ABSTRACT

MENG, LIPING. Investigation of the Role of Nuclear Factor of Activated T Cells (NFAT2) in T Regulatory Cell-induced Immune Suppression in Feline Immunodeficiency Virus Infection. (Under the direction of Dr. Mary Tompkins).

CD4+CD25+ T regulatory cells play an important role in maintaining the balance between protective immune responses and excessive inflammatory conditions. The molecular mechanisms by which Treg cells suppress the function of T helper and CD8+ cells remain to be elucidated. As Treg cells play a major role in lentivirus-induced immunodeficiency, the role of two transcription factors NFAT2 and FoxP3 as potential regulators in Treg-induced T helper cell suppression in FIV infection was examined. A human NFAT2 homologous protein was identified in the cat that plays a role in IL-2 induction by binding to the IL-2 promoter in activated lymphocytes. Further studies in FIV+ cats revealed that in the absence of in vitro stimulation, FoxP3, a suppressor of IL-2 transcription was expressed in CD4+CD25+ Treg but not in CD4+CD25- T helper cells. Moreover, PMA/ionomycin stimulation did not significantly influence FoxP3 expression, suggesting that it is constitutively expressed. In contrast, IL-2 mRNA was not expressed in unstimulated CD4+CD25- or CD4+CD25+ T cells but was expressed at high levels by both T cell subsets from FIV+ and control cats stimulated with PMA/ionomycin. NFAT2 mRNA was equally expressed in both subsets of unstimulated CD4+ T cells and was equally highly inducible by PMA/ionomycin. Co-culture of fresh CD4+CD25+ Treg cells isolated from FIV+ with T helper cells showed elevated expression of FoxP3 mRNA and inhibited IL-2 production in the T helper cells compared to T helper cells co-cultured with CD4+CD25- cells. However, while NFAT2 mRNA expression was increased in both co-cultures, there was no significant difference between the two co-cultures, suggesting that the decrease in IL-2 production in the
Treg-suppressor co-culture is not related to NFAT2 mRNA expression. As TGF-β has been implicated as a mediator of Treg suppression, the role of TGF-β in FoxP3 and NFAT2 expression and Treg-mediated suppression of CD4+CD25- T helper cells was assessed. ConA-stimulated CD4+CD25- T helper cells cultured in the presence of soluble TGF-β up-regulated FoxP3 and had a suppressed IL-2 response compared to cells cultured in the absence of TGF-β. NFAT2 mRNA expression was increased to similar levels in both cultures, suggesting that NFAT2 transcription was not affected by TGF-β mediated suppression. ChIP assay revealed that, while NFAT2 mRNA levels were similar in stimulated versus stimulated/suppressed cells, there was more NFAT2 bound to the IL-2 promoter in the nonsuppressed cells. These data suggest that FIV infection activates Treg cells. These activated Treg cells suppress T helper cell IL-2 production, by inducing FoxP3 expression which in turn interferes with NFAT2 binding to the IL-2 promoter. This Treg cell-induced T helper cell suppression is mediated via TGF-β signaling. These studies support the concept that FoxP3 functions as a suppressor molecule in a number of IL-2-dependent responses.
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Investigation of the Role of Nuclear Factor of Activated T Cells (NFAT2) in T Regulatory Cell-induced Immune Suppression in Feline Immunodeficiency Virus Infection

by
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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

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DEDICATION

To my parents Deming Meng and Baodi Han
BIOGRAPHY

Liping Meng was born and raised in Shanghai, China. She was awarded a B.S. in Biological Sciences from Fudan University (Shanghai, China) in July 2008. In the same year, Meng was admitted into the Comparative Biomedical Sciences Program at North Carolina State University in August, and then she transferred to Immunology Program and joined Professor Tompkins group in October. She has been studying the mechanisms of immune suppression in feline immunodeficiency virus infection as a model for HIV/AIDS.
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To Drs. Mary and Wayne Tompkins — thank you for offering me the opportunity to join your group, and for mentoring me how to be a good scientist.

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I. INTRODUCTION

HIV/FIV-induced immunodeficiency occurs early after HIV/FIV infection and is sustained throughout the whole course of the disease (Davidson et al., 1993). T cells become irreversibly unresponsive to viral specific antigen, recall antigens and mitogen stimulation during acute HIV/FIV infection (Clerici et al., 1989; Torten et al., 1991). Such early onset of immune dysfunction and hypo-responsiveness of T cells cannot be adequately explained by chronic T cell loss or specific clonal depletion (Aandahl et al., 2004; Kinter et al., 2004; Weiss et al., 2004).

CD4+CD25+ Treg cells are a unique population of T cells that are activated early after HIV/FIV infection and function as immune regulators (Sakaguchi et al., 1995; Mexas et al., 2008; Fogle et al., 2010a). Treg cells suppress CD4+CD25- T helper cells and CD8+ CTL responses by downregulating IL-2 production and inducing anergy in target cells (Sakaguchi et al., 1995; Thornton et al., 1998; Aandahl et al., 2004; Vahlenkamp et al., 2004b; Mexas et al., 2008). Normally, Treg cells return to a resting state after the pathogen is eliminated. However, following chronic infection such as HIV and FIV, Treg cells remain chronically activated. Therefore, Treg-mediated T cell suppression may explain the immune deficiency in lentiviral infection (Shevach et al., 2001).

One feature of Treg cells is the constitutive expression of FoxP3, a transcription regulator that determines specific Treg lineage development and suppressive activity. FoxP3 is known to suppress IL-2 gene expression in Treg cells, although the underlying molecular mechanism is ill defined (Fontenot et al., 2003; Hori et al., 2003; Fontenot et al., 2005;
Antons et al., 2008; Li et al., 2008). Activated Treg cells express membrane-bound TGF-β, which plays a crucial role in cell-contact dependent immunosuppression. And it has been demonstrated that Treg-mediated suppression is TGF-β/TGF-βRII signaling dependent (Nakamura et al., 2001; Annunziato et al., 2002; Chen et al., 2003b; Nakamura et al., 2004). The primary effect of TGF-β/TGF-βRII signaling is to suppress IL-2 production and thus inhibit IL-2 dependent proliferation of T cells (Kehrl et al., 1986). Moreover, TGF-β signaling also induces FoxP3 expression in T helper cells (Chen et al., 2003a; Fantini et al., 2004; Andersson et al., 2008). Therefore it is of interest to elucidate the interrelation between FoxP3 elevation and IL-2 suppression in suppressed T helper cells.

Recently it has been considered that a transcription factor NFAT2 may interact with FoxP3 and play a role in Treg-mediated T helper cell suppression (Bettelli et al., 2005; Torgerson et al., 2009). NFAT proteins were first identified as putative transcription factors that induce IL-2 gene expression in activated T cells, and were essential for establishing robust immune responses (Shaw et al., 1988; Peng et al., 2001). NFAT1 and NFAT2 are predominantly expressed by T cells and they are regulated by calcium-calcineurin signaling (Feske et al., 2003; Hogan et al., 2003). NFAT1 is constitutively expressed in naive T cells while expression of NFAT2 is induced upon activation of T cells (Chuvpilo et al., 1999; Zhou et al., 2002; Serfling et al., 2006). Due to the essential role of NFAT2 in IL-2 production and T cell activation, we ask the question if FoxP3 suppress IL-2 production through inhibition of NFAT2 expression in Treg cell-suppressed T helper cells.
In our study, we first identified a homologous protein in the cat which shares 85% nucleotide identity with human NFAT2. And we confirmed that this feline NFAT2 can be significantly up-regulated in activated T helper cells and in turn induces IL-2 production. However, we did not see significant difference of NFAT2 expression in T helper cells and Treg cells. When T helper cells are suppressed by Treg cells or soluble TGF-β, FoxP3 expression is highly elevated in association with inhibition of IL-2 production, yet NFAT2 expression remains unaltered. ChIP assays revealed that binding of NFAT2 to the feline IL-2 promoter is higher in activated T cells than in suppressed T cells. These observations indicate that although FoxP3 does not affect NFAT2 mRNA expression in suppressed T helper cells, binding avidity of NFAT2 protein to the promoter is decreased, which may contribute to the IL-2 inhibition and T helper cell suppression in feline lentiviral infection.
II. BACKGROUND AND LITERATURE REVIEW

A. SIV and FIV as Animal Models for HIV Pathogenesis Research

The acquired immune deficiency syndrome (AIDS), which is caused by human immunodeficiency virus (HIV), has been recognized as one of the most destructive pandemics in the world. It has killed more than 25 million people since its first identification in 1981. Although many prophylactic and therapeutic strategies have been developed to combat the disease, there are still millions of new infections every year and millions of annual deaths caused by AIDS. Due to economical and ethical issues involved, it has been important to find an animal model for HIV/AIDS studies.

Chimpanzees can be experimentally infected with HIV-1; however, they usually do not develop clinical AIDS-like syndromes (Ferrari et al., 1993; Juompan et al., 2008). Simian immunodeficiency virus (SIV) is most phylogenetically closely related to HIV, but SIV is more related to HIV-2 than HIV-1. Natural hosts of SIV, such as African green monkey and sooty mangabey, can be life-long carrier of SIV, yet never develop clinical AIDS syndromes despite high levels of viral replication (Broussard et al., 2001; Goldstein et al., 2000; Rey-Cuille et al., 1998). Similar to HIV, acute infection of SIV results in massive depletion of CD4+ cells; however, an effective anti-inflammatory milieu is rapidly developed to control aberrant systemic immune activation, which preserves the intact immune function in SIV natural hosts (Silvestri et al., 2003; Kornfeld et al., 2005; Pandrea et al., 2008). Asian macaques infected with SIV develop a similar disease course to HIV infection in humans during the acute phase. However, SIV infected Asian macaques are generally poorly
responsive to the virus and progress rapidly to opportunistic infections and wasting syndromes, culminating in death within a short period of time. Therefore, although the macaque is a valuable animal model in studying acute infection HIV and vaccine development, it is not an adequate model to study the complete pathogenetic course of HIV infection as they do not mirror the chronic asymptomatic stage of disease which occurs in human AIDS (Daniel et al., 1987; Kannagi et al., 1986; Letvin et al., 1990).

A new feline retrovirus was isolated in 1986 from domestic cats suffered from an AIDS like syndrome and was later named feline immunodeficiency virus (FIV) (Pedersen et al., 1987). Both FIV and HIV are members of genus Lentivirus, family of Retroviridae. Although FIV is not the most phylogenetic relative of HIV, it shares similarity to HIV in terms of morphology, genome organization, protein composition, and Mg$^{2+}$ dependent reverse transcriptase activity (Olmsted et al., 1989; Pedersen et al., 1989; Talbott et al., 1989). FIV and HIV have a somewhat similar cell tropism in that they both target T cells and macrophages. However FIV also infects B cells (Pedersen et al., 1987; Brunner et al., 1989; English et al., 1994; Clapham et al., 2001). Cats are the natural host of FIV and develop a similar course of disease progression as human infected with HIV (Pedersen et al., 1987; English et al., 1994). The initial infection is flu-like, associated with low-grade fever, leukopenia, and lymphadenopathy. Fever and leukopenia disappear after a few weeks while lymphadenopathy can last for months (Kinloch-de Loes et al., 1993). Massive T cell depletion occurs during the acute stage of infection. CD8 T cell numbers can rebound to close to normal level in a few weeks; however, CD4 T cell numbers only recover minimally,
leading to an inversion of the CD4:CD8 ratio, which is sustained throughout the infection (Tompkins et al., 1991). The acute infection lasts variably from weeks to months and transits to an asymptomatic chronic infection, which can last for many years. The asymptomatic stage of infection is characterized by down-modulation of viral replication, gradual decline in CD4 lymphocyte numbers, a persistent low CD4:CD8 ratio, and progressive immune function impairment. The development of AIDS is first signaled by appearance of “AIDS-related complex”, which is characterized by fever, weight loss, and generalized lymphadenopathy. The actual AIDS stage of disease occurs months later, which is manifested by high level of viral replication, concurrent with secondary opportunistic infections, chronic wasting diseases, recurring respiratory infections, cancer and/or neurologic disorders (Yamamoto et al., 1988; English et al., 1994). Besides natural infection, cats can also be experimentally infected with FIV by intravenous and trans-mucosal inoculations (Burkhard et al., 1997). Taken together that FIV shares the striking similarity with HIV in terms of genetic background, disease progression, pathogenesis, and the easy handling and universal availability of its natural hosts, FIV is an excellent model to study HIV pathogenesis (Pedersen et al., 1989; Vahlenkamp et al., 2006).

B. Immune Dysfunction in HIV/FIV Infection

Following HIV/FIV infection, robust humoral and cell-mediated anti-viral responses are induced in the acute stage of disease. Antibodies and cytotoxic CD8 T cells against HIV/FIV can be detected as early as 2 weeks post infection, and sustained throughout the
chronic infection (Ackley et al., 1990; Gotch et al., 1990; Egberink et al., 1992; Borrow et al., 1994; English et al., 1994; Beatty et al., 1996). However, immune responses fail to clear the virus, and immune dysfunction gradually develops. The underlying mechanism of HIV/FIV induced systemic immune dysfunction has been intensively studied yet remains controversial.

1. HIV/FIV-induced impairment of generative capacity of host immune system

The acute stage of HIV/FIV infection is featured by massive T cell depletion, especially memory and activated cells, due to virus-induced cell lysis, cytotoxic CD8 T cell-mediated killing, and bystander damage. This is followed by a continuing drop of CD4 T cells during the chronic stage of infection. At the same time the peripheral T cell pool is slowly declining, the regenerative capacity of the host immune system is greatly diminished by HIV/FIV infection. HIV/FIV targets almost all thymocytes at different stages of maturation, and attacks bone marrow, causing abnormalities in bone marrow architecture and impaired production of hematopoietic progenitor cells. Viral infection also causes pathologic damage of lymph nodes and disrupts the interaction of T cells and antigen presenting cells, resulting in the failure of CD4 T cell homeostasis. Over time, host compensatory mechanisms are overwhelmed, leading to eventual collapse of immune system (Bonyhadi et al., 1993; De Luca et al., 1993; Pantaleo et al., 1993; Su et al., 1995; Moses et al., 1998; Hasse 1999; Dai et al., 2001; McCune et al., 2001; Douek et al., 2003).
2. Cytokine dysregulation

CD4 T cells can be divided into two major groups, Th1 and Th2. Th1 cells mainly produce IL-2, IL-12 and IFN-γ, and are important for activation of cell-mediated immune responses, while Th2 cells mostly produce IL-4, IL-5, IL-6, IL-10 and promote B cell differentiation and humoral immune responses. Early reports showed that in HIV infection, IL-2 and IFN-γ production were diminished while IL-4 and IL-10 production were enhanced, suggesting an imbalance of Th1 and Th2 immune responses (Clerici et al., 1993; Meroni et al., 1996). However, other reports showed that there was no clear cut Th1 to Th2 switch (Graziosi et al., 1994). The production of IFN-γ, a profound Th1 cytokine has been found increasing in different compartments throughout FIV infection (Dean et al., 1998). Generally, the dramatic elevation of Th2 cytokines IL-10 and IL-4 is accompanied with a decline of Th1 cytokines, IL-2 and IL-12. An abnormally elevated IL-10: IL-12 ratio is correlated with the failure of a type 1 immune response following T.gondii challenge in FIV infected cats (Levy et al., 1998; Levy et al., 2004). Although there is no definite Th1 to Th2 switch, evidence has shown that the dysregulation of cytokines is one of the mechanisms for immunodeficiency in HIV/FIV infection.

3. Chronic systemic immune activation and activation-induced cell apoptosis.

It was first proposed in the late 1980s that abnormal systemic immune activation played a major role in CD4 T cell depletion and progressive immunodeficiency in HIV-1
infection (Ascher et al., 1988). Accumulating evidence has shown that systemic immune activation, rather than plasma viral load, is a better predictor of disease progression. In advanced HIV-1 infected patients whose peripheral CD4 T cell count fall below 50/mm³, elevated expression of CD38 on T cells is associated with shorter survival (Giorgi et al., 1999). A high turnover – activation of a small proportion of cells and subsequent expansion followed by activation-induced cell apoptosis – has been shown to affect both CD4+ and CD8+ T cells, resulting in a decline of T cell counts in HIV infection (Kovacs et al., 2001; Mohri et al., 2001). Accordingly, the percentage of HLA-DR+ CD3+ cells correlates with the decline of CD4 T cells (Leng et al., 2001). Examination of the expression of Ki67 – nuclear antigen existing only in proliferating cells – demonstrates that both CD4+ and CD8+ T lymphocytes in HIV-1 infected individuals are highly proliferative compared to healthy individuals (Sachsenberg et al., 1998; Hazenberg et al., 2000). And the half-life of circulating T cells in HIV infected patients is dramatically diminished compared to T cells in healthy individuals (Hellerstein et al., 1999; McCune et al., 2000). Systemic immune hyper-activation has also been found in FIV infection. Up to 80% of the total CD4+ T cells down-regulate surface expression of CD62L, a marker which is expressed on naive cells and lost on activated cells (Vahlenkamp et al., 2006). Flow cytometric analysis has revealed a progressive expansion of activated circulating CD8+ cells that are CD8^{low}CD62L^{-}CD44^{high}CD49d^{high}CD18^{high} (Bucci et al., 1998; Gebhard et al., 1999).

The abnormal chronic activation of the immune system is not just a simple result of a compensatory response of the immune system for loss of peripheral T cells, but the cause of
progressive immunodeficiency (Douek et al., 2003; Sodora et al., 2008). Evidence has shown that the proportion of CD4+ T cells that are infected by virus in the asymptomatic period is only about $1/10^4$. Productive infection during the chronic stage of infection is persistent but is only present in a very small fraction of cells, suggesting that virus cytopathicity and cytotoxic T cell killing are not major causes of massive depletion of CD4 T cells (Brinchmann et al., 1991; Chun et al., 1997; Anderson et al., 1998). Besides, HIV/FIV preferentially infect activated and/or memory cells which are already destined to activation induced apoptosis, while the majority of CD4 T cell death actually occur in uninfected cells (Haase et al., 1999).

One of the consequences of systemic immune activation is massive depletion of cells by activation-induced cell death. It has been reported that the high proportion of apoptotic cells in HIV-1 infected lymph nodes are bystander cells, and the abnormally elevated level of T cell apoptosis in LN from HIV-infected persons correlates with the global activation state of T cells (Finkel et al., 1995; Muro-Cacho et al., 1995; Gougeon et al., 1996; Badley et al., 2000). Several activation-induced apoptosis signaling pathways have been found to cause T cells depletion in HIV infection. A significantly higher frequency of T cells from HIV-infected persons express Fas, and when treated with anti-Fas antibodies in vitro, T cells from HIV-infected patients undergo a greater level of apoptosis in comparison with T cells from healthy individuals (Katsikis et al., 1995). Further investigation has revealed that CD4 T cell apoptosis occurs mainly in cells expressing both Fas and FasL (Dyrhol-Riise et al., 2001). Other than Fas-FasL signaling, it has been reported that in HIV-infected lymphoid tissues,
compared to uninfected lymphoid tissue, expression and production of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by antigen presenting cells and primary CD4 T cells are greatly enhanced, which can induce T cell apoptosis through TRAIL-DR4/5 signaling (Herbeuval et al., 2005a; Herbeuval et al., 2005b). Similar activation-induced apoptotic signaling has also been found in chronic FIV infection. B7 costimulatory molecules are normally expressed by antigen presenting cells. When B7 binds to CD28, naive/resting T cells are signaled for proliferation and differentiation. Activated T cells express surface CTLA-4. The avidity of B7 binding to CTLA-4 is much greater than to CD28, therefore, B7-CTLA-4 signaling regulates the T cell response, avoiding excess immune activation (Greenwald et al., 2005). In FIV+ cats, B7.1/B7.2 are expressed on a high proportion of T cells in lymph nodes, and the majority of CTLA4+ T cells is also B7+. Most importantly, enhanced spontaneous apoptosis of CD4+ T cells from FIV+ cats compared to control cats can be greatly inhibited by in vitro treatment of anti-B7.1 antibody. Such data suggest a T-T cell mediated apoptosis in chronic infection like HIV/FIV (Tompkins et al., 2002; Bull et al., 2004; Vahlenkamp et al., 2004a).

4. Immunological anergy

It has been reported that FIV acutely infected cats (18 weeks p.i.), when challenged with T.gondii, developed generalized toxoplasmosis with 75% mortality, while in normal cats, there is only a subclinical infection. This suggests that immune dysfunction occurs early after FIV infection, and can render the host more susceptible to secondary infections
In HIV infection, T cells from acutely infected patients first lose the response to viral specific antigen but respond normally to recall antigen such as PPD, tetanus, and CMV. However, with the progression of disease, T cells fail to respond to recall antigens and eventually become unresponsive to mitogen stimulation (Clerici et al., 1989; Torten et al., 1991; Aandahl et al., 2004). Such early onset of immune dysfunction and hypo-responsiveness of T cell against viral specific antigens cannot be adequately explained by chronic T cell loss, impairment of regenerative capacity of the immune system, or cytokine dysregulation. In addition, studies have shown that early loss of T cell responsiveness against viral specific antigen in HIV is not a result of specific clonal depletion, as CD4+ and CD8+ T cells from HIV infected individuals can produce IFN-γ, TNF-α and other cytokines but not IL-2 in response to HIV antigen stimulation in vitro (Aandahl et al., 2004; Kinter et al., 2004; Weiss et al., 2004). In the case of FIV infection, proliferative response of PBMCs from FIV+ cats, stimulated in vitro by mitogen and exogenous IL-2 were dramatically diminished (Davidson et al., 1993). Production of IL-2 by PBMCs from FIV+ cats was also significantly lower than PBMCs from FIV- cats (Seibelink et al., 1990). These diminished IL-2 responses are reminiscent of immunologic anergy, which is defined as the failure of T cells to produce IL-2 upon re-stimulation. How HIV/FIV infection induces anergy is not fully understood. However, a unique population of T cells, named T regulatory cells, has been proposed to mediate immune suppression during HIV/FIV infection.
C. The Role of Treg Cells in Lentivirus Infection and Disease Progression

1. Phenotypic and functional features of Treg cells

CD4+CD25+ Treg cells were identified as a unique population of T cells that function as suppressor cells, playing a major role in down-regulating autoimmune T cells and controlling potential inflammatory damage by antigen reactive CD4+ and CD8+ T cells (Sakaguchi et al., 1995). T regulatory cells were first reported to be generated in the thymus by recognition of tissue-specific antigens presented by thymic epithelial stromal cells (Modigliani et al., 1995a; Modigliani et al., 1995b; Modigliani et al., 1996; Coutinho et al., 2005; Salaün et al., 2005). However recently it has been found that T regulatory cells can also be induced in the periphery in cases of chronic infections that provide persistent immune stimulation (Iwashiro et al., 2001; Belkaid et al., 2002). Naturally thymic derived Treg cells (TregN) and peripheral induced Treg cells (TregI) are generated in different compartments, they are indistinguishable by their phenotype or suppressive activity; however, TregN are proposed to be responsible for maintaining self-tolerance, while TregI mainly suppress antigen-specific T cell response against foreign stimuli (Bluestone et al., 2003). Treg cells constitute 5~10% of the total peripheral T cell population. While Treg cells are activated through antigen specific TCR engagement, once activated, they suppress CD4+ T helper and CD8+ cytotoxic T cell immune responses in an antigen non-specific manner (Thornton et al., 1998). Naive Treg cells constitutively express surface markers, such as CD25, GITR, CTLA4, and CD45RO, which distinguish them from other naive T cells (Sakaguchi et al.,
1995; Read et al., 2000). They also constitutively express a member of the forkhead/winged-helix family of transcription factors, FoxP3, which distinguishes Treg cells from activated T helper cells as they also express CD25. Recently, it has been reported that FoxP3 expression can be elevated in human activated T helper cells; however, such elevation is only transient due to inherent epigenetic limitations, and does not enable T helper cells to acquire suppressive activity. In contrast, the expression of FoxP3 in Treg cells is stable and irrelevant to activation state (Hori et al., 2003; Fontenot et al., 2003; Fontenot et al., 2005; Li et al., 2008; Nagar et al., 2008).

Treg cells are unique in that they are unable to produce IL-2, require exogenous IL-2 for proliferation and are anergic to immune stimulation yet are relatively resistant to activation induced apoptosis (Maloy et al., 2001). While Treg cells fail to produce IL-2 and cannot proliferate, their suppressor function is dependent on an exogenous source of IL-2 (Shevach 2002). Activated Treg cells are well known for their ability to suppress IL-2 and IFN-γ production in T helper cells and CD8+ T cells respectively (Thornton et al., 1998; Fogle et al., 2010a). Such Treg-mediated suppression is mediated by membrane-bound TGF-β (Nakamura et al., 2001, see detailed discussion below).

2. Treg cells as susceptible target and productive reservoir for HIV/FIV infection

AIDS lentiviruses including HIV and FIV bind to and enter CD4+ target cells via virus specific cell surface receptors. HIV utilizes CCR5 or CXCR4 as co-receptors while FIV uses
CD134 and CXCR4 (Berger et al., 1999; Richiardson et al., 1999; Shimojima et al., 2004). The differential ability of HIV/FIV to enter populations of lymphocytes remains controversial. Some reports have shown that naive T cells are resistant to HIV infection and TCR engagement or stimulatory cytokine pre-treatment is required for naive T cells to become susceptible to HIV infection (Chou et al., 1997; Unutmas et al., 1999; Unutmas 2001). Others have shown that HIV can establish an efficient infection in resting T cells, but the infection is not productive and no viral release can be detected after cell activation (Tang et al., 1995). Such discrepancy may caused by the usage of virus strains with different co-receptor tropism. Generally, co-receptor CXCR4 is expressed on the surface of naive and activated T cells, while CCR5 on memory T cells (Berger et al., 1999). It has been found that Treg cells from humans or cats constitutively express high level of CCR5 or CXCR4, which render them highly susceptible to HIV or FIV infection (Oswald-Richter et al., 2004; Joshi et al., 2005a).

Virus replication subsequent to cell entry is dependent upon metabolic factors associated with the state of cell activation (Tang et al., 1999). HIV and FIV are unable to replicate in quiescent naive CD4+ T cells but do so in mitogen or antigen activated CD4+ T cells (Stevenson et al., 1990; Tang et al., 1995). Although both CD4+CD25- and CD4+CD25+ cells are found susceptible to FIV infection, only CD4+CD25+ Treg cells support virus replication even in the absence of mitogenic stimulation (Spina et al., 1997; Joshi et al., 2004; Joshi et al., 2005b; Borvak et al., 1995). During acute HIV or FIV infection, CD4+CD25+ T regulatory cells are more productively infected compared to
CD4+CD25- T helper cells (Jiang et al., 2008; Mexas et al., 2008). Transcription factors CEBP, ATF and AP-1 are known to be essential for FIV replication. They are constitutively expressed in Treg cells from FIV chronically infected cats and can be further enhanced in response to exogenous IL-2 (Joshi et al., 2005a). Thus while Treg cells do not produce IL-2 and cannot proliferate, they appear to express the necessary transcription factors for replication of AIDS lentiviruses. This, together with the facts that Treg cells are anergic (Kuniyasu et al., 2000; Gavin et al., 2002), unable to pass through the G1 cell cycle (Li et al., 2005), and are more resistant to activation-induced cell death (Fritzsching et al., 2005; Fritzsching et al., 2006), suggest that this small unique population of CD4+ cells may provide an important reservoir for lentivirus replication.

3. Treg-mediated immune suppression and disease progression in HIV/FIV infection

Normally, Treg cells return to a resting state after the pathogen is eliminated. However, following chronic infection such as HIV and FIV, Treg cells remain chronically activated. The role that Treg cells play in HIV and FIV infection is not yet fully understood. During acute stage of infection, Treg cells are activated and can suppress CD4+CD25- T helper and CD8+ CTL responses, allowing the virus to establish a persistent infection (Aandahl et al., 2004; Mexas et al., 2008; Fogle et al., 2010a). Persistent viral infection induces chronic Treg cell activation, resulting in suppression of immune responses to secondary infections. CD4 T cells from chronic HIV-infected patients regain proliferative ability in response to tuberculin, cytomegalovirus, and p24 when Treg cells are depleted.
Patients with higher frequency of Treg cells in peripheral blood are associated with lower Gag-specific CD4 T cell responses, and \textit{in vitro} depletion of Treg cells restores both CD4+ and CD8+ T cell responses as measured by intracellular production of IFN-\(\gamma\) (Baker et al., 2007). On the other hand, Treg cells appear beneficial to the host by limiting systemic hyper-activation of immune responses. Loss of Treg cells during chronic stage of HIV infection leads to massive T cell depletion due to activation-induced cell death, increase in viral replication and the development of AIDS (Eggena et al., 2005).

The complicated role that Treg cells play in lentiviral infection has triggered intensive studies. Unlike CD4+ T helper cells and CD8+ T cells which are prone to lysis, when infected by HIV/FIV, CD4+CD25+ Treg cells are more resistant to cell lysis than infected T helper cells (Joshi et al., 2005a). In the case of HIV/FIV infection, the survival of Treg cells may be problematic. It is recently reported that HIV infection results in Treg cell tissue redistribution (Ji et al., 2009). Expression of homing molecules CD62L and integrin \(\alpha_4\beta_7\) are elevated on Treg cells upon UV-inactivated-HIV-binding, directing Treg cell homing to and accumulating in peripheral lymphoid tissues. Interestingly, HIV-binding appears to promote Treg cell survival and make them less susceptible to homing induced apoptosis. More importantly, HIV-binding greatly enhances Treg cell suppressive activity, which is associated with elevated Treg cell molecular markers CTLA4, FoxP3, GITR, and mTGF-\(\beta1\). As an infected cell releases virus that efficiently infects only nearby targets, the high density of target cells in lymphoid tissues provides an ideal environment for propagation of infection, and Treg cells in those tissues can not only suppress local immune response, but also serve as
a source of infectious viral particles and facilitate disease progression (Andersson et al., 2005; Nilsson et al., 2006; Kinter et al., 2007; Ji et al., 2009).

**D. The Molecular Mechanism of Treg Cell-mediated Immune Suppression.**

CD4+CD25+ Treg cells are able to suppress both CD4+ T helper cell and CD8+ cytotoxic T cell immune responses. When purified CD4+CD25- T helper cells from HIV infected patients are cocultured with cognate CD4+CD25+ Treg cells *in vitro*, the ability of the T helper cell to produce IL-2 and proliferate upon stimulation are greatly impaired. Addition of IL-2 can overcome such suppression (Thornton et al., 1998; Shevach et al., 2001). Studies in FIV infection demonstrate that freshly isolated CD4+CD25+ Treg cells from FIV+ cats are activated *in vivo* and are capable of suppressing CD4+CD25- T helper cell proliferation and immune responses by down-regulating IL-2 production (Vahlenkamp et al., 2004b). As IL-2 is an essential cytokine to promote T cell clonal expansion, differentiation, and full activation, lack of IL-2 may lead to T cell anergy and apoptosis (Smith 1988). Therefore inhibition of IL-2 production by Treg cells may explain the immunodeficiency in lentiviral infection. The molecular mechanism by which Treg cell suppress immune responses has not been completely elucidated. Accumulating evidence has shed some light on how Treg cell development and suppressive activity are regulated as well as what molecules are involved in Treg cell-mediated suppression.
1. FoxP3 regulates Treg cell development and suppressive function

In mice, defects in the gene *scurfy* are associated with over-proliferation of CD4+ T cells, systemically elevated inflammatory cytokines, and excess autoimmune reactions (Brunkow et al., 2001). The human ortholog of mouse *scurfy* gene, *FoxP3* was identified in IPEX syndrome affected human, who suffered from multiple autoimmune disorders. Human FOXP3 is mapped to X-chromosome (Xq11.23-Xq13.3). It consists of 11 exons, and encodes 431 amino acids. FoxP3 protein contains a zinc finger domain, a leucine zipper motif, which is responsible for FoxP3 dimerization, and a forkhead/winged helix domain that is important for DNA binding. Mutations of human FoxP3 have been linked to at least half of the IPEX syndrome cases (Bennett et al., 2001; Kobayashi et al., 2001; Owen et al., 2003; Ochs et al., 2007; Van der Vliet et al., 2007).

It has been demonstrated that constitutive expression of Foxp3 determines specific Treg lineage development and suppressive activity. Flow cytometric analysis of lymph node cells and thymocytes from FoxP3+ mice and their FoxP3-/- littermates has confirmed that CD4+CD25+ Treg cell population is lost in scurfing-deficient mice, leading to rapid development of lymphoproliferative diseases. However, forced-expression of FoxP3 can restore the Treg cell population and delay the onset of diseases (Khattri et al., 2003). And Transfer of Treg cells into newborn FoxP3 deficient mice can successfully prevent lymphoproliferative disease (Fontenot et al., 2003).

Chromatin immunoprecipitation combined with microarray analysis enables investigators to study the genome wide target of FoxP3. Using FLAG-tagged FoxP3
transduced cells, it has been shown that FoxP3 binds to a large number of promoters, as much as 1% of the gene expression in murine cells is directly regulated by FoxP3, including CD25, GITR, Nrp1, and CCR4 (Marson et al., 2007). FoxP3 can act as a gene activator as well as a gene repressor. A large proportion of FoxP3 regulated genes are involved in membrane/intracellular signaling regulation or chromatin modification (Zheng et al., 2007). shRNA mediated FoxP3 knockdown in Treg cells dramatically down-regulates expression of Treg cell-associated cell surface markers, such as CD25, CTLA4, CD62L and HLA-DR, and significantly impairs Treg cell suppressive activity as measured by target T helper cell proliferation in co-culture (Antons et al., 2008). These studies have demonstrated that FoxP3 is the key regulator that determines unique Treg cell lineage development and suppressive function.

2. Treg cell-mediated suppression is membrane-bound TGF-β1 dependent

Several molecules such as TGF-β, CTLA-4 and FoxP3 have been found essential for Treg mediated suppression; however, the interrelation of these molecules has not yet been defined (Rubtsov et al., 2007). Although fully activated Treg cells produce high levels of suppressive cytokines IL-10 and TGF-β, their suppressive ability is cell-cell contact dependent rather than mediated by soluble suppressive cytokines (Nakamura et al., 2001). When Treg cells and T helper cells are separated by semi-permeable membrane, suppression is completely abolished, and when T helper cells are cultured in Treg supernatant, no suppression is observed (Thornton et al., 1998). Moreover, addition of antibodies against IL-
10 receptor, or IL-10 cannot block the suppression, while addition of anti-TGF-β1, anti-TGF-βR or anti-LAP is able to partially restore T helper cell proliferation in a dose dependent manner (Annunziato et al., 2002; Chen et al., 2003a; Nakamura et al., 2004). Flow cytometric analysis has revealed that both active and latent forms of TGF-β1 are highly expressed on activated Treg cells, while expression of TGF-βRII is significantly elevated on activated T helper cells surface (Chen et al., 2003b; Petty et al., 2008). When activated Treg cells are pre-treated with anti-TGF-β antibodies or activated T helper cells are pre-treated with anti-TGF-βRII antibodies, the suppression is greatly diminished (Petty et al., 2008). These studies suggest that Treg mediated suppression is cell-cell contact dependent and occurs via membrane-bound TGF-β1 expressed on activated Treg cells binding to the TGF-βRII on activated T helper cells. Interestingly, it has been found that TGF-βRII is also elevated on Treg cells, suggesting TGF-β/TGF-βRII signaling is not only essential for suppression of T effector cells but also for maintenance of Treg cells homeostasis (Nakamura et al., 2001; Chen et al., 2003b). In support of this, transferring CFSE-labeled CD4+CD25+ Treg cells from wild type or TGF-βR deficient mice into wild type mice has demonstrated that TGF-β signaling is critical for Treg cell expansion in vivo (Huber et al., 2004). Surprisingly, in vivo studies have shown that transfer of Tgfb1-/- splenocytes to Rag2/-/- mice induces inflammatory diseases, while co-transfer of Tgfb1-/- Treg cells clearly attenuates the severity of diseases, suggesting that Treg can suppress immune responses in the absence of TGF-β (Mamura et al., 2004). Further studies have revealed that Treg cells produce an
inhibitory cytokine IL-35, which is primarily responsible for contact-independent Treg-mediated cell suppression (Shevach et al., 2009; Chaturvedi et al., 2011).

CTLA-4 is another Treg cell-associated marker that is constitutively expressed at high levels. TGF-β treated CD4+CD25- T cells has been found to rapidly elevate surface expression of CTLA-4 and CD80. Blockade of CTLA-4 can down-regulate surface expression of TGF-βR on T effector cells and completely abolish the suppression mediated by Treg cells (Chen et al., 2003a; Zheng et al., 2006). Moreover, a recent study suggests that cross-linking of CTLA-4 could specifically enhance TGF-β1 production by Treg cells and facilitate accumulation of TGF-β1 at the cell-cell contact site, intensifying the TGF-β signaling (Oida et al., 2006). These studies suggest that CTLA-4 may be also involved in Treg cells mediated suppression. In summary, Treg-mediated suppression is a multi-step complicated process, and fully understanding of the mechanism needs further investigation.

3. T helper cells are suppressed and converted to Treg cells via TGF-β/TGF-βR signaling

When co-cultured with Treg cells, CD4+CD25- T helper cells not only become anergic, being nonresponsive to stimulation, they can actually be converted into T regulatory cells both phenotypically and functionally. It has been found that the same TGF-β/TGF-βR signaling that mediates suppression is critical for T helper cell conversion. CD4+CD25- T helper cells stimulated via TCR or anti-CD3/anti-CD28 and cultured in the presence of TGF-β1 produce markedly lower levels of IL-10 and little pro-inflammatory cytokines such like IL-4 and IFN-γ compared to cells cultured in the absence of TGF-β1 (Chen et al., 2003b).
Flow cytometric analysis reveals that TGF-β1 treated CD4+CD25- T cells become CD25+ and express high levels of FoxP3, TGF-β1 and intracellular CTLA-4. These cells become capable of functioning as Treg cells, suppressing proliferation of freshly isolated CD4+CD25- T cells in response to anti-CD3/anti-CD28 stimulation in vitro (Petty et al., 2008). These converted cells are also capable of functioning suppressive in vivo when co-transferred with CD4+CD25- T cells to RAG-/- mice, they can prevent the development of colitis (Chen et al 2003a; Fantini et al., 2004; Andersson et al., 2008). Furthermore, TGF-βR deficient CD4+CD25- T cells fail to be converted to Treg cells in the presence of TGF-β, demonstrating the critical role of TGF-β/TGF-βR signaling in T helper cell conversion (Liu et al., 2008). Additional studies have shown that exogenous IL-2 is required for TGF-β signal mediated CD4+CD25- T cells conversion (Davidson et al., 2007; Zheng et al., 2007).

However, the molecular changes in T helper cells that lead to conversion remain unclear. Considering that FoxP3 is the key regulator for Treg cell lineage development, it is of interest to determine if introduction of FoxP3 alone can convert T helper cells into Treg cells. Transfected FoxP3+GFP+ CD4+CD25- T helper cells became hypo-responsive to anti-CD3 stimulation and produced significantly lower level of IL-2, IFN-γ, IL-4 and IL-10 compared to control cells transfected with empty vector. These cells also expressed higher level of CD25, CTLA-4 and GITR than control cells. Importantly, they were able to dramatically suppress the IL-2 production and proliferative capability of fresh CD4+CD25- T cells in response to various stimuli (Hori et al., 2003). Therefore, FoxP3 alone appears to be sufficient to convert CD4+CD25- T helper cells into T regulatory cells.
4. Molecular mechanism of induction of FoxP3 in suppressed T helper cells

A number of studies have examined the molecular mechanism by which TGF-β/TGF-βR signaling induces FoxP3 in target T helper cells (Chen et al 2003b; Nakamura et al., 2004; Fogle et al., 2010a; Fogle et al., 2010b). TGF-β binds to a dimer of TGF-βRII, which recruits a dimer of TGF-βRI and thus forms a tetramer receptor complex. The two regulators Smad (RSMAD) proteins Smad2 and Smad3 are associated and phosphorylated by the TGF-βRI. The phosphorylated RSMADs have a high affinity for and form a heterodimer with Smad4 (Co-SMAD). This RSMAD/Co-SMAD complex translocates to nucleus where it binds to gene promoters and activates gene transcription including FoxP3. The TGF-β signaling is regulated by inhibitory SMADs (I-SMAD), SMAD6 and SMAD7. SMAD6 competes with RSMADs for binding to Co-SMAD, while SMAD7 prevents RSMADs from binding to TGF-βRI; therefore these two I-SMADs play a key role in negative regulation of the TGF-β signaling (Rubtsov et al., 2007). Using luciferase assay, it has been reported that FoxP3, as a gene repressor, down-regulates expression of Smad7, an inhibitory protein of TGF-β signaling, and thus renders FoxP3+ T cells more susceptible to TGF-β signaling. These data suggest that there is an auto-regulatory loop for maintaining FoxP3+ T cell homeostasis (Fantini et al., 2004; Rubtsov et al., 2007).

Additional experiments showed that while TGF-β signaling remains intact, manifested by normal phosphorylation of Smad3, no FoxP3 can be induced in TGF-β treated CD4+CD25- T cells cultured in the presence of anti-CTLA-4, suggesting that CTLA-4 ligation of CD80 is required for TGF-β-mediated FoxP3 induction (Chen et al., 2003a; Zheng
et al., 2006). Recently, using chromatin immunoprecipitation, it has been shown that Smad3 and Smad4 must bind to the FoxP3 enhancer for induction of FoxP3 in naive CD4+ T cells (Tone et al., 2008; Chen et al., 2010). However, as Smad proteins bind only to the FoxP3 enhancer and not the promoter, binding of Smads to DNA is usually weak, and there is a long lag time between phosphorylation of regulatory Smad proteins (which occurs immediately after ligation of TGF-βR) and production of FoxP3 mRNA, it has been argued that other co-activators are needed to activate FoxP3 transcription in suppressed/converted T cells (Chen et al., 2010). Studies have shown that T cell transcription factors such as STAT5, CREB/ATF, AP-1 and NFAT can bind to FoxP3 promoter and induce FoxP3 expression (Mantel et al., 2006; Burchill et al., 2007; Kim et al., 2007). Using a luciferase assay, it has been demonstrated that mutation of the AP-1 binding sequence in the FoxP3 promoter, which is adjacent to an NFAT binding element, greatly decreased the activity of the promoter (Mantel et al., 2006). Additional studies have shown that both NFAT2 and Smad3 binding to the FoxP3 enhancer are required for TGF-β-induced FoxP3 expression in T cells (Tone et al., 2008; Xu et al., 2010). Further studies have revealed that within FoxP3 promoter, there is an “enhanceosome” composed of a cluster of c-Rel-NFAT binding sites (Long et al., 2009; Ruan et al., 2009; Xu et al., 2010). These data suggest that NFAT2 may play a key role in induction of FoxP3. On the contrary, some other studies have shown that TGF-β signaling inhibits Tec kinase activity, Ca2+ influx, and impairs activation of NFAT proteins, indicating that NFAT activation is not associated with FoxP3 induction (Chen et al., 2003).
In summary, FoxP3 is critical for T helper cell anergy and conversion. More and more information have been collected, yet to fully understand the underlying mechanism of how FoxP3 is turned on, future investigation is needed.

E. The Role of NFAT in Treg-mediated T Helper Cell Suppression and Conversion

The primary effect of TGF-β/TGF-βRII signaling is to suppress IL-2 production and thus inhibit IL-2 dependent proliferation of T cells (Kehrl et al., 1986). FoxP3 is known to suppress IL-2 gene expression and proliferation of Treg cells, although the underlying molecular mechanism is ill defined. Recently it has been proposed that other transcription factors may interact with FoxP3 to induce T cells become anergic and gain regulatory phenotype and function. One of the key factors is NFAT2.

1. NFAT signaling in T cell activation

Since first identification as putative transcription factors present in the nuclear extract of activated T cells that bound to the human interleukin-2 (IL-2) promoter, NFAT proteins have been shown to play a key role in the immune system. NFAT binding sites are not only found in proximal promoters, but also distal regulatory elements of a large number of genes. Most cytokines produced by CD4+ T helper cells are influenced by NFAT activities (Shaw et al., 1988), and mice that are NFAT1 and NFAT2 double deficient have significantly impaired T cell and B cell immune responses (Peng et al., 2001).
The NFAT family has five members: NFAT1 (also named as NFATp or NFATc2), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3) and NFAT5. NFAT proteins are ubiquitously expressed by almost all types of immune cells, but NFAT1 and NFAT2 are predominantly expressed in mature T cells. Each NFAT protein has two or more alternatively spliced forms, which have variant N- and C- termini and a conserved core region composed of a NFAT-homology region (NHR) and a REL-homology region (RHR). The NHR has a transactivation domain containing many serine residues that are phosphorylated in resting T cells. It also includes the docking sites for calcineurin and NFAT kinase, which regulate the activation of NFAT proteins by determining the phosphorylation status of the serines. Although NHR domains among NFAT proteins are highly conservative, each of them also has its own features, suggesting that NFAT proteins may be regulated by overlapping but distinct sets of kinases depending on cell types and signal pathways that are activated (Hogan et al., 2003). The RHR is a highly conserved DNA-binding domain of the REL-family transcription factors and it also contains interacting regions for NFAT transcriptional partners, like AP-1, CEBP and FoxP3 (Chuvpilo et al., 1999; Chen et al., 1998; Baine et al., 2009).

All NFAT proteins, except for NFAT5, are regulated by calcium signaling. NFAT proteins exist mainly in an inactive phosphorylated state in the cytoplasm of resting T cells. The activation of NFAT proteins is induced by the engagement of receptors that are coupled to the calcium-signaling pathway, such as the antigen receptors that are expressed by T and B cells. Receptor engagement leads to the activation of phospholipase C-γ (PLC-γ), which
cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) into inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses through the cytosol and binds to IP3 receptor, particular calcium channels in the endoplasmic reticulum (ER). This leads to an increase in the cytosolic concentration of calcium. Calcium binds calmodulin, which in turn activates the calmodulin-dependent phosphatase calcineurin. NFAT proteins are dephosphorylated by activated calcineurin, resulting in their nuclear translocation and the induction of NFAT-mediated gene transcription (Feske et al., 2003; Hogan et al., 2003).

Among all 5 NFAT proteins, NFAT2 is considered the most important one that participates in cell activation, because the shortest isoform of NFAT2 (NFATc/A) is the only one that is subject to a positive autoregulatory loop. In naïve T cells, NFAT1 is the main isoform that pre-exists in the cytoplasm. When T cells are activated, pre-existing NFAT1 can act together with constitutively expressed low levels of NFAT2 to turn on expression of the inducible NFAT2 isoform NFATc/A, which is generated through a distinct promoter. Once the level of NFAT2 reaches a threshold; the self-sustaining positive autoregulatory loop works to maintain high levels of NFAT2 expression and protein activities. NFATc/A lacks C-terminal domain and has an alternative N-terminal domain, which enables NFATc/A to maintain cell activation and survive from programmed cell death (Chuvpilo et al., 1999; Chuvpilo et al., 2002; Zhou et al., 2002; Hogan et al., 2003; Serfling et al., 2006).
2. NFAT signaling in T cell anergy

As discussed above, NFAT proteins are considered as important factors for T cell activation. Therefore, it was surprising to find T cell and B cell hyperproliferation, aberrantly elevated immune response against *Leishmania major*, dysregulation of IL-4 production, and abnormally elevated serum IgE and IgG1 levels in NFAT deficient mice, suggesting that NFAT proteins also exert negative regulatory effects in immune responses (Xanthoudakis et al., 1996; Hodge et al., 1996; Ranger et al., 1998). Thus NFAT appears to not only regulate expression of cytokine genes that are responsible for T cell activation, but also control the expression of genes that program T cells anergy. The dual role that NFAT proteins play in T cell activation and anergy may be due to the capability of NFAT to form complexes with different transcription factor partners in response to different stimuli. Recently, NFAT1 has been shown to form a homodimer, which is critical for T cell anergy, by turning on expression of two anergy-inducing genes, *grail* and *caspase-3* (Soto-Nieves et al., 2009). It is not clear if NFAT itself is sufficient to repress gene expression in anergic T cells or if it cooperates with other transcription factors to do so.

3. The role of NFAT and FoxP3 in IL-2 suppression

T cell anergy is manifested by impaired IL-2 production, and as discussed above, transcription factor FoxP3 is known to suppress IL-2 expression. It has been shown that NFAT proteins are important for T cell anergy induction, but it is unclear as to what specific
role NFAT plays in FoxP3 mediated IL-2 suppression. At least three different mechanisms have been proposed.

One proposal states that FoxP3 suppresses IL-2 expression by cooperating with NFAT. Foxp3 is shown to associate with the REL family transcription factors, NFAT and NF-κB, and block their ability to induce the expression of key cytokines in T helper cells. Although the presence of FoxP3 does not affect the nuclear translocation or DNA binding activities of NFAT and NF-kB, their transcriptional abilities are blocked (Bettelli et al., 2005). Moreover, it has been shown that FoxP3 interrupts the formation of the AP1-NFAT transcription complex by competing with AP-1 for binding to NFAT, and the resulting FoxP3/NFAT complex inhibits IL-2 expression. When FoxP3 is mutated at its NFAT binding region, it no longer can suppress IL-2 expression. Accordingly, a FoxP3 binding site adjacent to the NFAT:AP-1 binding site on the IL-2 promoter has been identified. Additional experiment has shown that interaction between NFAT2 and Foxp3 is required for the suppressive ability of Treg cells (Wu et al., 2006). Chromatin immunoprecipitation assay demonstrates that although FoxP3 binds to a large set of gene promoters in both unstimulated and stimulated T cells, it represses gene expression more extensively in stimulated T cells, and the majority of FoxP3 binding sites are adjacent to consensus DNA binding sequences for NFAT proteins (Marson et al., 2007). Interestingly, although FoxP3 can constitutively bind to the promoters of CD25, CTLA4 and GITR, its binding to the IL-2 promoter is TCR signal-dependent and can be abolished by cyclosporine A treatment. Cyclosporine A can form a complex with cyclophilin, inhibit activation of calcineurin, and block the activation of NFAT signals (Chen
et al., 2006; Marson et al., 2007). These data suggest that NFAT is necessary for FoxP3 mediated IL-2 gene suppression.

A second mechanism by which FoxP3 may interrupt T cell activation proposes that NFAT2 expression is suppressed by FoxP3. Microarray analysis of cells transduced with wild type FoxP3 or mutant FoxP3 which lacks the forkhead DNA binding domain demonstrates that NFAT2 is an early target gene that is repressed by FoxP3 (Torgerson et al., 2009). Chromatin immunoprecipitation assay shows that FoxP3 can directly bind to the NFAT2 promoter and inhibit inducible expression of NFAT2 mRNA, resulting in less NFAT2 available for binding to the downstream cytokine gene promoters, thus reducing IL-2 production in activated T cells and rendering the cells anergic. In support, additional experiment has shown that exogenous expression of NFAT2 can restore IL-2 production in FoxP3+ T cells (Torgerson et al., 2009).

Some investigators have proposed a third mechanism, stating that FoxP3 itself is a putative repressor which binds to the IL-2 promoter and is alone sufficient for inhibiting gene expression by preventing promoter remodeling and transactivator binding (Schubert et al., 2001; Chen et al., 2006; Wells 2009). It has been reported that FoxP3-mediated gene suppression is trichostatin A sensitive, suggesting that FoxP3 may inhibit gene expression by recruiting HDAC. Besides, FoxP3 can also be associated with HMT, which can methylate lysine 27, resulting in silence of genes (Wells 2009).

Moreover, an analysis of the function of CD4-CD25+ Treg cells in mice that are deficient in NFAT1 and NFAT4 has shown that, even though these mice have functional
Treg cells, their CD4+CD25- cells are resistant to suppression by wild-type Treg cells (Bopp et al., 2005). Therefore, it seems that NFAT proteins play a key role in Treg cells-mediated immune suppression, not only through maintain Treg cell suppressive activity, but also render target T helper cells susceptible to suppressive signaling. Such evidence coordinates with the hypothesis that NFAT is important to IL-2 gene suppression in anergic T cells, both Treg and T helper.

In summary, FoxP3 is involved in suppression of cytokine gene expression in Treg cells as well as suppressed T helper cells, and NFAT proteins are involved in this complicated gene regulation process, although the underlying mechanism remains controversial. Most reports so far have used over-expression of FoxP3, which may overwhelm the endogenous cell signal. Thus further investigation that address the role of FoxP3 and NFAT2 in Treg cells anergy and suppressive function under physiological condition is needed.

Our aim is to examine the role of feline NFAT2 in Treg cell mediated T helper cell suppression, providing an explanation for immunodeficiency in chronic FIV infection. We aim at identifying the role of NFAT2 and FoxP3 in IL-2 expression in target CD4+CD25- T helper cells suppressed by CD4+CD25+Treg cells as effector cells.
III. MATERIALS AND METHODS

Cats

Specific pathogen free (SPF) cats were obtained from Liberty Research, Inc. (Waverly, NY), and housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. FIV infected cats were housed separately from FIV negative control cats. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

FIV Infection

The NCSU1 isolate of FIV was originally obtained from a naturally infected cat and has been described in detail elsewhere (Tompkins et al., 1991). Virus was grown as a single tissue culture passage in FCD4E cells (an IL-2 dependent feline CD4+ cell line) as described previously (Davidson et al., 1993). Cats were infected with $1 \times 10^5$ TCID$_{50}$ of cell-free virus and controls were sham inoculated with an equal volume of cell-free CD4E tissue culture media. At the time of this study, FIV+ cats had been infected for at least 1 year.

Lymph Node Biopsies and Purification of Lymphocyte Subsets

Lymphocytes were obtained either by biopsy of a peripheral lymph node or from cats following euthanasia. Lymph node biopsies were performed as described elsewhere (Mexas et al., 2008). Cells were then processed into single cell suspensions as described previously (Levy et al., 1998). Cells were stained with anti-CD4-PE and anti-CD25-FITC and sorted
into CD4+CD25- and CD4+CD25+ populations using a MoFlo cell sorter. Sorted populations were greater than 95% pure. For some experiments a third population was obtained that was depleted of CD4+ and CD8+ cells. This population was used as a source of antigen presenting cells (APC) in Treg cell suppression assays. (See detailed description below).

**Cell Culture and Activation**

T cells were cultured in CTL media (RPMI 1640 supplemented with 10% heat-inactivated FBS, 2mM L-glutamine, 0.01M Hepes, 0.075% NaBicarb, 100 units/ml Pen/100μg/ml Strep, 5 x 10^{-5} M β-ME and 0.1mg/ml Cipro). To activate lymphocytes, cells were cultured at 1 x 10^6/ml and treated with Ionomycin (1.5μM) (Sigma) alone; PMA (20ng/ml) (Sigma) plus Ionomycin (1.5μM); ConA (10mg/ml) or ConA (10mg/ml) plus Ionomycin (1.5μM) for various times. Non-treated cells were used as controls.

**Reverse Transcription and Real-Time PCR**

Relative quantification of FoxP3, IL-2, and NFAT2 mRNA was done by reverse transcription and real-time PCR. Total mRNA was extracted from 10^6 cells using RNeasy plus Mini Kit (Qiagen) and eluted in a final volume of 50μl RNase free water per reaction. 20μl of the RNA were reverse transcribed into cDNA using random primers and the Promega reverse transcription system according to manufacturers’ instructions. Reactions were incubated at 25°C for 10 min, 42°C for 15 min, 95°C for 5 min, 5°C for 5min, and 42°C for
60min before holding at 5°C. QuantiTect SYBR Green PCR kit (Qiagen) was used for quantification of FoxP3, IL-2, NFAT2 mRNA levels. 1.5μl of the cDNA obtained was used as template in each well. Reactions were run in triplicates in 96-well plates. All reactions were carried out using the same PCR program. They were denatured at 95°C for 20sec, annealed at 58°C for 20sec and elongated at 72°C for 30sec. Cycles were repeated for 40 times. GAPDH mRNA level was used as control. Data were calculated using delta delta Ct method (Livak et al., 2001). Specific primers for feline FoxP3, IL-2, NFAT2, and GAPDH amplification are shown in Table 1.

Table 1. Primers used in real-time PCR reactions for quantitation of feline FoxP3, IL-2 and NFAT2 mRNA levels.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>FoxP3</td>
<td>5’- cgggctgccacctgga-3’</td>
<td>5’- gcgtctttccgggttgc -3’</td>
</tr>
<tr>
<td>IL-2</td>
<td>5’- acagtgcacctgcagtc-3’</td>
<td>5’- ccggagagttggggtcag -3’</td>
</tr>
<tr>
<td>NFAT2</td>
<td>5’- accectacccggtcccgccagc-3’</td>
<td>5’- gagctggtgctcagctc-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’- ggagaaggctggtccac-3’</td>
<td>5’- ggtgcaagggacctgta-3’</td>
</tr>
</tbody>
</table>

**NFAT2 cDNA PCR**

Feline NFAT2 exons fragments were found in Genbank. Exon junctions were identified through PCR using cDNA generated the same way as described above. Primers are listed in Table 2.

Table 2. Primers used in PCR for identification of feline NFAT2 exon junctions.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>ex3-ex4</td>
<td>5’- catecggaaggggtcctctgc-3’</td>
<td>5’- gaggacccagtccagtc -3’</td>
</tr>
<tr>
<td>ex4-ex5</td>
<td>5’- tcaagagacggggaacctcg-3’</td>
<td>5’- caggtgctgcctgtagtc -3’</td>
</tr>
<tr>
<td>ex5-ex6</td>
<td>5’- cccgtgtcaccacgcctgagcag -3’</td>
<td>5’- ccttgccgacgctgcagtc -3’</td>
</tr>
<tr>
<td>ex6-ex7</td>
<td>5’- ccctgtgtaacctcgaac-3’</td>
<td>5’- gacetttctctccggcag -3’</td>
</tr>
<tr>
<td>ex7-ex9</td>
<td>5’- ttctgtcggccataatcttcgc -3’</td>
<td>5’- ggcgttatctgaccaccaacg -3’</td>
</tr>
</tbody>
</table>
Purification of Peripheral Blood Mononuclear Cells

28ml of blood were collected via jugular venipuncture into vacutainer tubes containing EDTA anticoagulant. Blood was spun at 12000rpm for 10 min and plasma was separated. Blood was diluted to twice the original volume in HBSS with 0.03% EDTA. PBMCs were separated using Ficoll-Histopaque-1077 (Sigma). 3ml of blood were layered above 3ml of Ficoll, followed by centrifugation at 400g for 30 min. Cells at the interface were collected, washed in HBSS and re-suspended in CTL media.

Western Blot

PMA+Ionomycin treated or non-treated feline PBMCs, feline kidney cells (CRFK) and human Jurkat cells were lysed in NP-40 lysis buffer (25ml 1M Tris-HCl, pH7.5; 15ml 5M NaCl; 2.5ml NP-40; 1.05g NaF; 452.5ml H2O) overnight at 4°C. Cell lysates were loaded onto SDS-Page gel (Invitrogen) followed by separation and transfer to PVDF membrane. The blots were incubated with anti-human NFAT2 (sc-13033, Santa Cruz Biotechnology, Inc.), followed by HRP-conjugated goat anti-rabbit IgG (sc-2004, Santa Cruz Biotechnology, Inc.) and detected by chemiluminescence (Pierce).

Chromatin Immunoprecipitation Assay

20x10^6 cells were fixed by addition of 37% formaldehyde to 1% final concentration, and were incubated on shaker for 10 minutes at room temperature. 2.5M glycine was added
to cells to 1% final concentration, and cells were shaken for an additional 5 minutes. Cells were pellet at 4°C at 1100rpm for 10min, washed once with 10ml PBS and re-pelleted. 5µl of 100mM PMSF and 5µl of 100 x PIC (Sigma) were added and the pellet stored at -80°C before use.

Frozen pellets from 40x10⁶ cells were pooled, resuspended in 1ml ice-cold lysis buffer (100µl 1M Tris pH7.5; 30µl 1M MgCl₂; 20µl 5M NaCl; 500µl 10%NP40; 9.35ml H₂O) plus 5 µl each of 100mM PMSF and 100xPIC and incubated on ice for 30 minutes. Cells were transferred to 1.7ml microfuge tubes, and nuclei were pelleted at 4°C for 10min at 5000rpm. Supernatant was removed and nuclei resuspended to a final concentration of 0.5mg/ml in MNase buffer (500µl 1M Tris pH7.5; 40µl 1M MgCl₂; 10µl 1M NaCl; 3.33ml 1M Sucrose; 50µl 1M Na Butyrate; 6.1ml H₂O) plus 2.5µl each of 100mM PMSF and 100xPIC, and incubated for 5 min at 37°C. 10U/ml MNase was added to digest nuclei by incubating for 7 min at 37°C with periodical vortex. 20µl/ml 0.5M EDTA was added to stop digestion, and nuclei were put on ice for 10 min then pelleted at 4°C for 10min at maximum speed. Supernatant was aliquoted and stored at -80°C before use.

5µl each of Protein A and Protein G magnetic beads (Invitrogen) were used per IP. Beads were washed twice in 100µl 1xRIPA/ssDNA (1ml 10xRIPA; 1ml 5mg/ml salmon sperm DNA; 8ml PCR grade water), resuspended in 50µl 1xRIPA/ssDNA plus 0.5µl each of 100mM PMSF and 100xPIC per IP, and incubated with separate Abs for 1h at 4°C. 10µg anti-human NFAT2 (sc-13033X, Santa Cruz) or 2µg of control rabbit IgG were used per IP.
Ab-beads conjugates were washed 3 times in 100µl 1xRIPA/ssDNA, and then resuspended in 80µl 1xRIPA/ssDNA plus 1µl each of 100mM PMSF and 100xPIC per IP and aliquoted to different tubes. 20µl chromatin was added to each tube and rotated for 2h at 4°C. Conjugates were washed 4 times in 180µl 1xRIPA plus 2 times in 180µl TE, pH8.0, and then resuspended in 100µl fresh elution buffer (100mM NaHCO₃/1%SDS), followed by 15 min light vortex. Tubes were placed on a magnet, and media were transferred to fresh tubes with 2µl 5M NaCl added. Reverse cross-linking was done by incubating tubes at 95°C for 15 min. 2µl 1mg/ml proteinase K was added and incubated at 37°C for 1h. DNA was purified using buffer PN (Qiagen) and buffer PE (Qiagen), and was eluted in 60µl of water. DNA samples were subject to real-time PCR. Specific primers for detecting NFAT binding to the feline IL-2 promoter are shown in Table 3. (A modified ChIP protocol is discussed in detail in Appendix B)

Table 3. Primers used in ChIP assay for detection of feline NFAT2 binding to the feline IL-2 promoter.

<table>
<thead>
<tr>
<th></th>
<th>forward</th>
<th></th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 1</td>
<td>5’- catccattcagtcattagggg -3’</td>
<td>5’- ctggaaaaatcttatggtggtc -3’</td>
<td></td>
</tr>
<tr>
<td>Set 2</td>
<td>5’- aactgccacctaagtgggc 3’</td>
<td>5’- tacctgttgccaaaaagcttacc -3’</td>
<td></td>
</tr>
<tr>
<td>Set 3</td>
<td>5’- agccactttgtatatccccaccccc -3’</td>
<td>5’- agcgtcacttagtgcagttg -3’</td>
<td></td>
</tr>
</tbody>
</table>

CD4+CD25+ Treg Cell Suppression Assay

Cats were euthanized, and all peripheral lymph nodes were collected and processed into a single cell suspension. Lymphocytes were stained with anti-CD4-Biotin-Strep Avadin PerCP, anti-CD25-FITC, and anti-CD8-PE. CD4+CD25- and CD4+CD25+ T cell
populations and CD4-CD8- cells were purified by MoFlo high speed sorting. 2x10^6 CD4+CD25- cells (target cells) were cultured with DiD Vybrant stained CD4-CD8- cells (antigen presenting cells) at a 1:1 ratio and treated with ConA (10mg/ml) for 1 hour. Cells were pelleted at 1500rpm for 10 minutes, and then resuspended in 2ml CTL media. 2x10^6 CD4+CD25+ cells (suppressor) were stained with DiD Vybrant stain (Molecular Probe) and added to the culture. A control culture consisted of target cells and APCs was co-cultured with DiD Vybrant stained CD4+CD25- cells. Cells were cultured for various times (3h, 5h, 1d, 2d and 4d) then DiD+ cells were removed by MoFlo sorting. 10^6 DiD- cells were collected and RNA extracted and subjected to Real-time PCR for measurement of IL-2, FoxP3 and NFAT2 mRNA levels. Culture supernatant was collected for quantification of IL-2 protein by ELISA.

**TGF-β Suppression Assay**

FIV+ cats were euthanized, and all peripheral lymph nodes were collected and processed into a single cell suspension. Lymphocytes were stained with anti-CD4-PE and anti-CD25-FITC and CD4+CD25- cells were purified by MoFlo high speed sorting. 1x10^6 CD4+CD25- cells were cultured in 1ml CTL media with ConA (10mg/ml) for 1 hour followed by addition of recombinant human rTGF-β1 (10ng/ml). Control groups consisted of ConA stimulated CD4+CD25- cells were cultured in the absence of rTGF-β1. Cells were cultured for various times (3h, 6h, 1d, 2d, 3d and 4d) then 10^6 cells from each group were harvest for RNA extraction and Real-time PCR analysis. Culture supernatant was collected.
for quantification of IL-2 protein by ELISA. In some experiments, cells were collected after 3 hours of culture and fixed for a ChIP assay.

**IL-2 ELISA**

Culture supernatants from the Treg cell suppression assays and TGF-β suppression assays were analyzed for IL-2 production using the DuoSet ELISA Development kit (R&D system) according to manufacturer’s manuscript. Briefly, a 96-well flat bottom plate was coated overnight with 100µl of anti-feline IL-2 capture antibody. The plate was washed 3 times with wash buffer (0.05% Tween® 20 in PBS, pH7.2-7.4) and blocked with 300µl of reagent diluent (1%BSA in PBS, pH7.2-7.4) for 1h at room temperature. 100µl of culture supernatant or serial diluted standards were added and the plate incubated for 2h at room temperature. The plate was then washed and 100µl of detection antibody were added and incubated for 2h at room temperature. Following a wash, 100µl of Streptavidin-HRP were added to each well, and incubated for 20 minutes in the dark at room temperature followed by addition of 100µl of substrate solution (1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine)). After 20 minute incubation in the dark at room temperature, 50µl of stop solution (2N H₂SO₄) was added to each well. Optical density of each well was immediately determined, using a microplate reader set to 450nm (BIO-TEK). Software used was KC Junior. Each sample was run in triplicate.
Statistical Analysis

Mann-Whitney rank sum tests were performed. P values of less than 0.05 were considered significant.
IV. RESULTS

A. Identification and Validation of Feline NFAT2

As NFAT2 is a major transcriptional activator of the IL-2 promoter, and as Treg cells suppress IL-2 transcription in CD4 T helper target cells, it is possible that Treg cell-mediated suppression of IL-2 transcription in T helper target cells is due to the inhibition of NFAT2 expression. To address this question, it was necessary to first identify a homologue of NFAT2 in the cat.

At least eleven exons have been identified in human and mouse NFAT2 gene respectively, and multiple NFAT2 transcripts have been reported (Macian 2005). The simplified schematic structure of NFAT2 gene is shown in Figure 1. Transcription of the NFAT2 gene is controlled by two different promoters, P1 and P2, and two poly A sites, pA1 and pA2 (Figure 1; Chuvpilo et al., 2002). The weak P2, which lies in front of exon 2, directs constitutive synthesis of longer isoforms of NFAT2 in human and murine primary T cells. The strong promoter P1, which lies in front of exon 1, is activated in effector T cells and directs synthesis of inducible NFAT2/A. Based on the published nucleotide sequences of human and mouse NFAT2 (NCBI Reference Sequence: NM_172390.1 and NM_001164109.1), several feline exon fragments that share high identities to the human and mouse gene were found. Exon junctions were then identified by PCR using primers that span different exons. Primer sequences are listed in Table 2. The schematic structure of the identified partial feline NFAT2 cDNA is shown in Figure 1. PCR products were sequenced and compared to sequences from Genbank database, and the results showed high identities.
Missing or mismatched nucleotides in exon junctions are highlighted in red (Figure 2a). The predicted partial nucleotide sequence and amino acid sequence of feline NFAT2 are shown in Figure 2b. Real-time PCR product sequence is compared with the predicted feline cDNA sequence (Figure 2c). 100% sequence match indicates that the PCR product is feline NFAT2, and this pair of primers can be used for detecting mRNA levels of feline NFAT2 in the following experiments.

Figure 1. Identification of partial feline NFAT2 cDNA. NFAT2 gene has at least eleven exons. The inducible NFAT2/A isoform contains eight exons. Based on the published cDNA sequence of human and mouse NFAT2/A, several exons of feline NFAT2 (partial ex3, ex4, ex5, ex6, ex7 and ex9) were found in GenBank. The exon junctions and ex8 were identified by PCR using primers that span different exons. Pairs of primers are shown in different color. Primer sequences are listed in Table 2. The pair of primers used for following real-time PCR analysis is indicated in black, and the primer sequence is listed in Table 1.
To further confirm this gene we identified is the NFAT2 homolog, protein alignment was performed among different species. Although the 1-85 amino acid sequence at the N-terminal of the target protein is missing, alignment shows that this feline protein shares high identities with human and mouse NFAT2. Isoforms of NFAT proteins are variable at N- and C- termini, yet contain a highly conserved core region, which consists of two tandem domains, Rel-homology region (RHR) and NFAT-homology region (NHR) (Park et al., 1996). RHR contains a Rel-similar DNA binding motif (RAHYETEG, Figure 2b and 2d, highlighted by overline and critical residues are shaded pink) which specifies the DNA binding characteristic of NFAT proteins (Jain et al., 1995). RHR also contains residues that interact with partner transcription factors, such as Fos and Jun (Figure 2b and 2d, residues highlighted by yellow and turquoise). Within NHR, there are two calcineurin-docking sites (Figure 2b and 2d, shaded green). The PxIxIT motif, which is consensus among all NFAT proteins, is highlighted by arrows, and the other site has only been found in NFAT2 and NFAT4. NHR also contains several serine-rich regions (SRR) and SPxx motifs that contain numerous serine residues that can be phosphorylated or dephosphorylated by kinase or phosphatase respectively, and the phosphorylation status of these residues determines the activity of NFAT proteins (Figure 2b and 2d, SRR and SPxx motifs are shaded blue, serine residues are indicated by stars) (Kiani et al., 2000). Moreover, there is a nuclear localization signal that locates in NHR (Figure 2b and 2d, the core of the NLS is shaded in red). All of these functionally critical motifs in feline NFAT2 are identical or highly conserved to human
and mouse NFAT2, suggesting that the protein encoded by the gene we identified in cats is a homolog of NFAT2.
**ex3-ex4:**
CATTCCGGAAGGGGCCTTCTGCGACCCATCTTCTTCGCGCATCTGCAGTCGGTGCCGCAGCACCCCTACCAGTGGGCCAGGCCCAGGTCCC------
CATCGGGAAGGGCCCTTCTGCGACCCATCTTCTTCGCGCATCTGCAGTCGGTGCCGCAGCACCCCTACCAGTGGGCCAGGCCCAGGTCCC
-------CCAGCCCGTCCCTCCCCGCTGGACTGGCAGGTGCCC(sequence from database)
CTTCGCCGCCGTCCTCCTCCCCGGCTGGACTGGCAGGTGCCC(sequencing result)

**ex4-ex5:**
CTACGAGACCGAGGGGGCTGACGCTGTGAAGGCGTCGGCCGGAGGACACCCCAGCACCTGACCTGTCATGCCG
ACGCGCGTACGTACAGCTGTTCATCGGGACCGCTGACGACCGCCTGCTGAGACCCCACGCCTTCTTACCAGGTGCATCGG
ACGAGCGCGTACGTACAGCTGTTCATCGGGACCGCTGACGACCGCCTGCTGAGACCCCACGCCTTCTTACCAGGTGCATCGG
ATACACGGCGAACAGCGT(sequence from database)
ATACACGGCGAACAGCGT(sequencing result)

**ex5-ex6:**
GTACAGACGAGCGCCATCTCTCCAACACCAACAGTCTGTTGGAGACCCACCCCTTGCTGCGCCCAAGGAGAT--TGACTG
GTACAGACGAGCGCCATCTCTCCAACACCAACAGTCTGTTGGAGACCCACCCCTTGCTGCGCCCAAGGAGAT--TGACTG
CGCCGGGATCTCTGAACTCGGACATCGACGGCTCCAAAGG(sequence from database)
CGCCGGGATCTCTGAACTCGGACATCGACGGCTCCAAAGG(sequencing result)

**ex6-ex7:**
CCCTGCAGTGCGCTCAACCACACTTGAGCCTCAGCGGAGGTCCCGGCCTTCTGTTGGAGAAGCAGAGCGCCTCG
CCCTGCAGTGCGCTCAACCACACTTGAGCCTCAGCGGAGGTCCCGGCCTTCTGTTGGAGAAGCAGAGCGCCTCG
AGCCTGCCGCCCTGCCGAGGGTAAAGATGGTC(sequence from database)
AGCCTGCCGCCCTGCCGAGGGTAAAGATGGTC(sequencing result)

**ex7-ex9:**
TCTCTGTCCGGCCTTCTGCAGAGACTCTCCAGGCTATTTTCTGTTGGAGAAGGACACCAG------------------------
TCTCTGTCCGGCCTTCTGCAGAGACTCTCCAGGCTATTTTCTGTTGGAGAAGGACACCAG------------------------
GAGACAAAAGAGACAGGAGGCCTCTGGCAGAGCCGAGGATGCTCGATTGGGACTTCGGCCTCGCCCGCCCTATCTGCTGCAGAGGAGCC
GAGACAAAAGAGACAGGAGGCCTCTGGCAGAGCCGAGGATGCTCGATTGGGACTTCGGCCTCGCCCGCCCTATCTGCTGCAGAGGAGCC
GAGACAAAAGAGACAGGAGGCCTCTGGCAGAGCCGAGGATGCTCGATTGGGACTTCGGCCTCGCCCGCCCTATCTGCTGCAGAGGAGCC
GAGACAAAAGAGACAGGAGGCCTCTGGCAGAGCCGAGGATGCTCGATTGGGACTTCGGCCTCGCCCGCCCTATCTGCTGCAGAGGAGCC

**Figure 2a. Sequencing results of PCR products identifying feline NFAT2 cDNA exon junctions.**
PCR products sequences are compared with sequences from Genbank database. Missing and mismatched nucleotides are highlighted in red.
Figure 2b. Partial predicted nucleotide sequence and amino acid sequence of feline NFAT2. Critical motifs are shaded different colors: calcineurin docking sites (green); SRRs and SPxx motifs (blue); the core of NLS (red); residues contact with DNA (pink), Fos (yellow) and Jun (turquoise). Rel-similar DNA binding motif is highlighted by black overline. qPCR primers are shown in frame.
NFAT2 qPCR product sequencing result:

```
ACCCCTACCAGTTGGGCCAGGCCCCAGGCTCCCAGTCCCTACGCAGGCCCCTGGCTCCCTCCCCGCCCTGGACTGGCAGGTGCCCTCG
```

CGCTCGGGCCCTACGAGCGGTGCAGACCTCTACGGGACGCGACGGCTC (predicted sequence)

CGCTCGGGCCCTACGAGCGGTGCAGACCTCTACGGGACGCGACGGCTC (sequencing result)

**Figure 2c. Real-time PCR product sequencing result.**
PCR product sequences are compared with the predicted NFAT2 cDNA sequences.
**Figure 2d. Protein alignment of predicted partial feline NFAT2 with human and mouse NFAT2.** Identities are shaded black, and similarities are shaded grey. Two calcineurin-binding regions are shaded green, the consensus PxIxIT motif is indicated by arrows; serine-rich regions and SPxx motifs are shaded blue, phosphorylated residues are indicated by stars; the core of the nucleus localization signal (NLS) is shaded red; NHR and RHR are highlighted by light orange and violet bar respectively. DNA binding motif (RAHYETEG) is highlighted by black overline. Residues that participate in contacts to DNA, Fos and Jun are shaded pink, yellow and turquoise respectively.
To determine if this feline NFAT2 gene transcription is regulated the same way as human and mouse NFAT2, primers were designed that span exon 3 and exon 4 of the cDNA sequence for analysis of mRNA expression (Figure 1 and Table 1). Human NFAT2 is expressed at basal levels in resting/naive T cells, but can be highly induced in activated T cells (Hogan et al., 2003). To test the inducibility of NFAT2 in feline T cells, purified CD4+CD25- T helper cells from FIV- cats were incubated with PMA (20ng/ml) and Ionomycin (1.5µM) (PMA/I) for various times, and NFAT2 mRNA expression was analyzed by quantitative real-time PCR. The NFAT2 mRNA level increased and peaked at 3 hours of PMA/I treatment, remained high at 6 hours, and returned to basal level after 23 hours (Figure 3a). To verify if feline NFAT2 is primarily regulated by calcium signaling (calcium/calcineurin pathway) as is human NFAT2, T helper cells from FIV- cats were treated with Ionomycin alone. NFAT2 mRNA levels were also elevated and peaked at 3 hours of Ionomycin stimulation. However, the level of mRNA was not as great as with combined PMA and Ionomycin treatment and it declined by 6 hours (Figure 3b), suggesting that both the PMA and calcium signaling pathways are important for sustained NFAT2 transcriptional activation. Notably, the primers we chose for NFAT2 mRNA expression analysis span exon 3 and exon 4, which are located in the highly conserved NHR region of NFAT2, thus we are measuring the total mRNA levels of all NFAT2 isoforms, including constitutively and inducibly expressed NFAT2.

In this part, we have found a NFAT2 homolog in cats. The partial cDNA of this feline NFAT2 is identified and the protein alignment demonstrates that feline NFAT2 is highly
conserved with human and mouse NFAT2. The transcription of feline NFAT2 gene is highly inducible in activated T cells. Feline NFAT2 is regulated by the same PKC and calcium signaling pathways as human and mouse NFAT2.

**Figure 3. Induction of feline NFAT2 mRNA expression.**
Lymph node cells from an FIV- cat were stained with anti-CD4-PE and anti-CD25-FITC. The CD4+CD25- T cell population was purified using a MoFlo high speed sorter. Cells were cultured at a concentration of 10^6 cells/ml and treated with a) PMA (20ng/ml) and Ionomycin (1.5µM); or b) Ionomycin (1.5µM) for different times. Non-treated cells were used as control groups. RNA was extracted from 10^6 cells from each group and NFAT2 mRNA analyzed by Real-time PCR. Expression of the housekeeping gene GAPDH was used as a control, and results were normalized to untreated control cells using 2^(-ΔΔCt). Error bars indicate ± S.D. of triplicate PCR reactions. Data shown is representative of 2 independent experiments.
B. The role of feline NFAT2 in IL-2 expression

NFAT2 was first reported as a putative IL-2 gene transcription activator in human T cells. As we are the first to identify the NFAT2 homologous gene in cats, we wished to determine if this feline NFAT2 plays a key role in activating IL-2 gene transcription in T cells as does human and mouse NFAT2.

Association of NFAT2 and IL-2 mRNA expression in activated feline T helper cells

As NFAT2 expression is associated with IL-2 transcription and dependent on the same signaling pathways (Ca$^{2+}$/PKC) in activated T cells, studies were designed to determine if a correlation existed between activation of NFAT2 and IL-2 transcription. Purified CD4+CD25- T cells from FIV- cats were incubated in media alone or with PMA/I for 3 hours and total mRNA extracted. NFAT2 and IL-2 mRNAs were measured by quantitative real-time PCR. Both NFAT2 (Figure 4a) and IL-2 (Figure 4b) mRNA transcription increased significantly in activated cells as compared to controls, suggesting that there is a correlation between NFAT2 and IL-2 transcriptional activation in feline T helper cells.
Figure 4. Correlation between NFAT2 and IL-2 mRNA induction in activated feline T helper cells.

Purified CD4+CD25- lymphocytes from an FIV- cat were cultured at a concentration of $10^6$ cells/ml and treated with PMA (20ng/ml) and Ionomycin (1.5µM) for 3 hours or left untreated as control. RNA was extracted and analyzed by Real-time PCR for a) NFAT2, and b) IL-2 mRNA expression. Expression of housekeeping gene GAPDH was used as control, and results normalized to untreated control cells using $2^{-\Delta\Delta Ct}$. Error bars indicate ± S.D. of triplicate PCR reactions. Data shown is representative of 2 independent experiments.
Analysis of NFAT2 protein binding at the feline IL-2 promoter site

To confirm a role for NFAT2 in IL-2 transcription in activated feline T cells, the binding of NFAT2 to the IL-2 promoter was analyzed by ChIP assay. It has been reported that the consensus binding sequence for human and murine NFAT proteins is GGAAAA (Rooney et al., 1995). Using the online Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess), 3 potential NFAT binding sites in the feline IL-2 promoter were found (Figure 5). One is located at -104 bp to -97 bp upstream of the transcription start site and is adjacent to an AP-1 binding site. The other two are located at -147bp to -140bp and -294bp to -287bp respectively. NFAT2 protein was analyzed for its binding to the feline IL-2 promoter.
-500bp
cattgtgtagatcaaaaggtaaaaaccatcctgaacaggaaccaatattcttctgtgtatcaacaaatctaa
aattttatcttttgcaactatatttactcttgtccaccatacaccacagtgctcaccatgttcaaacacagttttatga
caagagagaaaaattttcatgagccacttttgtatccccccccccttaaagagagggagggagaaaaatggttcat
agagaagggctttaggtgttgtcgaattacaactgccccactttaagaagagggagggagaaaaat
acatccattcagtcggtttatggggtttaagaatctccaaagagtcatacagaagagaaaaat
gctttttgccacacaggtgagatttttttcttgtaatat
-1bp

consensus NFAT binding site 1: -104bp to -97bp (bold and underlined) which is adjacent to AP-1 binding site (shown in gray and italic)

consensus NFAT binding site 2: -147bp to -140bp (bold and underlined)

consensus NFAT binding site 3: -294bp to -287bp (bold and underlined)

**Figure 5. Predicted NFAT binding sites in the feline IL-2 promoter.**
-500bp to -1bp 5’ upstream sequence of the feline IL-2 promoter is shown. The predicted NFAT binding sites are underlined and shown in bold. NFAT binding site 1 is adjacent to the AP-1 binding site (shown in gray and italic).
Identification of antibody that recognizes feline NFAT2

Prior to performing a ChIP assay for detection of NFAT2 binding to the IL-2 promoter in feline cells, it was necessary to identify and validate antibody that recognizes feline NFAT2. A polyclonal rabbit anti-human NFAT2 antibody was selected based on the following criteria: 1) the epitope does not map to the internal region of the protein; 2) does not recognize other NFAT proteins; 3) recognizes not only human but murine and rat NFAT2; 4) has been used for both Western blot and ChIP assays (Balkan et al. 2009). Feline PBMCs and a kidney derived fibroblast cell line (CRFK) were used for feline NFAT2 detection, and Jurkat, a human T cell line, was used as a positive control. Cells were treated with PMA/Ionomycin for 4 hours or left untreated as control groups. Consistent with reports of multiple isoforms of NFAT2 in human T cells, weighing between 80KDa ~ 140KDa (Northrop et al., 1993; Northrop et al., 1994; Lyakh et al., 1997; Chuvpilo et al., 1999), Figure 6, lanes 3 and 4 reveal several bands weighing between 80KDa ~ 110KDa, representing NFAT2 isoforms in Jurkat cells. Additionally, there is a similar pattern of upregulation of NFAT2 in stimulated Jurkat cells (lane 4) compared to non-stimulated cells (lane 3) as reported by Lyakh et al (1997) and Chuvpilo et al (1999). In CRFK cells, the antibody detects several bands (indicated by arrows) at the similar molecular weight (80KDa ~110KDa) as human NFAT2 in Jurkat cells, suggesting that this antibody may be able to recognize multiple isoforms of feline NFAT2 (Figure 6, lanes 1 and 2). The expressions of feline NFAT2 isoforms are not altered by PMA/Ionomycin, which was expected as CRFK are not immune cells. To explore the expression of NFAT2 in feline immune cells, PBMCs
were isolated from blood from two different cats. Interestingly, in non-stimulated PBMCs, one dominant band is observed at ~65KDa (Figure 6, lanes 6 and 8, indicated by triangles), however, in stimulated cells, this small band disappears and one sharp band at ~90KDa is observed (Figure 6, lanes 7 and 9, indicated by stars). We speculate that the expression of feline NFAT2 may be regulated by alternative splicing with addition of extra exons in activated immune cells compared to non-activated cells, which is quite different from the regulation of NFAT2 in human and mouse.

Figure 6. Western blot identifying antibody that recognizes feline NFAT2. Equal numbers of feline kidney cells (CRFK), human Jurkat cells and PBMCs isolated from two different cats were cultured in the presence or absence of PMA (20ng/ml) and Ionomycin (1.5µM) for 4 hours. Cells were lysed with NP-40 lysis buffer. Protein was separated by SDS-PAGE and analyzed by immunoblot using anti-human NFAT2 followed by HRP-conjugated goat anti-rabbit secondary antibody. Arrow indicates the alternative isoforms of feline NFAT2 (80KDa~110KDa). The isoform that presents in non-treated PBMCs (~65KDa) are highlighted by triangle, and the isoform that presents in the treated PBMCs (~90KDa) are highlighted by stars.
ChIP assay to detect NFAT2 binding

Having identified antibody that recognizes feline NFAT2, 3 sets of primers were designed to amplify the NFAT binding sites described above (See Table 3 in Materials and Methods). The efficacy of the primers was tested and set 2 primers were chosen for the following ChIP assay. To perform the ChIP assay, unfractionated lymphocytes collected from two FIV negative cats were stimulated for 4 hours with PMA/I, fixed, and the chromatin isolated and digested. Following immunoprecipitation with NFAT2 antibody, the resulting DNA was amplified by real-time PCR. As shown in Figure 7, binding of NFAT2 to the IL-2 promoter was enhanced in PMA/I treated cells as compared to untreated control cells. These data suggest that as in human and mouse, NFAT2 may be a major factor in regulating IL-2 transcription by binding to the IL-2 promoter upon activation of feline immune cells.
Figure 7. Induction of feline NFAT2 binding to the IL-2 promoter in activated feline lymphocytes.
Peripheral lymph nodes were collected and processed into a single cell suspension. Total lymphocytes were either untreated (ctl) or treated with PMA (20ng/ml) and Ionomycin (1.5µM) (PMA/Iono) for 4 hours, fixed, and chromatin harvested for a chromatin immunoprecipitation assay using anti-NFAT2, or rabbit IgG as a control. Resulting DNA samples were amplified by real-time PCR amplifying the NFAT binding sites as listed in Table 3. Error bars indicate ± S.D. of triplicate PCR reactions. Data shown is representative of 3 independent experiments.
C. Association of FoxP3 with NFAT2 and IL-2 mRNA Expression in Activated Feline CD4+ T Cells

CD4+CD25+ Treg cells but not CD4+CD25- Th cells constitutively express FoxP3, which is a transcription regulator known to suppress IL-2 production. It has been reported that FoxP3 inhibits IL-2 production in Treg cells by competing with NFAT1 for binding to and activating the NFAT2 promoter, thus inhibiting NFAT2 transcription (Torgerson et al., 2009). To determine if this was the case in feline lymphocytes, the expression of FoxP3, NFAT2, and IL-2 mRNA in resting and activated CD4+ T cells were examined.

Constitutive expression of FoxP3, NFAT2 and IL-2 mRNA in CD4+ cells from FIV- and FIV+ cats

Analysis of these molecules in freshly isolated CD4+CD25- and CD4+CD25+ cells from FIV negative cats revealed that, as expected, FoxP3 was expressed in CD4+CD25+ cells but not in CD4+CD25- cells (Figure 8a). There was little expression of IL-2 message in either population (Figure 8b), which was expected as these cells were not activated. Both populations expressed similar levels of NFAT2 mRNA (Figure 8c). As FIV infection leads to in vivo functional activation of Treg cells (Vahlemkamp et al., 2004b), FoxP3, NFAT2 and IL-2 mRNA expression in CD4+ cells from FIV+ cats was also examined. Similar to FIV- cats, freshly isolated Treg cells from FIV+ cats express significantly higher levels of FoxP3 (Figure 8a) but lower level of IL-2 mRNA (Figure 8b) than T helper cells. As with the FIV- cats, mRNA levels of NFAT2 were similar in both Treg and Th cells from FIV+ cats (Figure
8c). When the constitutive mRNA levels in Th or Treg cells from FIV- cats are compared to Th or Treg cells from FIV+ cats, there is no difference in FoxP3 and NFAT2 mRNA expression. However, in the case of IL-2 mRNA expression, CD4+CD25- Th cells from FIV+ cats express higher levels than the FIV- cats, suggesting *in vivo* infection of cats with FIV partially activated T helper cells and up-regulates IL-2 but not NFAT2 mRNA expression.
Figure 8. Evaluation of constitutive expression of FoxP3, IL-2 and NFAT2 mRNA in freshly isolated T helper and Treg cells from FIV- and FIV+ cats.
RNA was extracted from $10^6$ freshly isolated, purified CD4+CD25- and CD4+CD25+ T cells and analyzed by Real-time PCR to measure a) FoxP3, b) IL-2 and c) NFAT2 mRNA expression. Expression of the housekeeping gene GAPDH was used as control, and results were calculated as $2^{(Ct_{\text{GAPDH}} - Ct_{\text{target gene}})}$. Each dot represents a single cat. Mann-Whitney Rank Sum Test was used for statistic calculation, * $p<0.05$, ** $p<0.01$
Effect of *in vitro* stimulation on expression of FoxP3, NFAT2, and IL-2 mRNA in CD4+ cells from FIV- and FIV+ cats

As FIV infection of cats activates Treg suppressor function, yet, in this study, did not increase FoxP3 or NFAT2 mRNA expression, it was of interest to determine if additional *in vitro* stimuli would lead to transcriptional induction of these genes. To test this, T cells from FIV- and FIV+ cats were stimulated *in vitro* with known activation stimuli.

**PMA/Ionomycin stimulation:**

Following stimulation for 3 hours with PMA/I, there was no change in the expression of FoxP3 in either Th or Treg cells from FIV- cats (Figure 9a). In contrast, IL-2 mRNA was significantly elevated in T helper cells as well as in Treg cells following activation. Although activated Treg cells produced lower level of IL-2 mRNA compared to activated T helper cells, the difference was not significant (Figure 9b). NFAT2 mRNA expression was highly induced in both T cell populations stimulated with PMA/I compared to unstimulated control cells from FIV- cats (Figure 9c). Thus, both Treg and Th cells expressed increased levels of NFAT2 and IL-2 mRNA, but no change in FoxP3 message following stimulation with PMA/I. As seen in the FIV- cats, FoxP3 was expressed at higher levels in Treg cells than in T helper cells from FIV+ cats, and it was not altered under stimulation. Both IL-2 and NFAT2 mRNA levels were significantly elevated in activated Th and Treg cells compared to control cells. As in FIV- cats, Treg cells from FIV+ cats express lower level of IL-2 mRNA than T helper cells, while no difference of NFAT2 mRNA levels between these
two activated T cell populations was observed (Figure 9a-c). In summary, comparing the
response of cells from FIV- versus FIV+ cats to PMA/I stimulation, IL-2 and NFAT2 were
highly induced, whereas FoxP3 was not increased in either CD4+CD25- or CD4+CD25+ T
cell populations from FIV- or FIV+ cats (Figure 9a-c).
Figure 9. Evaluation of expression of FoxP3, IL-2 and NFAT2 mRNA in activated T helper and Treg cells from FIV- and FIV+ cats

Purified CD4+CD25- and CD4+CD25+ lymphocytes from FIV- and FIV+ cats were incubated with PMA (20ng/ml) and Ionomycin (1.5µM) for 3 hours, RNA purified and analyzed by Real-time PCR to measure a) FoxP3, b) IL-2 and c) NFAT2 mRNA expression. Expression of the housekeeping gene GAPDH was used as control, and results were calculated as $2^{(\text{Ct}_{\text{GAPDH}}-\text{Ct}_{\text{target gene}})}$. Each dot represents a single cat. Mann-Whitney Rank Sum Test was used for statistic calculation, * p<0.05, ** p<0.01
We were surprised by the fact that PMA/I activated Treg cells significantly up-regulated IL-2 mRNA expression, as Treg cells are considered anergic and not able to produce IL-2 when activated via their TCR. To address the question if PMA/I activated Treg cells can produce IL-2 protein, T helper cells and Treg cells were activated by PMA/I for 1d, and the supernatant collected and analyzed for IL-2 production by ELISA. Figure 10 reveals that activated Treg cells do produce IL-2 but at a much lower level compared to activated T helper cells. As we separate the two T cell populations only based on expression of CD25, which is not only expressed on Treg cells but also activated T helper cells, it is possible that the Treg population we have is not pure, and the production of IL-2 in CD4+CD25+ population is due primarily to activated T helper cells. Also, it is important to note that PMA/I stimulation bypasses membrane signaling, which may result in overwhelming of intracellular signal events.
Figure 10. Evaluation of IL-2 protein production in activated T helper and Treg cells from FIV-cat.

Purified CD4+CD25- and CD4+CD25+ lymphocytes from an FIV-cat were incubated with PMA (20ng/ml) and Ionomycin (1.5µM) for 1 day, and supernatants were analyzed for IL-2 protein production by ELISA. Supernatants collected from non-treated cells were used as control. Error bars indicate ± S.D. of triplicate reactions.
ConA stimulation:

As the induction of IL-2 and NFAT2 mRNA in CD4+CD25+ Treg cells by PMA/I treatment was unexpected, the response to a membrane stimulus was examined. Activation of lymphocytes with PMA/I results in direct activation of protein kinase C and calcium influx, thus by-passing membrane signaling, whereas ConA, a lectin, activates cells through cell surface receptors, as do TCR. As previous studies have demonstrated that ConA alone will not activate purified CD4+CD25- or CD4+CD25+ cells (Mexas et al., 2008), FoxP3, NFAT2, and IL-2 responses in Th and Treg cells stimulated with ConA or ConA plus Ionomycin were examined. Ionomycin or PMA/Ionomycin stimulation was used as control.

Purified CD4+CD25- and CD4+CD25+ cells from FIV+ cats were incubated for 3 hours in media alone, ConA (10mg/ml) alone, ConA (10mg/ml) plus Ionomycin (1.5µM), Ionomycin(1.5µM) alone, and PMA (20ng/ml) plus Ionomycin (1.5µM) followed by mRNA extraction and real-time PCR analysis. As shown in Figure 11a, non-stimulated Treg cells express significantly higher levels of FoxP3 than T helper cells, and FoxP3 mRNA expression was not altered by stimulation. Stimulation with ConA alone resulted in little IL-2 mRNA production, whereas IL-2 mRNA levels in both T helper and Treg cells were increased when Ionomycin was added to the ConA (Figure 11b). ConA/I stimulated T helper cells express significantly higher levels of IL-2 mRNA than ConA/I stimulated Treg cells, whereas ConA/I caused a similar elevation of NFAT2 mRNA levels in both T helper and Treg cells.
These data (Figure 9 and 11) demonstrate that T helper cells, when activated \textit{in vitro}, express higher level of IL-2 mRNA than Treg cells, but the increase in NFAT2 mRNA expression in these two cell population is similar, suggesting that IL-2 mRNA expression is not simply correlated with NFAT2 mRNA up-regulation.
Figure 11. Evaluation of expression of FoxP3, IL-2 and NFAT2 mRNA in T helper and Treg cells from FIV+ cat under different stimulating conditions.

Purified CD4+CD25- and CD4+CD25+ lymphocytes from FIV+ cats were incubated in media alone; Ionomycin (1.5µM) alone; PMA (20ng/ml) plus Ionomycin (1.5µM); ConA (10µg/ml); ConA (10µg/ml) plus Ionomycin (1.5µM) for 3 hours. mRNA from 10^6 cells of each group was purified and Real-time PCR performed to measure a) FoxP3, b) IL-2 and c) NFAT2 mRNA expression. Expression of the housekeeping gene GAPDH was used as internal control. Results were normalized to untreated CD4+CD25- control cells using 2^-∆∆Ct. Each dot represents a single cat. Mann-Whitney Rank Sum Test was used for statistic calculation, * p<0.05, ** p<0.01
ChIP analysis of NFAT2 binding to the IL-2 promoter in CD4+ T helper and Treg cells

As there is a significant increase in IL-2 mRNA expression in activated T helper cells compared to activated Treg cells when stimulated with ConA plus Ionomycin, but the increase in NFAT2 mRNA expression is similar, it is possible that there is less binding of NFAT2 protein at the IL-2 promoter in the Treg cells compared to the T helper cells. To address this, purified CD4+CD25- T helper cells and CD4+CD25+ Treg cells from FIV+ cats were incubated with ConA plus Ionomycin for 5 hours, fixed, and chromatin extracted for a ChIP assay to analyze the binding of NFAT2 to the IL-2 promoter. As shown in Figure 12, the binding of NFAT2 is much higher in the T helper cells than that in activated Treg cells. These data suggest that although NFAT2 mRNA expression level is similar in activated T helper cells and Treg cells, NFAT2 protein binds to the IL-2 promoter more efficiently in T helper cells and may explain the higher level of IL-2 mRNA expression.
Figure 12. Binding of feline NFAT2 to the IL-2 promoter is higher in activated CD4+CD25- T helper cells than in activated CD4+CD25+ Treg cells

Two FIV+ cats were euthanized, and all peripheral lymph nodes were biopsied and processed into a single cell suspension. Lymphocytes were stained with anti-CD4-PE and anti-CD25-FITC. CD4+CD25- T helper and CD4+CD25+ Treg cell populations were purified by MoFlo high speed sort. Both cell populations were first treated with ConA (10mg/ml) for 2 hours, followed by addition of Ionomycin (1.5µM), and cells were cultured for 3 more hours, harvested and fixed. Cells from two cats were pooled together and chromatin immunoprecipitation based on a modified protocol (appendix B) was performed using anti-NFAT2. Samples were subject to real-time PCR analysis using primers amplifying NFAT binding sites (Table 3) or GAPDH genomic DNA (Table 5). Data were calculated as described in appendix B. Error bars indicate ± S.D. of triplicate PCR reactions. Data A and B shown here are results from duplicate ChIP reactions.
D. The Role of FoxP3 and NFAT2 in IL-2 Expression in Functionally Suppressed CD4+CD25- T Helper Target Cells

Although Treg cells suppress IL-2 message and protein production by CD4+CD25- T helper cells, the mechanism by which this occurs is unclear. This mechanism, in part, may relate to NFAT2 binding to the IL-2 promoter. Data in Figures 11 and 12 indicate that Treg cells and T helper cells express similar amount of NFAT2 mRNA but the level of NFAT2 protein binding to the IL-2 promoter in Treg cells is lower, perhaps due to the presence of FoxP3.

FoxP3, IL-2 and NFAT2 mRNA expression in Treg cell-suppressed CD4+CD25- T helper cells

To determine the effect of Treg cells on expression of these genes in T helper target cells, lymphocytes from one FIV+ cat were sorted into CD4+CD25- T helper, CD4+CD25+ Treg cells, as well as a CD4-CD8-depleted cell population (antigen presenting cells). The CD4+CD25- T helper cells were cultured with ConA and the DiD Vybrant stained CD4-CD8-depleted cells added at a 1:1 ratio to act as antigen presenting cells as described elsewhere (Mexas et al., 2008). After 1 hour, the cells were washed to remove excess ConA, and DiD Vybrant stained CD4+CD25+ cells (suppressor) or DiD Vybrant stained CD4+CD25- cells (control suppressor cells) were added to the CD4+CD25- target cells at a suppressor to target cell ratio of 1:1. Following co-culture for various times, DiD negative cells (target cells) from each co-culture (suppressor or control) were collected and RNA
purified for detection of FoxP3, IL-2 and NFAT2 mRNA. Culture supernatant was collected and IL-2 protein determined by ELISA.

T helper cells co-cultured with Treg suppressor cells expressed similar levels of FoxP3 mRNA as the T helper cells co-cultured with control (T helper) cells at 3h and 5h of co-culture. However, after 1 day of co-culture, expression of FoxP3 in the target T helper cells co-cultured with Treg cells was markedly elevated compared with the non-suppressor CD4+CD25- control group and remained high through 4 days of co-culture (Figure 13a). IL-2 mRNA was detected in target cells from both co-cultures by 3 hours, increased at 5 hours, but was gone by 1 day of culture. However, the level of IL-2 mRNA was much lower in the T helper cells co-cultured with Treg suppressor cells compared to T helper cells co-cultured with control T helper cells. When IL-2 protein was measured, there was also a significant difference between the Th/Treg co-culture and the Th/Th control cell co-culture. IL-2 in the supernatant of the control co-culture reached 700pg/ml at 1 day of co-culture, while in the Treg co-culture, IL-2 production peaked at only 100pg/ml (Figure 13b and 13c). Interestingly, there was no difference in NFAT2 mRNA expression in T helper target cells co-cultured with suppressor or control cells. NFAT2 message increased and was highest at 3 hours of culture in both groups and gradually returned to basal levels by 1 day (Figure 13d).

As an additional control, the same suppression assay was also performed using cells from an FIV negative cat. Although the pattern of responses of FoxP3, IL-2 and NFAT2 in the T helper cells co-cultured with Treg cells was similar to that seen with cells from an FIV+ cat, the magnitude of the responses were much lower (Figure 14). While FoxP3
mRNA expression was elevated in the Th/Treg co-culture (Figure 14a), it did not sustain for 4 days of culture, in contrast to the significant elevation of FoxP3 seen in T helper cells co-cultured with Treg cells from FIV+ cats (Figure 13a). IL-2 mRNA level in T helper cells co-cultured with Treg cells is lower than the T helper cells in the control co-culture, but interestingly even in the control group, IL-2 mRNA expression was not as high as that of the control group from the FIV positive cat (Figure 14b). Although the cells co-cultured with Treg cells produced a lower amount of IL-2 protein than the control group, the difference was not as marked as with the FIV+ cat (Figure 14c). While NFAT2 mRNA expression increased in the target cells of both co-cultures, there was no difference in the levels between the Treg and the control co-cultures (Figure 14d).
Figure 13. Evaluation of FoxP3, IL2, and NFAT2 levels in T helper cells co-cultured with Treg cells from FIV+ cats

CD4+CD25-, CD4+CD25+ T cells and CD4-CD8- depleted antigen presenting cells from FIV+ cats were purified by MoFlo high speed sort. 2x10⁶ CD4+CD25- cells were cultured with DiD Vybrant stained CD4-CD8- cells at 1:1 ratio and treated with ConA (10mg/ml) for 1 hour. Cells are washed and 2x10⁶ DiD Vybrant stained CD4+CD25- cells (suppressor) or CD4+CD25+ cells (control suppressor) were added to the culture. Cells were collected at different time points and separated by MoFlo high speed sort. 10⁶ DiD- cells of each group were used for RNA extraction and Real-time PCR to measure a) FoxP3, b) IL-2 and d) NFAT2 mRNA expression levels. Expression of housekeeping gene GAPDH was used as control, and results were calculated as 2⁻ΔΔCt. Error bars indicate ± S.D. of triplicate PCR reactions. c) supernatant was collected for IL-2 ELISA. Data is representative of 4 independent experiments.
Figure 14. Evaluation of FoxP3, IL2 and NFAT2 levels in T helper cells co-cultured with Treg cells from FIV- cats
CD4+CD25-, CD4+CD25+ T cells and CD4-CD8- depleted antigen presenting cells from an FIV- cat were purified by MoFlo high speed sort. 2x10^6 CD4+CD25- cells were cultured with DiD Vybrant stained CD4-CD8- cells at 1:1 ratio and treated with ConA (10mg/ml) for 1 hour. Cells are washed and 2x10^6 DiD Vybrant stained CD4+CD25+ cells (suppressor) or CD4+CD25- cells (control suppressor) were added. Cells were collected at different time point and separated by MoFlo high speed sort. 10^6 DiD- cells of each group were used for RNA extraction and Real-time PCR to measure a) FoxP3, b) IL-2 and d) NFAT2 mRNA expression levels. Expression of housekeeping gene GAPDH was used as control, and results were calculated as 2^(-ΔΔCt). Error bars indicate ± S.D. of triplicate PCR reactions. c) supernatant was collected for IL-2 ELISA.
Role of TGF-β in regulating Foxp3, IL-2 and NFAT2 expression in T helper target cells

Several studies, including those in this laboratory, suggest that Treg suppression of T helper cells is mediated by membrane-associated TGF-β (mTGF-β) on the Treg cell and TGF-β receptor (TGF-βR) on the target cell (Thornton et al., 1998; Nakamura et al., 2001; Annunziato et al., 2002; Chen et al., 2003b; Nakamura et al., 2004). As it has been shown that signaling through the TGF-βR leads to FoxP3 expression in activated T helper cells, it is possible that TGF-β-induced FoxP3 suppresses IL-2 message by interfering with NFAT2 function. To address the possible role of TGF-β/TGF-βR signaling, an in vitro suppression assay of ConA-activated CD4+CD25- T helper cells (to up-regulate TGF-βR (Petty et al., 2008)) was performed using recombinant human TGF-β1 (10ng/ml) and FoxP3, IL-2 and NFAT2 mRNA quantified. Treatment of ConA-stimulated CD4+CD25- T helper cells from an FIV positive cat with TGF-β resulted in increased FoxP3 mRNA expression, decreased IL-2 mRNA and protein expression compared to T helper cells stimulated with ConA alone (Figure 15). Increase in NFAT2 mRNA expression was similar in the ConA/TGF-β1 treated T helper cells compared to the T helper cells treated with ConA only with the same kinetics as seen in suppression assays using Treg cells (Figure 13). These data suggest that the Treg suppression of T helper cells may be mediated by mTGF-β but does not correlate with NFAT2 mRNA up- or down-regulation.
Figure 15. Evaluation of FoxP3, IL-2 and NFAT2 levels in T helper cells \textit{in vitro} suppressed by TGF-β.

1x10^6 purified CD4+CD25- cells from an FIV+ cat were cultured with ConA (10mg/ml) for 1 hour followed by addition of recombinant human TGF-β1 (10ng/ml). Cells were collected at various time points and RNA was extracted and subject to real-time PCR to measure a) FoxP3, b) IL-2 and d) NFAT2 mRNA expression levels. Expression of housekeeping gene GAPDH was used as control, and results were calculated as 2^{-\Delta \Delta Ct}. Error bars indicate ± S.D. of triplicate PCR reactions. c) Culture supernatant was collected for IL-2 ELISA. Data is representative of 2 independent experiments.
Is there a difference in NFAT2 binding to the IL-2 promoter in TGF-β suppressed versus non-suppressed CD4+CD25- T helper cells?

As ConA/TGF-β treated T helper cells show an increase in FoxP3 and a decrease in IL-2 mRNA levels but a similar increase in NFAT2 mRNA levels compared to T helper cells treated with ConA alone, experiments were designed to determine if inhibition of IL-2 production in TGF-β1 suppressed T helper cells is due to decreased NFAT2 binding to the IL-2 promoter. As this experiment employed a ChIP assay using purified CD4+CD25- T helper cells, Ionomycin was used in place of APCs in combination with ConA to stimulate the T helper cells. Prior to performing a ChIP assay, an abbreviated TGF-β-mediated suppressor assay was done to document the mRNA expression of IL-2 and NFAT2 in the T helper cells using this altered stimulation. Purified CD4+CD25- T cells from FIV+ cats were treated with ConA for 1 hour, which leads to up-regulation of TGF-βR on cell surface (Petty et al., 2008). Recombinant hTGF-β1 was then added, followed by Ionomycin 1 hour later. Cells were cultured for an additional 3 hours then mRNA was extracted for real-time PCR analysis. As shown in Figure 16, IL-2 mRNA expression was lower in the TGF-β treated group compared with the non-treated group while there was no difference in NFAT2 mRNA expression between the two groups. The suppression assay was repeated and the target cells fixed, chromatin harvested and NFAT2 binding to the IL-2 promoter was analyzed by ChIP. As shown in figure 17, binding of NFAT2 to IL-2 promoter in non-TGF-β treated (control) cells is much higher than that in TGF-β treated (suppressed) cells. This result is from one ChIP experiment, repeat of this ChIP experiment would be highly desirable to confirm the
change we see here. Overall, these data suggest that, as with Treg-mediated T helper cell suppression (Figure 13), there is an inverse correlation between FoxP3 over-expression and inhibition of IL-2 mRNA expression with NFAT2 protein binding to the IL-2 promoter, suggesting that Treg-mediated suppression of T helper function may involve FoxP3 inhibition of NFAT2-dependent activation of the IL-2 promoter.

Figure 16. TGF-β suppresses ConA/Ionomycin-induced IL-2 mRNA expression in T helper cells.
Purified CD4+CD25- T cells from FIV+ cat were first treated with ConA (10mg/ml) for 1 hour, then recombinant human TGF-β1 (10ng/ml) was added to suppression group for another 1 hour, then Ionomycin(1.5µM) was added to both groups and cells cultured for 3 more hours. 10⁶ cells of each group were used for RNA extraction and Real-time PCR to measure IL-2 and NFAT2 mRNA expression. Expression of housekeeping gene GAPDH was used as control, and results were calculated as 2^(Ct_{GAPDH}-Ct_{target~gene}). Error bars indicate ± S.D. of triplicate PCR reactions.
Figure 17. The level of NFAT2 binding to IL-2 promoter is higher in activated T helper cells than in suppressed T helper cells.

Two FIV+ cats were euthanized, and all peripheral lymph nodes were biopsied and processed into a single cell suspension. Lymphocytes were stained with anti-CD4-PE and anti-CD25-FITC. CD4+CD25- T helper cells were purified by MoFlo high speed sort. Cells of both the activation and suppression groups were first treated with ConA (10ng/ml) for 1 hour, and then recombinant human TGF-β1 (10ng/ml) was added to the suppression group for another 1 hour. Ionomycin (1.5µM) was then added to both groups and cells cultured for 3 more hours, harvested, and fixed. Cells from two cats were pooled and chromatin immunoprecipitation based on the modified protocol (appendix B) was performed using anti-NFAT2. Samples were subject to real-time PCR using primers amplifying NFAT binding sites (Table 3) or GAPDH genomic DNA (Table 5). Data are calculated as described in appendix B. Error bar indicates ± S.D. of triplicate PCR reactions.
V. DISCUSSION

Lentiviral-induced immune deficiency is manifested by loss of CD4+CD25- T helper cell numbers and responses to primary as well as recall antigens (Clerici et al., 1989; Torten et al., 1991). Numerous mechanisms have been proposed for this loss, including cytokine dysregulation, activation-induced apoptosis, and immunologic anergy. One of the primary mediators of immunologic anergy is the CD4+CD25+ T regulatory cell. Treg cells are a small subpopulation of T cells that can be generated either in the thymus or induced in the periphery (Modigliani et al., 1995a; Belkaid et al., 2002). Treg cells constitutively express surface markers, such as CD25, GITR, CTLA-4, CD45RO and intracellular FoxP3, which distinguish them from other T cell subsets (Sakaguchi et al., 1995; Hori et al., 2003). Although activated through antigen specific TCR engagement, Treg cells function as immune suppressors in an antigen non-specific manner (Thornton et al., 1998). Activated Treg cells express membrane-bound TGF-β, which may mediate suppression of T and B cell immune responses through signaling via binding to the TGF-βR on target cells (Chen et al., 2003b). Treg cells suppress both CD4+ T helper cell and CD8+ cytotoxic T cell immune responses by inhibiting production of cytokines, such as IL-2, thus suppressing T cell proliferation and activation (Thornton et al., 1998).

Treg cells play a prominent role in the pathogenesis of lentivirus infection. This laboratory has demonstrated that Treg cells are chronically activated in FIV+ cats and suppress proliferation of mitogen-activated CD4+CD25- T helper cells and CD8+ cytotoxic T cells (Vahlemkamp et al., 2004b; Mexas et al., 2008; Fogle et al., 2010a). This
suppression is cell-cell contact-dependent and is the result of decreased IL-2 and IFN-γ expression by the target cells (Mexas et al., 2008; Fogle et al., 2010a). However, the molecular mechanism by which Treg cell/T helper cell interaction leads to decreased IL-2 gene transcription in the target T helper cell is unclear. IL-2 is a cytokine essential for T cell survival and expansion; lack of IL-2 leads to T cell anergy and apoptosis (Smith 1988). IL-2 is produced by T cells immediately in response to TCR engagement and CD28 co-stimulation and is regulated by a number of transcription factors, including the nuclear factors of activated T cells (NFAT) proteins, established as activators of IL-2 gene transcription. NFAT binding elements have been mapped in both human and murine IL-2 promoters (Rooney et al., 1995; Macián et al., 2001). The importance of these transcription activators is evidenced by the fact that site mutations of NFAT binding sequences almost completely abolishes IL-2 gene transcription in stimulated T cells (Rooney et al., 1995). The NFAT family of proteins is composed of 5 members. Mature T cells predominantly express NFAT1 and NFAT2. NFAT1 pre-exists in the cytoplasm of resting T cells, while NFAT2 is induced in activated cells, and highly correlates with the activation of IL-2 expression (Northrop et al., 1994). The study reported herein examined the transcriptional expression and promoter binding of the inducible IL-2 transcription activator NFAT2 in T helper target cells co-cultured with activated Treg cells to better understand the mechanisms of Treg-induced T helper cell immuno-suppression.

We identified a NFAT2 homologue in cats. The partial cDNA of feline NFAT2 gene shares 85% identities in nucleotide sequences with human NFAT2. Protein alignment
revealed that this feline NFAT2 contains the same functional motifs as human and mouse NFAT2, including identical DNA binding motif, calcineurin docking regions, serine-rich regions, SPxx motifs, nuclear localization signal and highly conserved residues that participate in contacts to transcription partner c-Fos and c-Jun (Figure 2), suggesting that this feline NFAT2 may be regulated and function the same way as its homologous in human and mouse. As reported in human and murine cells (Lyakh et al., 1997), NFAT2 is constitutively expressed at basal levels in feline T cells but is highly induced when cells are activated by PMA plus ionomycin (Figure 3). Western blot reveals that as in human and mouse, there are multiple isoforms of NFAT2 in cats. Different from the expression pattern of NFAT2 in human that two smaller isoforms weighing around 80~90 KDa are highly induced in activated Jurkat cells (Figure 6, lanes 3 and 4), one smaller isoform of feline NFAT2 weighing ~65KDa presents in resting PBMCs, while a larger isoform weighing ~ 90KDa predominantly presents in activated PBMCs (Figure 6, lanes 6, 7, 8 and 9). This data suggests that alternative splicing of NFAT2 may exist in feline immune cells while the regulation pattern may be different from what have been known in human and mouse. Based on the sequence of another isoform of human NFAT2 (NCBI Reference Sequence: NM_172389.1), extra exon pieces other than what we have identified in figure 1 and 2 are found in cat genome, indicating the existence of multiple feline NFAT2 isoforms, but further experiments like gene cloning and Northern blot would be needed to confirm this hypothesis and reveal the detailed regulation of NFAT2 in cats.
Induction of NFAT2 mRNA correlated with the activation of IL-2 mRNA expression in feline T cells. ChIP assay revealed that in PMA/Ionomycin activated lymphocytes, binding of NFAT2 to the IL-2 promoter was strongly enhanced (Figure 7), suggesting that in feline cells, NFAT2 may play an important role in activating IL-2 expression and immune cell activation. However, the role of NFAT2 in binding to the IL-2 promoter and activating gene expression remains controversial in other species. Lyakh et al (1997) reported that NFAT2 protein in nuclei extracts from human peripheral blood T cells activated with PMA/Ionomycin failed to bind to the IL-2 promoter probe containing multiple NFAT binding elements, whereas NFAT1 binding was demonstrated. In contrast, Rooney et al (1995) found that NFAT1 and NFAT2 were identical in their ability to bind to the IL-2 promoter in the presence of AP-1. Such a discrepancy may be due to the fact that NFAT2 needs to cooperate with AP-1 in order to form a stable tertiary complex with DNA, as supported by the crystal structure analysis reported by Chen et al. (1998); whereas NFAT1 may bind to DNA binding elements itself by forming homodimers (Hogan et al., 2003). Although NFAT2 contains homology domains suggesting it can also form homodimers, there are no reports of it doing so in the literature.

NFAT2 transcription is regulated by the calcium-calcineurin signaling pathway, but must cooperate with AP-1, a heterodimer composed of c-Fos and c-Jun, which is activated through the PKC/Ras pathway, and known to induce IL-2 gene expression (Macián, et al 2001). Thus IL-2 transcription is dependent upon both pathways. In support of a requirement for a dual signaling pathway, we found that feline IL-2 mRNA production was
limited in T cells activated by ionomycin (an activator of the calcium-calcineurin pathway) alone; whereas T cells activated by PMA (which activates PKC pathway) plus ionomycin induced significantly higher levels of IL-2 mRNA. However, NFAT2 mRNA levels were increased almost equally by the two activation methods (Figure 11), suggesting that in addition to NFAT2 transcriptional activation, post-transcriptional regulation of NFAT2 may be required for full activation of IL-2 transcription.

Prior to address the molecular changes leading to Treg cell-induced IL-2 suppression in T helper cells, the expression of NFAT2 and FoxP3 and how they may regulate IL-2 production in purified T helper and Treg cells from FIV- and FIV+ cats were examined. In support of previous studies in our laboratory (Petty et al., 2008; Mexas et al., 2008), constitutive FoxP3 mRNA expression in Treg cells was significantly higher than that in T helper cells. This is similar to reports by Petty et al. (2008) and Mexas et al. (2008), who also found high levels of FoxP3 mRNA in Treg cells compared to T helper cells. Activation of Treg and T helper by ConA or PMA/I did not result in an increase in FoxP3 mRNA (Figures 9 and 11). In studies with human T cells, FoxP3 has been found to be temporarily elevated in activated T helper cells (Li et al., 2008); however, in our study, we found no change in Foxp3 mRNA level in T helper cells despite the activation state. Although the level of FoxP3 mRNA was not significantly different in Treg cells from FIV- cats and FIV+ cats, there was a trend of higher FoxP3 mRNA expression in the FIV+ cats (Figure 9). Previous data from Mexas et al. (2008) showed significant up-regulation of FoxP3 mRNA and protein levels in CD4+CD25+ T cells from the blood and lymph nodes during acute FIV
infection. It is possible that during acute infection, the presence of high levels of virus may aggressively activate Treg cells, leading to significant elevation of FoxP3 expression; whereas chronic infection supports low levels of virus, and could result in lower levels of Treg activation.

In contrast to FoxP3, constitutive NFAT2 mRNA expression was similar in T helper and Treg cells and there was no difference between FIV- and FIV+ cats. However, activation (PMA/I) significantly increased NFAT2 mRNA expression equally in both T helper and Treg cells. These results are different from those reported by Torgerson et al. (2009) who found that NFAT2 mRNA expression was significantly lower in FoxP3+ T cells than FoxP3- T cells. They also reported that FoxP3 suppresses NFAT2 promoter activity by competing with NFAT1 binding to the NFAT2 promoter, thus inhibiting NFAT2 transcription. However, in this study, NFAT2 mRNA expression was not affected by FoxP3 expression. As the protein level of NFAT2 was not evaluated in this study, it is possible that NFAT2 protein levels in FoxP3+ Treg cells are lower than T helper cells, or they are not functionally activated in the presence of FoxP3.

Feline Treg cells are similar to human and murine Treg cells in that they are anergic and fail to produce IL-2 upon immune stimulation (Maloy et al., 2001; Vahlenkamp et al., 2004b). In this study, freshly isolated T helper and Treg cells from control cats expressed low levels of IL-2 and FIV infection did not result in significantly greater IL-2 mRNA expression (Figure 8). Interestingly, activation of Treg cells from both naïve and FIV+ cats by PMA/ionomycin resulted in significant up-regulation of IL-2 mRNA expression as in
activated T helper cells (Figure 9). However, IL-2 protein production by Treg cells was dramatically lower than T helper cells (Figure 10). We were surprised to see that Treg cells can also produce IL-2, although in a much lower level. As we separated T helper cells and Treg cells only based on the expression of CD25, which is also expressed by activated T helper cells. The production of IL-2 in CD4+CD25+ T cell population may be attributed to activated T helper cells. Besides, it is possible that, as PMA and ionomycin bypass the receptor signaling, the PKC and calcium pathways could be activated intracellularly. To assess the IL-2 mRNA expression in T helper and Treg cells under a more physiological condition, T helper and Treg from FIV+ cats were stimulated with ConA/Ionomycin. Although the increases in NFAT2 mRNA levels were still similar in T helper and Treg, IL-2 mRNA was significantly higher in T helper than in Treg cells, suggesting that Treg cells are anergic, possibly due to over-expression of FoxP3. While FoxP3 is known to suppress IL-2 expression (Marson et al., 2007), its role in regulating IL-2 transcription factors such as NFAT2 remains to be clarified.

At least three mechanisms by which FoxP3 and NFAT2 may be involved in IL-2 suppression have been proposed. In activated conventional T cells, transcription factors, such as NFAT, AP-1 and NFkB, generated by various signaling triggered through TCR and CD28 crosslinking, synergistically activate IL-2 transcription (Figure 18a). Schubert et al. (2001) and Wells (2009) suggested that in Treg cells, FoxP3 may function as a repressor of the IL-2 promoter and is alone sufficient for inhibiting IL-2 gene expression (Figure 18b). They proposed that FoxP3 competes with other transcription activators, such as NFAT, AP-1.
and NFκB; and binding of FoxP3 to the IL-2 promoter results in gene repression. Different from the first mechanism, Bettelli et al. (2005) reported that FoxP3 may physically associate with NFAT and block its transcriptional activities. Later, Wu et al. (2006) proposed that FoxP3 cooperates with NFAT in suppressing gene expression by competing with AP-1 for forming a complex with NFAT (Figure 18c). A FoxP3 binding site has been located adjacent to NFAT binding sequence in the murine IL-2 promoter. Besides, NFAT interacting region has been found in FoxP3, the mutation of which decreases the ability of FoxP3 to suppress IL-2 gene transcription. In a third mechanism, Torgerson et al. (2009) reported that FoxP3 inhibited inducible NFAT2 expression, resulting in suppression of NFAT2-dependent gene expression such as IL-2 and induction of T cell tolerance and anergy (Figure 18d). In the studies reported herein, NFAT2 mRNA levels were similar in T helper and Treg cells, suggesting that rather than FoxP3 inhibiting NFAT2 transcription, FoxP3 may block binding NFAT2 to the IL-2 promoter. In this study, ChIP data supported this by revealing that in activated T helper cells, binding of NFAT2 to the IL-2 promoter is much higher than that in activated Treg cells (Figure 12). Thus, it is reasonable to speculate that although mRNA levels of NFAT2 are similar in T helper and Treg cells, less NFAT2 protein is binding to the IL-2 promoter, leading to suppressed IL-2 expression in activated Treg cells. While there is no direct evidence that the lower NFAT2 binding to the promoter is the result of FoxP3 competition, the data show a strong correlation between increased FoxP3 expression, decreased NFAT2 binding to the IL-2 promoter, and declined IL-2 production.
While much attention has been paid to the molecular characteristics defining the anergy and functions of Treg cells, less is known about the mechanisms regulating the suppression and anergy of the T helper and CD8+ target cells. A number of studies suggest that Treg cell-induced suppression of T helper cells is mediated by Treg membrane-associated TGF-β binding to TGF-β receptor on T helper cells (Chen et al., 2003b). One of the consequences of TGF-β binding to its receptor is the induction of FoxP3 expression in the T helper target cell (Chen et al., 2003a). As FoxP3 has been found to have a role in the suppression of IL-2 production in Treg cell, possibly through the suppression of NFAT2 binding to the IL-2 promoter (Figure 12), the question of how FoxP3 and NFAT2 may be involved in regulating IL-2 expression in T helper cells suppressed by activated Treg cells was addressed. The mRNA expression of FoxP3, IL-2 and NFAT2 in Treg-suppressed T helper cells from FIV+ cats was examined. Similar to previous studies in our lab by Vahlenkamp et al. (2004b) and Mexas et al. (2008), IL-2 production was reduced in target T helper cells when co-cultured with Treg cells. Also FoxP3 was up-regulated and sustained at high level in T helper cells co-cultured with Treg cells, suggesting that up-regulation of FoxP3 in target cells correlated with inhibition of IL-2 production. In support of these studies, Fogle et al. (2010b) demonstrated that FoxP3 expression was increased in CD8+ target cells co-cultured with FIV-activated Treg cells. This study demonstrated a decrease in IL-2 production by Treg-suppressed T helper target cells; however, NFAT2 mRNA was equally increased in both Treg-suppressed and non-suppressed cells. Additionally, in vitro TGF-β treatment revealed a similar pattern of molecular changes as those seen in the Treg-
induced suppression assay in that FoxP3 mRNA expression was up-regulated and IL-2 production inhibited in TGF-β treated cells compared to untreated cells, while NFAT2 transcription was equally elevated in both cell cultures. While this study demonstrates the involvement of TGF-β signaling in Treg-mediated T helper suppression, the role of NFAT2 in IL-2 suppression in suppressed T helper cells is not clear. As data suggest that NFAT2 plays a major role in IL-2 induction (Figure 4 and 7), yet transcription of the NFAT2 gene is not different in activated and Treg-suppressed T helper cells, studies were designed to evaluate NFAT2 at the protein level, particularly its ability to bind to the IL-2 promoter. These studies revealed that binding of NFAT2 to the IL-2 promoter in suppressed T helper cells is lower than that in non-suppressed T cells. Importantly, these data (Figure 13 and 15) suggest that while FoxP3 does not inhibit NFAT2 mRNA expression in suppressed T helper target cells, it may suppress IL-2 production by decreasing NFAT2 binding to the IL-2 promoter. While the exact mechanism by which FoxP3 suppresses NFAT2-dependent cytokine expression is unclear, the data presented in this study suggest that FoxP3 may inhibit binding of NFAT2 to the IL-2 promoter resulting in suppression of IL-2 production and T helper immune responses. More importantly, these data may provide a mechanism by which AIDS lentiviruses mediate T cell immunodeficiency.

Different from the mechanism proposed by Torgerson et al (2009), although upregulation of FoxP3 is observed in suppressed T helper cells, the suppression of IL-2 in target cells co-cultured with Treg cells is not due to the FoxP3-inhibited inducible NFAT2 expression. The NFAT2 mRNA levels are similar in T helper cells from control and
suppression co-culture. However, the question remains that if the decreased NFAT2 binding to the promoter is due to the competition of FoxP3 binding. We have searched the feline IL-2 promoter for consensus Forkhead binding sequences. We found one suspect which locates at 600bp upstream of transcription start site, and several other possible binding elements at 1500bp upstream of transcription start site. None of them is adjacent to NFAT binding sequences, contrary to the mechanism reported by Bettelli and Wu. We speculate that the inhibition of IL-2 in suppressed T cells in cats is due to the binding of repressor FoxP3, although further investigation is needed to prove this hypothesis.

In summary, this study demonstrated that elevation of NFAT2 mRNA is similar in activated T helper and Treg cells. However, ChIP assays revealed that the binding of NFAT2 protein to the feline IL-2 promoter in T helper cells and non-suppressed target cells is higher than in Treg cells and Treg-suppressed target cells respectively. These data suggest that suppression of IL-2 expression in Treg cells and Treg-suppressed T helper target cells is caused by decreased NFAT2 binding to the IL-2 promoter. To answer the question if reduction of NFAT2 protein binding to the IL-2 promoter is caused by FoxP3 binding is the issue of future studies. This is the first study to show that NFAT2 binding to the IL-2 promoter is decreased in Treg-suppressed target cells and that this decrease is associated with an increase in FoxP3 expression. These studies provide important information to our understanding of how the IL-2 gene is regulated and how Treg cells induce suppression in target cells.
Figure 18. Proposed mechanisms by which IL-2 transcription is suppressed in Treg cells.
a). Signals that lead to IL-2 induction in activated T helper cells.
b). FoxP3 directly binds to IL-2 promoter, competes with transcription activators, results in IL-2 gene repression. (Schubert et al., 2001; Wells 2009)
c). FoxP3 binds to IL-2 promoter and suppress IL-2 expression in cooperation with NFAT (Bettelli et al., 2005; Wu et al., 2006)
d). FoxP3 suppresses activation-induced NFAT2 expression, consequently inhibits IL-2 expression (Torgerson et al., 2009)
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Appendix A

Repeat of Treg Suppression Assay in FIV+ Cats

The expression of FoxP3, IL2, and NFAT2 mRNA and IL2 protein in CD4+CD25- Th cells following co-culture with CD4+CD25+ Treg cells was analyzed using cells from 3 FIV+ cats. Due to the limited amount of cells we got from those cats, we could only perform 2 different time points from each cat. In Cat 1, the molecular change during short-term of suppression was examined. After 3 hours of co-culture, the expression of IL-2 mRNA in T helper cells co-cultured with Treg cells was much lower than T helper cells co-cultured without Treg cells. In Cat 2 and Cat 3, the molecular change during long-term of suppression was examined. IL-2 production was measured by ELISA, and in support of the change seen in short-term suppression, Treg-suppressed T helper cells produced dramatically lower level of IL-2 than non-suppressed T helper cells. FoxP3 mRNA was up-regulated in suppressed T helper cells compared to non-suppressed cells, while no significant difference of NFAT2 mRNA expression was observed in non-suppressed versus suppressed T helper cells. These results are similar to those shown in Figure 13.
Cat 3
Appendix B

Trouble Shooting of Feline ChIP

In our original ChIP experiments, rabbit IgG control antibody gave an unacceptably high background. Only in the PMA/I activation studies did the induced NFAT2 binding to the IL-2 promoter in activated lymphocytes produced a strong enough signal over the high IgG background (Figure 7). The two ConA/Ionomycin stimulation studies (Figure 12 and Figure 17) produced an inducible signal that was below IgG background. To find out the cause of the high IgG background and to get rid of it, we did following experiments.

1. Check PCR product contamination

The first step was to determine if the chromatin had been contaminated with PCR product, which may result in the amplification in IgG pull down. To do that, we designed a new set of primers amplifying a new PCR product which did not overlap with the original one. These primers are less than 100bp downstream of NFAT2 binding sites and include the beginning of IL-2 mRNA, therefore they are close enough to NFAT2 binding sites to detect the binding signals if there is any. Primers are shown in Table 4. The real-time PCR result shows that IgG still gives a high background signal, which rules out the possibility of original PCR product contamination.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>GAPDH</td>
<td>5’-taacctcaactctgccacc-3’</td>
<td>5’-ccattcaaaagcaacegtaatcc -3’</td>
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Table 4. New primers used to detect feline NFAT2 binding to the feline IL-2 promoter
2. Check the integrity of chromatin

During the preparation of chromatin, if MNase digestion is not complete, then the chances are that long chromatin may give a higher IgG background. To check if the chromatin was properly digested, 50 µl chromatin, 8 µl 5M NaCl and 1 µl RNase A (10 mg/ml) were added to 150 µl H₂O, vortexed and incubated 4 hr at 65°C. 2 µl 5 mg/ml Proteinase K was added followed by incubation for 90 min at 42°C. DNA was extracted with equal volume of 50:50 phenol:chloroform and then equal volume of chloroform. 600 µl 100% EtOH was added to DNA extraction followed by vortexing and precipitation for 20 min at -20°C, then centrifugation for 15 min at 4°C at maximum speed. The pellet was washed in 500 µl 70% EtOH, vortexed, and centrifuged 5 min at 4°C at maximum speed. The pellet was air-dried for 5 min at RT, resuspended in 20 µl TE, and run on 2% agarose gel. The majority of DNA ran as ~300 and ~450 bp bands, suggesting that chromatin were properly digested.

3. Use a modified ChIP protocol

It is possible that the wash steps in the original protocol were not stringent enough to remove non-specific bound IgG. Therefore, the wash protocol was modified. 10µl of Protein G magnetic beads (Invitrogen) were used per IP. Beads were washed twice in 100µl 1xRIPA/ssDNA (1ml 10xRIPA (100mM Tris-HCl, pH 7.5; 1.5M NaCl; 10mM EDTA; 5mM EGTA; 10% Triton X-100; 1% SDS; 1% NaDeoxycholate); 5ml 5mg/ml salmon sperm
DNA; 400µl 10% BSA; 3.6ml PCR grade water), resuspended in 50µl 1xRIPA/ssDNA plus 0.5µl each of 100mM PMSF and 100xPIC, and incubated with separate Abs for 1h at 4°C. 5µg anti-human NFAT2 (sc-13033X, Santa Cruz) or 5µg of control rabbit IgG were used. Ab-beads conjugates were washed 3 times in 100µl 1xRIPA/ssDNA, and then resuspended in 85µl 1xRIPA/ssDNA plus 1µl each of 100mM PMSF and 100xPIC and aliquoted to different tubes. 15µl chromatin was added to each tube and rotated overnight at 4°C. Conjugates were washed 2 times in 180µl 1xRIPA/ssDNA (warm at 37°C prior to use), 2 times in 180µl high salt wash solution (500mM NaCl; 0.1% SDS; 1% Triton X-100; 2mM EDTA; 20mM Tris-HCl, pH 8.0, warm at 37°C prior to use), 2 times in 180µl LiCl wash solution (250mM LiCl; 1% NP40; 1% NaDeoxycholate; 1mM EDTA; 10mM Tris-HCl, pH 8.0, warm at 37°C prior to use), plus 2 times in 180µl TE, pH8.0, and then resuspended in 100µl fresh elution buffer (100mM NaHCO₃/1%SDS), followed by 15 min light vortex. Tubes were placed on magnet, and media were transferred to fresh tubes with 2µl 5M NaCl added. Reverse cross-linking by incubating tubes at 95°C for 15 min. 2µl 1mg/ml proteinase K was added and incubated at 37°C for 1h. DNA was purified using buffer PN (Qiagen) and buffer PE (Qiagen), and was washed in 60µl of water. With this new protocol, the signal of IgG binding remained high. A high salt solution containing higher NaCl concentration (750mM and 1M) was also tried. However, the signal of IgG binding was not altered.
4. Check the non-specificity of rabbit IgG antibody

To answer the question if rabbit IgG antibody binding to feline chromatin with high avidity is specific or non-specific, we performed a ChIP reaction using three different antibodies, rabbit polyclonal anti-NFAT2 (specific antibody in our experiment), rabbit polyclonal IgG antibody, and rabbit polyclonal anti-RAG-1 antibody as a non-specific control, as RAG-1 should mainly be expressed in immature T cells and not present in mature T cells or kidney cells (due to the extremely limited amount of T cell chromatin available, we chose to do all the test experiments on CRFK chromatin). In addition to feline chromatin, we also used chromatin from a mouse T cell line as a control. The signal of NFAT2 binding to the IL-2 promoter in CRFK and mouse T cell line were similarly low compared to input, which was expected as CRFK cells don’t make IL-2, and the mouse T cells used were not stimulated. IgG and anti-RAG-1 gave a lower background signal compared to NFAT2 in the mouse ChIP, however, they showed equally high level of background in the feline ChIP. These experiments suggest that rabbit polyclonal antibody may non-specifically bind to feline chromatin with high avidity for some unknown reason.

5. Use a different normalization method

As we cannot get rid of the high IgG background in our experiment, we decide to use a different normalization method in which binding of the specific antibody to the target elements is normalized to somewhere in genome that is known for not containing the binding elements (Sikes et al., 2009; Seitan et al., 2011). In our experiment, we chose GAPDH as
our non-specific binding site. The primers that amplify GAPDH mRNA, but not span two exons are chosen, and the efficacy of primer was tested. Based on this normalization method, Figure 12 and Figure 17 are generated.

Table 5. Primers used to amplify feline GAPDH genomic DNA

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<tr>
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