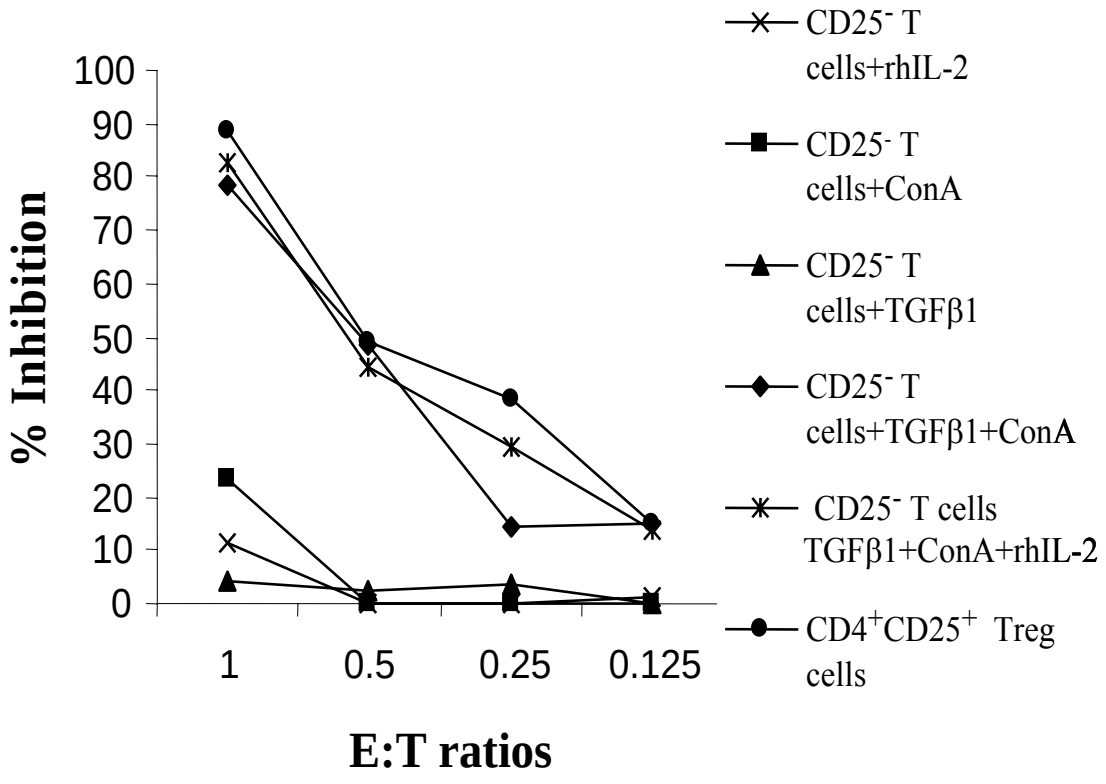
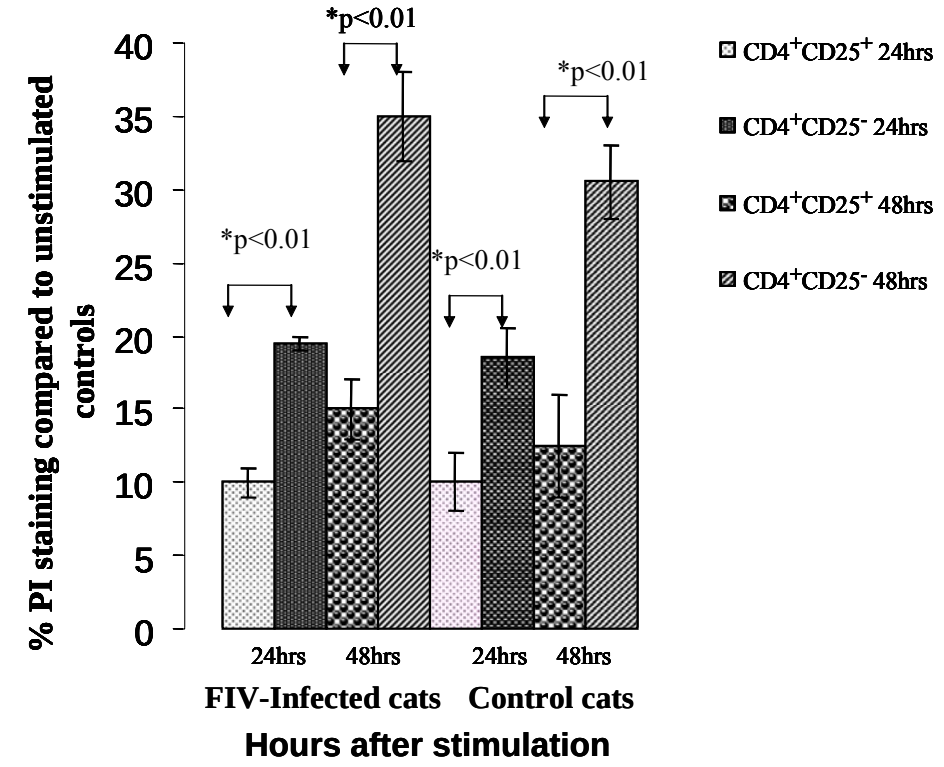

% Inhibition
100
90
80
70
60
50
40
30
20
10
0
1
0.5
0.25
0.125
E:T ratios
CD25- T cells+rhIL-2
CD25- T cells+ConA
CD25- T cells+TGFβ1
CD25- T cells+TGFβ1+ConA
CD25- T cells TGFβ1+ConA+rhIL-2
CD4+CD25+ Treg cells

Figure4. Resistance to activation induced apoptosis. **(A)** Flow cytomery sorted pure $CD4^+$ populations from FIV-infected and control cats were stimulated with or without 5μg/ml Con-A for 24hrs or 48hrs. Propidium Iodide was added to the cells ($1*10^5$) at 24hrs or 48hrs time points to analyze cell viability. Percent PI uptake by ConA stimulated cells was calculated by subtracting percent cell death of unstimulated controls of that $CD4^+$ T cell subset at that time point. **(B)** $CD4^+CD25^-$ T cells were stimulated with rhIL-2 , or TGFβ1, or ConA +/- TGFβ1 for 2 days. Stimulated ($1*10^5$) cells were stained with anti-TGFβ1 antibody and propidium iodide at 48hrs time point to analyze cell viability. % PI positive cells were determined from the total $CD4^+CD25^-$ T cell population for $CD4^+CD25^-$ T cells stimulated with TGFβ1 or rhIL-2, or ConA stimulations **(left)**. But for $CD4^+CD25^-$ T cells stimulated with TGFβ1 plus ConA percentage PI staining on only TGFβ1 positive $CD4^+$ T cells was determined which relates to percentage cell death of only converted Treg like cells **(right).** *p-value < 0.01 was considered significant.

A


*p<0.01
*p<0.01
*p<0.01
*p<0.01
% PI staining compared to unstimulated controls
40
35
30
25
20
15
10
5
0
CD4+CD25+ 24hrs
CD4+CD25- 24hrs
CD4+CD25+ 48hrs
CD4+CD25- 48hrs
24hrs
48hrs
24hrs
48hrs
FIV-Infected cats
Control cats
Hours after stimulation

B

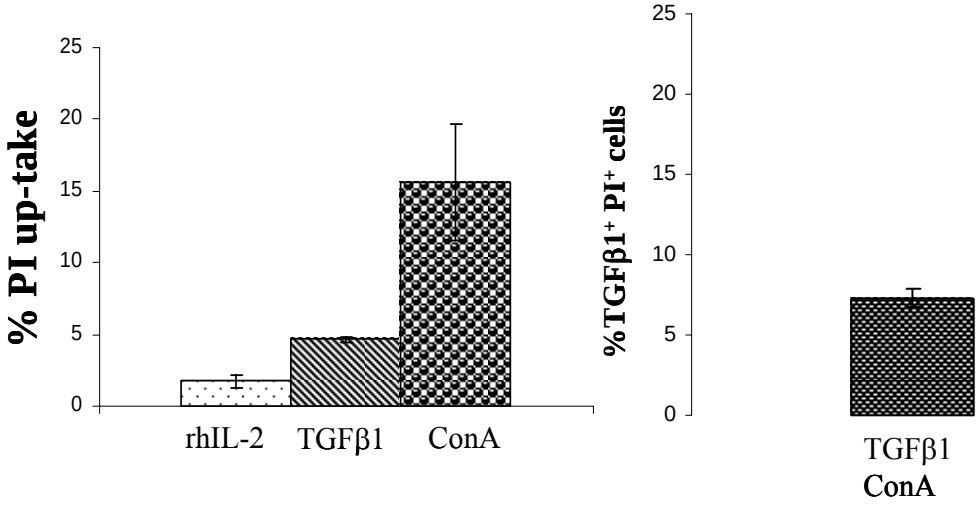
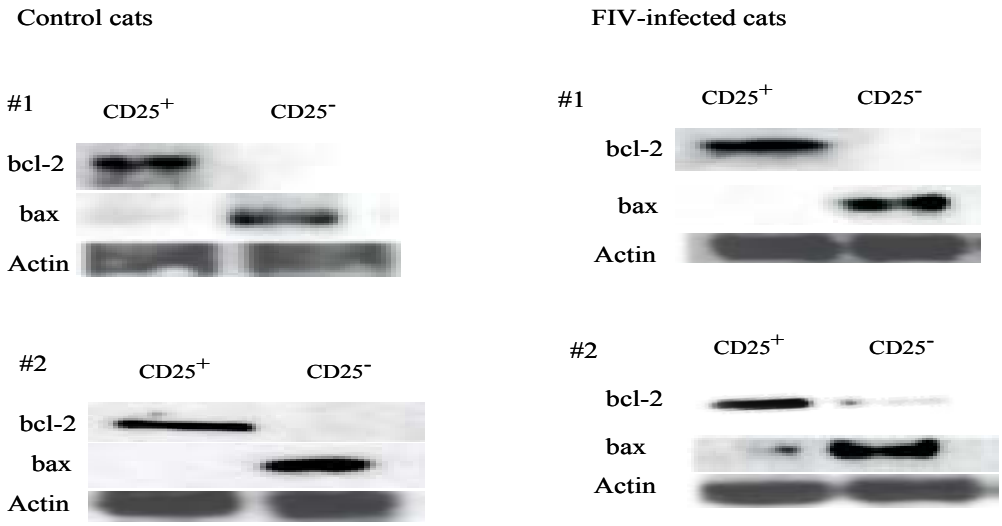

% PI up-take
25
20
15
10
5
0
rhIL-2
TGFβ1
ConA
%TGFβ1+ PI+ cells
25
20
15
10
5
0
TGFβ1
ConA

Figure5. Differential expression of pro-apoptotic and pro-apoptotic proteins in the $CD4^+$ T cell subsets. **(A-B)** Equal number of purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells ($2x10^6$) **(A)** or $CD4^+CD25^-$ T cells were stimulated with or without ConA +/- TGFβ1, or TGFβ1, or rhIL-2 for 4 days **(B),** from control cats (n=2) and FIV-infected cats (n=2) were lysed with NP-40 lysis buffer to obtain total cellular protein for the detection of bcl-2 and bax expression. Total cell lysates from FIV-infected and control cats were separated by SDS-PAGE and analyzed by immunoblot using specific primary antibodies against pro-apoptotic and anti-apoptotic proteins (bax and bcl-2), followed by HRP-conjugated goat anti-mouse IgG1 secondary antibody and detected by chemiluminescence. The blots were stripped using stripping buffer and reprobed with actin primary antibody and HRP-conjugated goat anti-mouse IgG1 secondary antibody followed by chemilumeniscence detection.

A


Control cats
FIV-infected cats
#1
CD25+
CD25-
bcl-2
bax
Actin
#2
CD25+
CD25-
bcl-2
bax
Actin

B

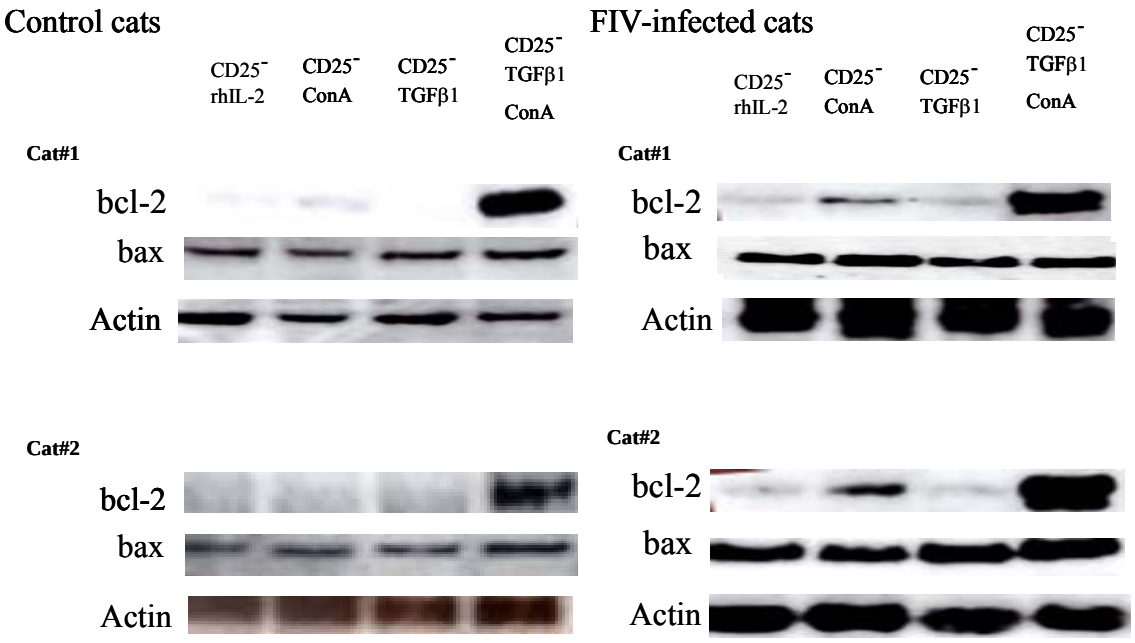
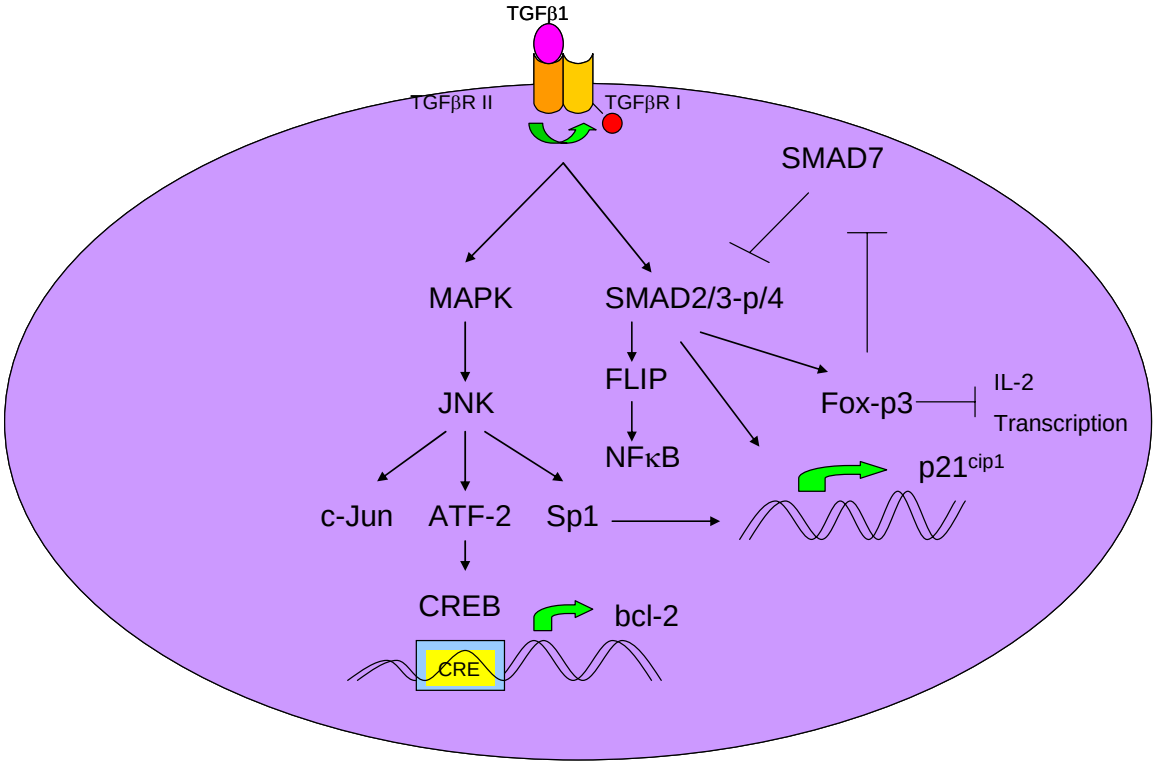

Control cats
FIV-infected cats
CD25- rhIL-2
CD25- ConA
CD25- TGFβ1
CD25- TGFβ1 ConA
Cat#1
Cat#2
bcl-2
bax
Actin

Figure 6. Schematic diagram of TGFβ1/TGFβR signaling pathway in Treg cells

Binding of TGFβ1 to its receptor leads to the formation of TGFβRI/II complex that in turn phosphorylates receptor-activated Smad 2/3 (R-Smads). R-Smads form complexes with Smad4 which allows their translocation into the nucleus where the complex interacts with Smad-binding elements. R-Smad complex promotes the transcription of several target genes including $p21^{cip1}$, and transcription factors such as Foxp3 and NFκB. Foxp3 in turn down regulates Smad7 which induces a positive auto-regulatory loop of TGFβ-Smad signaling. On the other hand Foxp3 inhibits IL-2 transcription. TGFβ1 signaling can also activate MAP-Kinase pathway that induces expression of several transcriptional factors such as ATF, Sp1, and c-Jun. Transcription factors of the Sp1 family along with Smads can activate $p21^{cip1}$ transcription. ATF transcription factor induces phosphorylation of cyclic AMP response element binding proteins (CREB) which inturn targets induction of several genes including bcl-2.


TGFβ1
TGFβR II
TGFβR I
SMAD7
MAPK
SMAD2/3-p/4
FLIP
JNK
Fox-p3
IL-2
Transcription
NFκB
p21cip1
c-Jun
ATF-2
Sp1
CREB
bcl-2
CRE

REFERENCES

1. Banz, A., C. Pontoux, et al. (2002). "Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells." J Immunol **169**(2): 750-7.
2. Belkaid, Y., C. A. Piccirillo, et al. (2002). "CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity." Nature **420**(6915): 502-7.
3. Belkaid, Y. and B. T. Rouse (2005). "Natural regulatory T cells in infectious disease." Nat Immunol **6**(4): 353-60.
4. Bucci, J., R. English, et al. (1998). "Mucosally transmitted feline immunodeficiency virus induces a CD8+ antiviral response that correlates with reduction of cell-associated virus." J Infect Dis **177**: 18-25.
5. Chen, W., W. Jin, et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-86.
6. Chen, W. and S. M. Wahl (2003). "TGF-beta: the missing link in CD4+CD25+ regulatory T cell-mediated immunosuppression." Cytokine Growth Factor Rev **14**(2): 85-9.
7. Christ, M., N. L. McCartney-Francis, et al. (1994). "Immune dysregulation in TGF-beta 1-deficient mice." J Immunol **153**(5): 1936-46.
8. Dennler, S., M. J. Goumans, et al. (2002). "Transforming growth factor beta signal transduction." J Leukoc Biol **71**(5): 731-40.
9. Dieckmann, D., H. Plottner, et al. (2005). "Activated CD4+ CD25+ T cells suppress antigen-specific CD4+ and CD8+ T cells but induce a suppressive phenotype only in CD4+ T cells." Immunology **115**(3): 305-14.
10. English, R. V., P. Nelson, et al. (1994). "Development of clinical disease in cats experimentally infected with feline immunodeficiency virus." J Infec Dis **170**: 543-52.
11. Fantini, M. C., C. Becker, et al. (2004). "Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7." J Immunol **172**(9): 5149-53.

12. Hisaeda, H., Y. Maekawa, et al. (2004). "Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells." Nat Med **10**(1): 29-30.
13. Hori, S., T. L. Carvalho, et al. (2002). "CD25+CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by Pneumocystis carinii in immunodeficient mice." Eur J Immunol **32**(5): 1282-91.
14. Irmler, M., M. Thome, et al. (1997). "Inhibition of death receptor signals by cellular FLIP." Nature **388**(6638): 190-5.
15. Iwashiro, M., R. J. Messer, et al. (2001). "Immunosuppression by CD4+ regulatory T cells induced by chronic retroviral infection." Proc Natl Acad Sci U S A **98**(16): 9226-30.
16. Jackson, S. K., A. DeLoose, et al. (2001). "Induction of anergy in Th1 cells associated with increased levels of cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1." J Immunol **166**(2): 952-8.
17. Jackson, S. K., A. DeLoose, et al. (2002). "The ability of antigen, but not interleukin-2, to promote n-butyrate-induced T helper 1 cell anergy is associated with increased expression and altered association patterns of cyclin-dependent kinase inhibitors." Immunology **106**(4): 486-95.
18. Joshi, A., H. Garg, et al. (2005). "Different thresholds of T cell activation regulate FIV infection of CD4+CD25+ and CD4+CD25- cells." Virology **335**(2): 212-21.
19. Joshi, A., T. W. Vahlenkamp, et al. (2004). "Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation." Virology **321**(2): 307-22.
20. Kataoka, T., R. C. Budd, et al. (2000). "The caspase-8 inhibitor FLIP promotes activation of NF-kappaB and Erk signaling pathways." Curr Biol **10**(11): 640-8.
21. Kee, B. L., R. R. Rivera, et al. (2001). "Id3 inhibits B lymphocyte progenitor growth and survival in response to TGF-beta." Nat Immunol **2**(3): 242-7.
22. Kulkarni, A. B., C. G. Huh, et al. (1993). "Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death." Proc Natl Acad Sci U S A **90**(2): 770-4.
23. Maloy, K. J. and F. Powrie (2001). "Regulatory T cells in the control of immune pathology." Nat Immunol **2**(9): 816-22.

24. Marie, J. C., J. J. Letterio, et al. (2005). "TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells." J Exp Med **201**(7): 1061-7.
25. Massague, J. (2004). "G1 cell-cycle control and cancer." Nature **432**(7015): 298-306.
26. Montagnoli, C., A. Bacci, et al. (2001). "The plasticity of dendritic cells at the host/fungal interface." Immunobiology **204**(5): 582-9.
27. Moustakas, A., K. Pardali, et al. (2002). "Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation." Immunol Lett **82**(1-2): 85-91.
28. Papiernik, M., M. L. de Moraes, et al. (1998). "Regulatory CD4 T cells: expression of IL-2R alpha chain, resistance to clonal deletion and IL-2 dependency." Int Immunol **10**(4): 371-8.
29. Powell, J. D., J. A. Ragheb, et al. (1998). "Molecular regulation of interleukin-2 expression by CD28 co-stimulation and anergy." Immunol Rev **165**: 287-300.
30. Prehn, J. H., V. P. Bindokas, et al. (1994). "Regulation of neuronal Bcl2 protein expression and calcium homeostasis by transforming growth factor type beta confers wide-ranging protection on rat hippocampal neurons." Proc Natl Acad Sci U S A **91**(26): 12599-603.
31. Romagnoli, P., J. Tellier, et al. (2005). "Genetic control of thymic development of CD4(+)CD25(+)FoxP3(+) regulatory T lymphocytes." Eur J Immunol **35**(12): 3525-3532.
32. Saile, B., N. Matthes, et al. (2001). "The bcl, NFkappaB and p53/p21WAF1 systems are involved in spontaneous apoptosis and in the anti-apoptotic effect of TGF-beta or TNF-alpha on activated hepatic stellate cells." Eur J Cell Biol **80**(8): 554-61.
33. Saile, B., N. Matthes, et al. (1999). "Transforming growth factor beta and tumor necrosis factor alpha inhibit both apoptosis and proliferation of activated rat hepatic stellate cells." Hepatology **30**(1): 196-202.
34. Sanchez-Capelo, A. (2005). "Dual role for TGF-beta1 in apoptosis." Cytokine Growth Factor Rev **16**(1): 15-34.

35. Schuster, N., H. Bender, et al. (2003). "Transforming growth factor-beta and tumor necrosis factor-alpha cooperate to induce apoptosis in the oligodendroglial cell line OLI-neu." J Neurosci Res **73**(3): 324-33.
36. Shevach, E. M. (2002). "CD4+ CD25+ suppressor T cells: more questions than answers." Nat Rev Immunol **2**(6): 389-400.
37. Shi, Y. and J. Massague (2003). "Mechanisms of TGF-beta signaling from cell membrane to the nucleus." Cell **113**(6): 685-700.
38. Shi, Y., Y. F. Wang, et al. (1998). "Crystal structure of a Smad MH1 domain bound to DNA: insights on DNA binding in TGF-beta signaling." Cell **94**(5): 585-94.
39. Shull, M. M. and T. Doetschman (1994). "Transforming growth factor-beta 1 in reproduction and development." Mol Reprod Dev **39**(2): 239-46.
40. Taams, L. S., J. Smith, et al. (2001). "Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population." Eur J Immunol **31**(4): 1122-31.
41. Thornton, A. M. and E. M. Shevach (1998). "CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production." J Exp Med **188**(2): 287-96.
42. Tompkins, M. B., D. H. Gebhard, et al. (1990). "Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat." Vet. Immunol. Immunopathol. **26**: 305-317.
43. Tompkins, M. B., G. K. Ogilvie, et al. (1987). "Induction of IL-2 and lymphokine activated killer cells in the cat." Vet Immunol Immunopathol **16**(1-2): 1-10.
44. Vahlenkamp, T. W., M. B. Tompkins, et al. (2004). "Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells." J Immunol **172**(8): 4752-61.
45. Wahl, S. M., J. M. Orenstein, et al. (2000). "TGF-beta influences the life and death decisions of T lymphocytes." Cytokine Growth Factor Rev **11**(1-2): 71-9.
46. Wolfraim, L. A., T. M. Walz, et al. (2004). "p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness." J Immunol **173**(5): 3093-102.

47. Xiao, M., B. K. Oppenlander, et al. (2001). "Transforming growth factor-beta(1) induces apoptosis in CD34(+)CD38(-/low) cells that express Bcl-2 at a low level." Exp Hematol **29**(9): 1098-108.
48. Yaswen, L., A. B. Kulkarni, et al. (1996). "Autoimmune manifestations in the transforming growth factor-beta 1 knockout mouse." Blood **87**(4): 1439-45.

5. SUMMARY

a) Feline $CD4^+CD25^+$ Treg cells are functionally anergic as they do not proliferate upon mitogenic stimulation.

b) Activated feline $CD4^+CD25^+$ Treg cells are potent suppressors as they can suppress proliferation of mitogen stimulated $CD4^+CD25^-$ T cells.

c) CdkI's such as $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$ are differentially expressed in the total peripheral blood lymphocytes from FIV-infected and control cats.

d) $CD4^+CD25^+$ Treg cells from FIV-infected cats but not control cats express elevated levels of CdkI proteins compared to $CD4^+CD25^-$ T cells. In contrast cyclin D and phosphorylated retinoblastoma protein (pRb) are expressed at elevated levels in $CD4^+CD25^-$ T cells compared to $CD4^+CD25^+$ Treg cells.

e) The differential expression of CdkI's in the two purified $CD4^+$ T cell subsets was observed even at RNA level.

f) $CD4^+$ T cells from FIV-infected cats are activated in-vivo as detected by reduced levels of CD62L-selectin compared to control cats.

g) $CD4^+CD25^+$ Treg cells are more activated in FIV-infected cats than in control cats.

h) Differential expression of the CdkI's between the two $CD4^+$ T cell subsets is activation dependent.

i) TGFβ1 is expressed at significantly elevated levels in $CD4^+CD25^+$ Treg cells from FIV-infected cats compared to autologous $CD4^+CD25^-$ T cells. No significant difference in TGFβ1 expression was observed between the two $CD4^+$ T cell subsets from control cats.

j) TGFβRII is also expressed at significantly elevated levels in $CD4^+CD25^+$ Treg cells from FIV-infected and control cats compared to $CD4^+CD25^-$ T cells.

k) $CD4^+CD25^+$ Treg cells from FIV-infected express significantly more surface TGFβRII compared to $CD4^+CD25^+$ Treg cells from control cats.

l) TGFβ1 along with ConA could induce functional anergy in $CD4^+CD25^-$ T cells.

m) These converted cells are potent suppressors as they can suppress proliferation of other T cells.

n) In correlation with functional anergy, TGFβ1 plus ConA stimulated $CD4^+CD25^-$ T cells express phenotypic molecules such as CdkI's ($p21^{cip1}$) similar to that of naturally occurring $CD4^+CD25^+$ Treg cells.

o) Similar to naturally occurring $CD4^+CD25^+$ Treg cells, TGFβ1 plus ConA treated $CD4^+CD25^-$ T cells from FIV-Infected and control cats are more resistant to activation induced apoptosis compared to rhIL-2 stimulated $CD4^+CD25^-$ T cells.

p) In conjunction with the reduced apoptotic potential, TGFβ1 induced $CD4^+CD25^-$ T cells similar to the professional $CD4^+CD25^+$ Treg cells also express enhanced levels of anti-apoptotic proteins (bcl-2).

q) Naturally occurring $CD4^+CD25^+$ Treg cells express decreased levels of pro-apoptotic proteins (bax) compared to their counterparts $CD4^+CD25^-$ T cells. Mitogen along with TGFβ1 stimulation of $CD4^+CD25^-$ T cells had no effect on bax expression.

6. CONCLUSIONS

Cell cycling from G0/G1 to the S phase is regulated by a family of cyclin-dependent kinases (Cdk) and their inhibitors (CdkI's). $CD4^+CD25^+$ T regulatory cells (Treg) are arrested in the G0/G1 phase of the cell cycle and fail to produce IL-2 and proliferate in response to TCR engagement. The expression profile of the Cdk and CdkI genes in $CD4^+CD25^+$ Treg cells and the role they play in maintaining the anergic state has not been studied. We previously reported that $CD4^+CD25^+$ Treg cells from FIV-infected but not uninfected cats are activated in-vivo and possess potent immuno-suppressor function in the absence of additional in-vitro stimulation. In this study we assessed the expression profile of the CdkI's in Treg cells from FIV-infected and uninfected control cats.

We report that while $CD4^+CD25^+$ Treg cells from both FIV-infected and naïve cats are functionally anergic, as manifested by their inability to produce IL-2 and proliferate upon mitogenic stimulation, only those from FIV-infected cats are activated for strong suppressor function in vivo. Functional activation of Treg cells from FIV-infected cats correlates with up-regulation of TGFβ1 and TGFβRII on their surface and over expression of the CdkI's, $p21^{cip1}$, $p27^{kip1}$, and $p16^{ink4}$. As TGFβ1/TGFβR signaling is known to induce G0/G1 cell-cycle arrest by up-regulation of CdkI's, it can be speculated that the other phenotypic and functional responses observed in Treg cells from FIV-infected cats derive from over expression of the TGFβ1/TGFβR signaling pathway.

Although $CD4^+CD25^+$ T regulatory (Treg) cells are anergic and arrested in the G0/G1 phase of the cell cycle, they are programmed for cell survival. However, the molecular mechanism(s) involved in G0/G1 cell cycle arrest and cell survival signaling has not been defined. As mentioned earlier $CD4^+CD25^+$ Treg cells from chronically FIV-infected cats as opposed to those from control cats are functionally activated in vivo and express high levels of TGFβ1 on their surface and elevated levels of intercellular cyclin-dependent kinase inhibitors (CdkI's). To learn more of the potential role of cell surface expressed TGFβ1 in regulating the phenotypic and functional characteristics of Treg cells, we examined the role of exogenous TGFβ1 in conversion of $CD4^+CD25^-$ T cells to a Treg phenotype.

Herein we report that TGFβ1 stimulation along with TCR- engagement can induce functional and phenotypic anergy in converted Treg like cells, as manifested by their inability to proliferate upon mitogenic stimulation and expression of CdkI, $p21^{cip1}$ respectively. TGFβ1 converted Treg like cells, similar to naturally occurring Treg cells, are more resistant to activation induced apoptosis compared to mitogen stimulated $CD4^+CD25^-$ T cells, which correlates with the expression of anti apoptotic proteins (bcl-2) and pro-apoptotic proteins (bax). Therefore, TGFβ/TGFβR signaling may be one of the mechanisms by which Treg cells could induce G0/G1 cell cycle arrest by up-regulation of CdkI's, yet be resistant to apoptosis by maintaining anti-apoptotic and pro-apoptotic protein ratios for survival. Further, molecular characterization of $CD4^+CD25^+$ Treg cells from FIV chronically infected cats may be helpful in providing experimental basis for understanding the immunopathogenic potential of activated Treg cells and

developing immunotherapeutic strategies in treatment for chronic diseases such as HIV/FIV infection.