ABSTRACT

FOGLE, JONATHAN EDWARD. T regulatory Cell Suppression of $CD8^+$ Lymphocyte Responses During FIV Infection. (Under the direction of Mary Tompkins.)

The action of activated $CD8^+$ lymphocytes is critical to the control and elimination of viral pathogens. Impaired $CD8^+$ immune responses are well recognized in lentiviral infections; however, the mechanisms underlying $CD8^+$ impairment are incompletely understood. Using the FIV model for human AIDS, we reported previously that $CD4^+CD25^+$ Treg cells in both the acute phase and long-term, asymptomatic phase of infection are constitutively activated and suppress $CD4^+CD25^-$ T cell immune responses. Building upon these observations, we tested the hypothesis that $CD4^+CD25^+$ Treg cells suppress $CD8^+$ responses to immune stimulation during both the acute and chronic, asymptomatic stages of FIV infection. SPF cats were infected with $NCSU_1$ FIV. During the acute stage of infection, plasma viremia as well as PBMC and LN lymphocyte phenotype was assessed at regular intervals. Unfractionated lymph node, $CD4^+CD25^+$ depleted lymph node, and $CD8^+$ / $CD4^+CD25^+$ co-cultures were assayed for IFNγ production via a feline specific ELISpot. During the chronic, asymptomatic phase of infection, IFNγ mRNA in $CD8^+$ lymphocytes was assessed using real time RT-PCR following $CD8^+$ co-culture with $CD4^+CD25^+$ lymphocytes. Our results demonstrated that the $CD8^+$ nadir at 14 days corresponds to peak plasma viremia and is followed by an increase in $CD8^+$ number to greater than pre-infection values. Ex-vivo depletion of $CD4^+CD25^+$ lymphocytes from lymph node suspensions significantly enhanced the production of IFNγ during the acute phase of infection. Furthermore, co-culture of $CD8^+$ lymphocytes with $CD4^+CD25^+$ lymphocytes results in suppression of $CD8^+$ IFNγ

production during both the acute and chronic stages of infection. The same observations were not evident in uninfected cats evaluated in an identical manner. These results demonstrate the profound suppressive effect of $CD4^+CD25^+$ T regulatory cells on the $CD8^+$ immune response during the acute and chronic stages of FIV infection.

Although the mechanism of $CD4^+CD25^+$ T cell-mediated suppression is controversial, there is strong evidence to suggest that, at least in some models, it occurs via a TGFβ / TGFβRII signaling pathway. We hypothesize that during the early acute stage of FIV lentiviral infection, TGFβ is up-regulated on the plasma membrane of Treg cells (mTGFβ), which engages TGFβRII on the surface of antigen activated $CD8^+$ cells thus transducing a signal through the Smad pathway for G1 cell cycle arrest (anergy) and effectively aborting $CD8^+$ T cell expansion and a sustained $CD8^+$ immune response. The experiments that follow demonstrate up-regulation of mTGFβ in the $CD4^+CD25^+$ subset and up-regulation of TGFβRII in the $CD8^+$ subset of FIV^+ cats as assessed by FACS analysis. Furthermore, we demonstrate Smad 2 phosphorylation in $CD8^+$ targets following $CD4^+CD25^+$ / $CD8^+$ co-culture.

T regulatory Cell Suppression of $CD8^+$ Lymphocyte Responses During FIV Infection.

by
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BIOGRAPHY

Jonathan Edward Fogle was born in Columbia, South Carolina. Prior to his senior year of high school, Jonathan moved to Scottsdale, AZ. He graduated with a degree in veterinary science from the University of Arizona (Tucson, AZ) in 1996. In 1997, he attended Ross University School of Veterinary Medicine (Basseterre, St. Kitts, West Indies). Sponsored by Ross University, he completed his clinical training at Louisiana State University in Baton Rouge, Louisiana and received his Doctorate of Veterinary Medicine from Ross University in 2000. In 2002, Jonathan completed a rotating internship in Small Animal Medicine and Surgery at Auburn University (Auburn, AL). He was accepted into the clinician investigator program (a combined small animal internal medicine residency and PhD program) at North Carolina State University College of Veterinary Medicine in 2002. He completed his residency in 2005 and has been a member of the American College of Veterinary Internal Medicine since 2005.

Jonathan's parents, Robbie and Genie, are residents of Brevard, NC. Jonathan has one older sister, Marie and one younger sister, Susannah. He was married to Callie Anne Stanley of Stonewall, Louisiana in 2002. Callie is currently an assistant professor of equine surgery at North Carolina State University College of Veterinary Medicine.

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LIST OF ABBREVIATIONS

AIDS.....Acquired Immunodeficiency Syndrome
ANA.....Anti-Nuclear Antibody
APC.....Allophycocyanin
ATF.....Activated Transcription Factor
CCR5.....Chemokine Receptor 5
CFSE.....Carboxyfluorescein Succinimidyl Ester
ConA.....Concanavalin A
CTLA-4.....Cytotoxic T Lymphocyte Antigen 4
CXCR4.....Chemokine Receptor 4
dnTGFβRII.....dominant negative TGFβ Receptor II
EBV.....Epstein-Barr Virus
FACS.....Fluorescent Activated Cell Sorting
FITC.....Fluorescein Isothiocyanate
FRV.....Friend Virus
GITR.....Glucocorticoid Induced Tumor Necrosis Factor Receptor
HCV.....Hepatitis C Virus
HIV.....Human Immunodeficiency Virus
HSV.....Herpes Simplex Virus
IFNγ.....Interferon Gamma
KO.....Knock Out
LN.....Lymph Node
mTGFβ.....membrane TGFβ
NFAT.....Nuclear Factor of Activated T cells
PE.....Phycoerythrin
PerCp.....Peridinin chlorophyll protein C complex
RV.....Rota Virus
SIV.....Simian Immunodeficiency Virus
SFC.....Spot Forming Cell
SPF.....Specific Pathogen Free
TGFβ.....Transforming Growth Factor Beta
TGFβRII.....Transforming Growth Factor Beta Receptor II
Treg.....T regulatory cell

I. INTRODUCTION

In the early 1980s, physicians in New York and San Francisco began to notice unusual clusters of diseases in homosexual men. They had observed an increase of *Pneumocystis carinii* pneumonia in both cities and at the same time, doctors in New York noted an increase in Kaposi's Sarcoma (7). This type of cancer was rare and typically associated with elderly patients. As more and more unusual diseases began to occur among homosexual men, blood transfusion patients, and intravenous drug users, an acquired immune deficiency syndrome (AIDS) became recognized and named. As we well know today, these diseases are a result of immunodeficiency caused by infection with the human immunodeficiency virus (HIV). The human immunodeficiency virus was discovered by two French virologists, Francoise Barré-Sinoussi and Luc Montagnier in 1983; and an American virologist, Robert Gallo in 1984. In 2008, Barré-Sinoussi and Montagnier won the Nobel Prize in Medicine for their discovery and Robert Gallo is credited with establishing the link between the virus and the development of AIDS.

Today the estimated number of people worldwide living with HIV / AIDS has reached an all time high of 33 million. Approximately 2.7 million new HIV infections were recorded in 2007 worldwide (7). The majority of HIV / AIDS infected people are in sub-Saharan Africa, but Southeast Asia and Eastern Europe have experienced an alarming proliferation of newly diagnosed cases. On a local level, the state of North Carolina had an estimated number of 21,593 persons living with HIV / AIDS at the end of 2007 (90). Prevention remains the most effective strategy at combating the spread of HIV, however it is

estimated that prevention services reach less than 10% of the individuals at risk worldwide (83).

Retroviral drug therapy has proven highly effective in treating HIV infections and many people in industrialized countries have access to therapy. However, access to anti-retroviral therapy in developing nations is problematic due to the expense and more importantly due to the lack of health care infrastructure (7, 33). It is important to recognize that for every new patient placed on anti-retroviral therapy, between two and three new infections have occurred (7, 33). The World Health Organization and the United Nations have reported that aside from the humanitarian crisis, the AIDS pandemic also presents an economic crisis by eliminating much of the viable adult workforce in developing nations (83). Experts worldwide agree that controlling the HIV / AIDS pandemic involves a combination of prevention, education and anti-retroviral therapy in the short term. Long term strategies include all of the short term strategies plus the search for a vaccine, better anti-retroviral therapies, and however unlikely, perhaps even a cure. In order to meet the long term goals, we must continue to dedicate time and resources to the study of AIDS lentiviral pathogenesis. Just as controlling HIV / AIDS requires a combination of strategy, so does studying HIV / AIDS pathogenesis. We should utilize all tools available to study this disease during both the acute and chronic stages of infection and the well characterized FIV model continues to be an important tool in studying HIV / AIDS pathogenesis.

Access to healthcare, fear, denial, and even the stigma of an AIDS diagnosis are obstacles to studying acute HIV infections. The route of inoculation, the amount of virus

exposure and the presence of other infections also varies between HIV infected individuals. We believe that key events dictating the course of lentiviral infections occur during the first few weeks of infection and that animal models enable us to study these early events under controlled conditions of infection.

Primates are more closely related to humans and SIV is more closely related to HIV. However, SIV causes disease only in macaques, which are not its normal host, and does not cause disease in its natural African primate hosts such as green monkeys and chimps. FIV is an infection that results in a progressive disease process in its natural host (the domestic cat) that is identical to that of HIV in humans, including early $CD4^+$ and $CD8^+$ cell dysfunction, progressive loss of $CD4^+$ cells, and development of secondary infections, wasting disease, neurologic dysfunction, and B cell lymphomas. Cats can be housed, maintained, and handled much more easily and cost effectively when compared to non-human primates. This allows greater numbers of animals to be used in experiments, which increases the probability of obtaining reproducible and statistically meaningful data. As with any animal model, the time of infection and dose of virus can be carefully controlled, allowing us to study the very early period (hours to a few days) post infection which is not possible in HIV infections. Infection of specific pathogen free cats allows for the study of the interaction between the virus and the host's immune system without confounding effects of secondary infections, which cannot be done in non-human primate models or in HIV infections.

II. BACKGROUND AND LITERATURE REVIEW

A. SIV and FIV as Animal Models for HIV pathogenesis

Simian immunodeficiency virus (SIV) collectively describes a large family of lentiviruses whose natural hosts are African primates such as chimpanzees and African green monkeys. SIV has not been reported to cause disease in African primates, likely due to a long co-evolutionary history, and different species have evolved different responses to infection. For example, African green monkeys maintain a high viral load, but infection appears to be asymptomatic, whereas chimpanzees completely clear the virus following infection (110). In 1985 researchers discovered that SIV infection of Asian primates such as rhesus macaques, who are not natural hosts, causes disease similar to HIV-1 infection in humans (31). The two most common SIV isolates utilized in macaque studies are designated SIVmac 251 and SIVmac 239, and will be collectively referred to as SIV or SIVmac for discussion purposes. One of the major arguments for the use of SIV is that non-human primates are more closely related to humans phylogenetically, and thus their physiologic and immunologic responses to the virus are more similar than other animal models. SIV is also more closely related to HIV and shares somewhat more structural and sequence homology than other lentiviruses such as FIV (20, 68, 136).

Rhesus macaques can be infected intravenously, rectally, vaginally and even orally with SIVmac (70). The disease course is similar to that of HIV in that inoculation leads to an acute viremic phase characterized by generalized malaise, fever and lymphadenopathy that then transitions into an asymptomatic phase. The asymptomatic phase is characterized by a

progressive decline in $CD4^+$ T cell counts and an overall loss in lymphocyte function. Loss of lymphocyte function leads to the development of opportunistic infections that characterize the progression to AIDS, and eventually death (110).

Inoculation with SIVmac results in peak viremia 7-10 days post infection and at approximately three weeks post infection, there is a strong CTL response that correlates with a decline in viral load (91). Seroconversion with the production of neutralizing antibody is typically observed at week four. The viral set point is reached at approximately seven weeks and is dictated by the quality of the $CD8^+$ and humoral immune responses (37). SIV and HIV both utilize CD4 as a primary receptor, and CCR5 as a co-receptor to facilitate cell entry. SIV does not use CXCR4 which is a co-receptor utilized by both HIV and FIV for cell entry (110).

The neutralizing antibody titer to SIV has been shown to be predictive of disease outcome and animals with the lowest neutralizing antibody titers exhibit rapid disease progression, while animals with high neutralizing antibody titers exhibit the longest survival times, similar to HIV-1 infection (37). Approximately 25% of infected macaques are characterized as rapid progressors- they exhibit little to no neutralizing antibody production, rapid loss of T cell function, the development of neurological symptoms and death within a few months of infection. Intermediate progressors are characterized by an intermediate neutralizing antibody response and survive an average of 3 years before the progression to AIDS. A small percentage of macaques are long term non-progressors, these animals are characterized by the highest neutralizing antibody response, which probably indicates a more

functional immune system overall (37, 110). Long term non-progressors may offer particular insights into genetic determinants, such as MHC variability, that confer protection to a small proportion of individuals in SIV and HIV.

Feline immunodeficiency virus is a natural infection of domestic cats and was first described by Peterson and colleagues in 1987 (97). FIV is most closely related to the lentiviruses of small ruminants however, the course of disease is very similar to that of HIV infection (136). Like HIV, transient clinical signs such as pyrexia and lymphadenopathy are noted during the early stages of acute FIV infection. The acute stage of infection lasts from 2-6 months and is followed by an asymptomatic phase that can last many years. The progression of HIV / FIV is signaled by the development of one or more AIDS related diseases, collectively known as AIDS related complex (ARC) phase, which typically lasts a few weeks or months before the development of fulminant AIDS (11, 136).

Although the natural route of transmission is by bite wounds, FIV can be transmitted experimentally by intravenous and trans-mucosal routes (11, 19, 97). Peak plasma viremia occurs 2-3 weeks post infection and is accompanied by profound lympholysis resulting in generalized B and T lymphocyte depletion (11, 20). In HIV, SIV and FIV, an early rebound in $CD8^+$ cell count and CTL activity corresponds with a decline in plasma viremia (136, 145). This early increase in the peripheral blood $CD8^+$ cell count combined with the decreased $CD4^+$ cell count, leads to the inverted $CD4^+$ to $CD8^+$ ratio that is associated with lentiviral infections. HIV infection of $CD4^+$ lymphocytes was identified early in HIV pathogenesis studies. Our laboratory was the first to report that FIV infects $CD4^+$ T cells,

$CD8^+$ T cells and B cells as well as monocytes and dendritic cells and subsequent to this report, HIV was likewise shown to infect $CD8^+$ lymphocytes and B lymphocytes (41, 42). We have also reported that $CD4^+CD25^+$ T regulatory cells appear to support a productive FIV infection and the same has since been demonstrated for HIV (66, 84). Unlike HIV, FIV does not use CD4 to facilitate cell entry. However, CXCR4 is utilized as a co-receptor by HIV, and is the primary receptor utilized by most strains of FIV to facilitate cell entry (66, 144, 146). Evidence suggests that CXCR4 up-regulation in activated T cells renders them more susceptible to viral entry (102). Co-receptor usage by FIV has not been fully characterized, but CCR5 and CCR3 have been implicated as possible co-receptors in facilitating viral entry. FIV infection results in a strong neutralizing antibody response but the role of neutralizing antibody in controlling infection is unclear, and unlike SIV, this response does not appear to correlate with disease progression (8, 131).

The development of AIDS is the dramatic culmination of insidiously progressive immune dysfunction and it is quite clear that immune dysfunction begins early during the course of lentiviral infections. For example, early in HIV infection, $CD4^+$ lymphocytes exhibit reduced proliferative capacity in response to HIV recall antigens, although $CD4^+$ proliferative capacity against other recall antigens appears to be intact (101). Conversely, the response to previously unencountered infectious organisms appears to be severely impaired. Using a feline ocular toxoplasmosis model developed by Davidson and colleagues, our laboratory showed that asymptomatic, FIV^+ cats, eighteen weeks post FIV infection exhibited profound respiratory disease and high mortality when challenged with a mild strain

toxoplasmosis. In contrast, FIV negative, control cats displayed modest clinical signs including transient anorexia and lethargy along with chorioretinitis that resolved in about three weeks (32). One of the more puzzling aspects unique to lentiviral infections is the development of chronic, generalized immune activation in the face of obvious immune dysfunction. In both HIV and FIV, $CD4^+$ and $CD8^+$ lymphocytes display an activated phenotype (129, 136). Our laboratory reported that the presence of $CD8\alpha^{hi}\beta^{lo}CD62L^-$ lymphocytes correlated with a reduction in plasma viremia early during the course of FIV (17). The progressive replacement of naïve $CD8^+$ lymphocytes with the $CD8\alpha^{hi}\beta^{lo}CD62L^-$ activated phenotype suggests chronic immune activation. Furthermore, FIV infection resulted in increased $CD4^+$ and $CD8^+$ lymphocyte apoptosis associated with up-regulation of the costimulatory molecules CD80/86 (B7) and their ligand CTLA-4 (134). A constant cycle of immune activation and subsequent apoptosis helps to explain, in part, the eventual immune exhaustion and lymphopenia that defines the development of AIDS and the eventual progression to death.

B. A Historical Perspective of T regulatory cells.

The phenomenon of suppressor T cells was first introduced in the 1970's by R. K. Gershon, when populations of T cells were observed to inhibit the responses of cytotoxic T cells and B lymphocyte antibody production (115). Because the mechanism of suppression was unclear, and initial explanations were dismissed, T suppressors were all but ignored for the next ten years. As different murine models of autoimmune disease were developed and studied, it became evident to many researchers that autoimmune T cells were escaping

thymic deletion. These autoimmune T cells could remain silent in the periphery for long periods of time, or could be activated by various means to produce autoimmune disease. Different groups theorized that there were also subpopulations of T cells which were suppressing these autoreactive T cells. These groups of researchers began systematically depleting different T cell subsets in an effort to produce autoimmune disease in different murine models. One particular subset was defined by constitutive expression of CD25, the alpha chain of the IL-2 receptor. Sakaguchi et al (108) demonstrated that $CD4^+CD25^+$ T cells were able to prevent the development of autoimmunity. They harvested splenic and LN $CD4^+$ T cells from BALB/c nu/+ mice and then depleted $CD25^+$ cells using mAb and complement. The $CD25^+$ depleted fraction was then administered to BALB/c nu/nu mice in three different doses- $1x10^8$, $5x10^7$, or $1x10^7$ cells per mouse. The group that received the highest dose ($1x10^8$) died within 3 weeks of a severe wasting disease. After 3 months, the other two groups developed a constellation of autoimmune inflammatory lesions such as insulinitis / diabetes mellitus, adrenalitis, gastritis, thyroiditis, oophiritis, sialoadenitis, glomerulonephritis and arthritis. They then convincingly proved that co-administration of $CD4^+CD25^+$ lymphocytes along with the $CD4^+CD25^+$ depleted fraction prevented the development of inflammatory lesions in a dose-dependent fashion.

Shortly thereafter, using a murine model of autoimmune gastritis, Shevach's group demonstrated that $CD4^+CD25^+$ T cells were essential to inhibiting autoimmune gastritis (118). They first demonstrated that there were autoreactive T cells against H/K ATPase in normal (unaffected) BALB/c mice. They then showed that $CD4^+CD25^+$ cells from adult

mice, injected into 3 day thymectomized mice, prevented the development of autoimmune gastritis. In a second, more elegant experiment, H/K ATPase autoreactive T cell lines were transferred into nu/nu recipients, inducing autoimmune gastritis. Co-transfer of $CD4^+CD25^+$ cells purified from normal spleen prevented the development of gastritis in nu/nu recipients. Similar to the findings by Sakaguchi et al, they clearly demonstrated that co-transfer of $CD4^+CD25^+$ cells prevented the development of autoimmune inflammatory lesions.

Shevach's group also showed in two different experiments, that once activated via their TCR, $CD4^+CD25^+$ inhibition of polyclonal T cell activation was antigen non-specific. The first experiment used T cells harvested from transgenic mice with TCRs specific for either pigeon cytochrome C (PCC) or influenza hemagglutinin (HA). When HA specific $CD4^+CD25^-$ cells were stimulated with HA in the presence of HA specific $CD4^+CD25^+$ cells, the anti-HA response was effectively inhibited. More importantly, when HA specific $CD4^+CD25^-$ cells were HA stimulated in the presence of activated, PCC specific $CD4^+CD25^+$ cells, the response was also effectively inhibited. The second experiment demonstrated that $CD4^+CD25^+$ lymphocytes stimulated with anti-CD3 and IL-2 markedly suppressed $CD4^+CD25^-$ proliferative responses with a major mechanism of suppression was via the inhibition of IL-2 production in the $CD4^+CD25^-$ targets (126). They also showed that these $CD4^+CD25^+$ cells were anergic. As stated above, the $CD4^+CD25^+$ cells were stimulated with anti-CD3 and IL-2 and unlike their $CD4^+CD25^-$ counterparts, these cells did not proliferate after 7 days in culture. However, stimulation did promote potent suppressor function in the $CD4^+CD25^+$ subset (125, 126). In the span of a few short years, several of the major

defining characteristics of T regulatory cells were discovered including, the $CD4^+CD25^+$ phenotype, the ability to inhibit T cell activation (particularly in autoimmune models) in an antigen independent fashion, the inhibition of IL-2 production by target cells, and the anergic response to CD3 and IL-2 stimulation.

C. Phenotypic and Functional Characteristics of T regulatory Cells

In 2001, researchers identified mutation in the FOXP3 gene as the cause for scurfy in mice and IPEX in humans. Scurfy and IPEX are characterized by marked immunoproliferation leading to autoimmune polyendocrinopathy and enteropathy. Schubert and others (112) showed that FOXP3 is a forkhead transcription factor which binds DNA adjacent to NFAT regulatory sites and represses NFAT mediated IL-2 transcription in $CD4^+$ T cells. They also demonstrated that over expression of FOXP3 attenuated T cell responses to inflammatory signals. Mutations of the forkhead region reduced FOXP3 mediated suppression (112). FOXP3 was eventually shown to be essential to the development of $CD4^+CD25^+$ regulatory T cells by different groups of researchers. Similar independent experiments demonstrated that retroviral transduction of the FOXP3 gene into FOXP3 defective mice converted the transduced T cells into a regulatory phenotype; and more importantly these cells were fully capable of suppressing $CD4^+CD25^-$ responses (47, 58). In one such experiment, FOXP3 was transduced into $CD4^+CD25^-$ lymphocytes using a MIGR1 retroviral vector with a GFP reporter (58). GFP positive cells proliferated poorly with anti-CD3 and IL-2 stimulation and the higher the amount of GFP (corresponding to higher levels of FOXP3), the greater the surface expression of CD25, GITR, and CTLA-4. The transduced

cells prevented $CD4^+CD25^-$ proliferation in vitro in a cell contact dependent manner. Most importantly, the FOXP3 transduced cells prevented autoimmune gastritis and colitis in vivo in a mouse model of autoimmune disease.

Structurally, FOXP3 has four distinct domains (21). The forkhead domain, as described earlier, is responsible for DNA binding. The leucine zipper domain is responsible for homo- and hetero-dimerization. There is a zinc finger domain whose function has yet to be fully defined. The fourth domain is found at the amino terminus and is rich in proline residues. This region physically interacts with NFAT, and both FOXP3 and NFAT bind DNA at the IL-2 promoter region (149). Alterations of all but the zinc finger domain have been shown to cause IPEX in humans (21). The downstream cascade of TCR signaling in T regs has been incompletely defined; however, FOXP3 appears to reduce recruitment of Zap70, and IL-2 treatment (in $FOXP3^+$ cells) leads to activation of STAT-5 (132). The alterations in the TCR signaling pathway likely account, at least in part, for the lack of proliferation and activation of suppressor function following TCR stimulation. Finally, Chen et al (22) demonstrated that ectopically expressed FOXP3 could bind to CD25, CTLA-4 and GITR promoter regions and induce transcription.

T regulatory cell phenotype has been more difficult to define, particularly because activated T helper cells and Tregs share many common surface markers. Several markers including: CD25, CTLA-4, CD80 / CD86 (B7.1 and B7.2), and GITR help to define the T regulatory phenotype. The most well recognized marker, CD25 (the high affinity IL-2 receptor alpha) is constitutively expressed by T regulatory cells and is integral to T

regulatory function (124, 135). In a mouse model, antibody neutralization of IL-2 led to an overall decrease in regulatory T cells and more importantly led to the development of autoimmunity similar to that found with $CD4^{+}CD25^{+}$ T cell depletion (114). The primary source of IL-2 required for in vivo activation of Tregs is activated lymphocytes that are responding to inflammatory signals. In response to IL-2 stimulation, the T regulatory cell then exhibits heightened suppressor function and is able to temper inflammatory responses in a negative feedback loop (107). Similar to CD25, T regulatory cells also constitutively express CTLA-4, with T helper cells displaying CTLA-4 only after activation. CTLA-4 binds CD80 and CD86 (B7.1 and B7.2) on activated T cell surfaces and delivers an inhibitory signal via CD80 / CD86 which halts the proliferative response (64). What is uncertain, however, is whether CTLA-4 is one of the primary mechanisms of T regulatory mediated suppression or if it is a co-inhibitory molecule that helps to strengthen the primary inhibitory signal. CTLA-4 as a suppressive mechanism will be discussed in more detail below. In FIV infection, T regulatory cells also exhibit increased expression of CD80 / CD86, indicating a chronic activation state. However, the significance of B7 signaling to T regulatory function remains unclear (135). Glucocorticoid induced tumor necrosis factor receptor (GITR) is also constitutively expressed on T regulatory cell surfaces and like CD25 and CTLA-4, is also expressed by activated T cells. This receptor seems to play a dual role in mediating T regulatory cell activity. GITR ligand (GITR-L) is expressed on the surface of immature dendritic cells, macrophages and B cells and GITR signaling may be one of the "tolerizing" mechanisms contributing to T regulatory activation, as GITR-L expression is

greatly reduced upon maturation of the aforementioned cell types (106). GITR is also up-regulated on activated T effector cell surfaces and its ligation renders the effector T cell resistant to Treg mediated suppression, which again is likely another feedback loop (137). More recently, we have examined membrane bound TGFβ on T regulatory cell surfaces as a phenotypic and functional marker of activation and its role will be thoroughly discussed below. A subtle but important point is that $CD4^+$ T regulatory cells constitutively express CD25, CTLA-4, and GITR, while $CD4^+$ T helper cells can also express these molecules upon activation; and both subsets express the B7 antigens upon activation. This plasticity can make isolation and evaluation of T regulatory cells by surface phenotype difficult, particularly during acute stages of infection when the T helper compartment is activated and mobilized.

T regulatory mediated suppression of target $CD4^+$ and $CD8^+$ effector cells is incompletely understood. As in other signaling modalities in the immune system, Tregs probably rely on a combination of signals to effectively induce anergy and there are also differences between in vitro and in vivo mechanisms of inhibition (137). No matter what mechanism or mechanisms are utilized by Tregs, three important facts are apparent. First, the target of T regulatory mediated suppression is to reduce the transcription of a cluster of important cytokines such as IL-2 and IFNγ in effector cells (137). Second, Tregs behave like other T cells in that they have homing and chemokine receptors attracting them to sites of inflammation and as stated earlier, Tregs appear to be activated by smaller amounts of antigen than T effector cells. Third, cell to cell contact is essential for Treg mediated

suppression in most in vitro and in vivo experimental models.

Different experimental models have yielded a variety of possible mechanisms for T regulatory mediated suppression. The interaction between CTLA-4 on Tregs and CD80/CD86 has been investigated as a possible mechanism of T effector suppression. As has been mentioned previously, the role CTLA-4 plays in suppression is quite complex. There are at least three possible scenarios for the CTLA-4 and CD80/86 interaction, two of which result in T regulatory mediated suppression of T effector cells and one in which T - effector cells modulate each other, becoming refractory to T regulatory mediated suppression. However complex this interaction may be, the components consist of a T regulatory cell, a T effector cell, and an APC (137). The first and simplest scenario is that an APC activates a T regulatory cell and a T effector cell via antigen presentation- these two cells are activated and in close proximity. Then CTLA-4 on the T regulatory cell surface is then able to ligate CD80/86 on the T effector surface and shut down activation (137). In a second more complex scenario, activated T effector cells display both CTLA-4 and CD80/86 and are therefore able to shut down the activation of neighbor T effector cells and vice-versa. In this scenario the T effector cells are less vulnerable to T regulatory mediated suppression and activated T effector cells may actually be *inhibiting* the T regulatory cell if the T effector ligates CD80/86 on the Treg cell surface (64, 134). In the third scenario, a Treg is activated by the APC. Constitutively expressed CTLA-4 on the Treg surface then ligates CD80/86 on the APC, increasing indoleamine 2, 3 dioxygenase within the APC, therefore reducing available levels of tryptophan necessary for T effector activation. In vitro and in vivo studies

have suggested that CD80/CD86 ligation by CTLA-4 accounts for at least partial T effector suppression, but the dual role this mechanism plays in the relationship between Treg and T effector probably relegates it to a secondary pathway whose purpose is to strengthen the primary suppressive signal (96, 122).

Granzyme A and B dependent T regulatory suppression has also been described. Granzymes are serine proteases whose main action is to activate intracellular caspases, initiating programmed cell death and they function more efficiently when released in conjunction with perforin, which polymerizes in target cell membranes to form open channels (64). Grossman and others (52) reported that after CD3/CD46 stimulation, $CD4^+CD25^+FOXP3^+$ lymphocytes isolated from human blood showed increased granularity caused by accumulation of cytoplasmic Granzyme A. In co-culture experiments, these stimulated T regulatory cells were able to kill autologous $CD4^+$, $CD8^+$ and $CD14^+$ target cells in a Granzyme A – perforin- dependent fashion, as perforin inhibitors blocked target cell killing. Interestingly, activated $CD4^+$ and $CD8^+$ target cells were more susceptible to T regulatory killing than non-activated controls. In a mouse model, Gondek et al (49) reported that treatment of activated Tregs with anti-GITR antibody caused down-regulation of Granzyme B in vitro. Following these observations, they also demonstrated that Treg function was reduced in Granzyme B knockout mice, suggesting that one mechanism of T regulatory suppression was contact mediated, Granzyme B secretion with apoptosis of the target cell. Granzyme mediated killing of activated $CD4^+$ and $CD8^+$ targets is probably more relevant to autoimmune and transplantation models, because it appears that in many

infectious disease models, effector cells are anergized and not destroyed by Treg interactions (49, 52).

Anti-GITR antibody treatment has also been utilized to block regulatory T cell function. In a series of experiments, Shimizu et al (116) reported that treatment of murine $CD4^+CD25^+$ T regulatory cells with anti-GITR antibody blocked in vitro suppression. They also reported that treatment of spleen cell suspensions with anti-GITR antibody, prior to adoptive transfer, was equivalent to the level of autoimmunity observed by $CD25^+$ depletion. They then went on to show that in vivo administration of anti-GITR antibody to mice did not deplete $CD4^+CD25^+$ T regulatory cells, but did inhibit T regulatory cell function, resulting in autoimmunity. Using a similar strategy, Dittmer and colleagues (36) showed that administration of anti-GITR antibody to mice persistently infected with Friend virus (FV) inhibited T regulatory cell function. Treatment with anti-GITR antibody, in combination with adoptive transfer of $CD8^+$ lymphocytes resulted in a significant reduction in viral load.

T regulatory cells may also trap T effector cells in the lymph node by inducing a reduction in T effector chemokine receptor expression. In a murine autoimmune diabetes model, Tregs were shown to reduce IFNγ production by $CD4^+CD25^-$ effector cells (109). Interferon gamma promotes expression of CXCR-3, and reduction of IFNγ led to a marked reduction in the level of effector T cell CXCR-3 expression. Without CXCR-3 driven chemotaxis, the pancreatic T cell inflammatory infiltrate was markedly reduced, essentially trapping the effector cells in the pancreatic lymph nodes.

Another intriguing strategy that T regulatory cells employ is the conversion of T

effector cells into T regulatory cells, thereby amplifying the suppressive response. Work in our laboratory and by others has demonstrated that mitogenic stimulation of $CD4^+CD25^-$ T cells in the presence of soluble TGFβ can convert these cells into fully functional $CD4^+CD25^+FoxP3^+$ T regulatory cells (1, 25, 98). This mechanism may be important for tempering the inflammatory response to pathogens, thus preventing collateral damage associated with uncontrolled inflammation.

As early as 2001, Nakamura and others (88) reported that in mice, $CD4^+CD25^+$ lymphocytes but not $CD4^+CD25^-$ lymphocytes displayed high levels of TGFβ on their surface and also produced soluble TGFβ in response to CD3 and CTLA-4 stimulation . They reported that inhibition of $CD4^+CD25^-$ proliferation and B cell immunoglobulin production by $CD4^+CD25^+$ cells was contact dependent. Treatment of $CD4^+CD25^+$ cells with anti-TGFβ antibody prevented contact mediated suppression suggesting that surface TGFβ was responsible for contact mediated suppression. In contrast to Nakamura's findings, Piccirillo et al (100) showed in a series of experiments, using a mouse autoimmune model, that TGFβ was not essential for T regulatory cell suppressor function. Neutralization of TGFβ using either anti-TGFβ antibody or soluble TGFβ receptor did not reverse in vitro suppression. Furthermore $CD4^+CD25^+$ Tregs inhibited proliferation of $CD4^+CD25^-$ and $CD8^+$ lymphocytes from both Smad3 deficient and TGFβRII dominant negative receptor (dnTGFβRII) mice, and $CD4^+CD25^+$ T regulatory cells from TGFβ deficient mice were as effective in suppressing proliferation as wild type Tregs.

Expanding their findings in a follow-up study, Nakamura and others again demonstrated the importance of TGFβ to regulatory T cell activity. They reported that blocking TGFβ with recombinant latency associated peptide (rLAP) blocked both human and mouse $CD4^+CD25^+$ function. They also showed that T regulatory cells from TGFβ deficient mice exhibited suppressor function in vitro, however these cells were incapable of protecting mice from the development of colitis in a SCID transfer model (87). More recently, in vitro and in vivo inhibition of regulatory T cell activity has been achieved using a TGFβ specific peptide inhibitor known as P17 (48). Gil Guerrero et al reported that P17 reversed T regulatory mediated inhibition in vitro, restoring the proliferative capacity of mouse and human effector cells in response to CD3 and antigen specific stimulation. More importantly, they demonstrated that in vivo P17 treatment reduced the overall number of $CD4^+FOXP3^+$ cells. Furthermore, BALB/c mice immunized with an anti-tumor peptide and treated with P17 had strong anti-tumor immunity when compared to mice immunized with the peptide alone. Finally they reported that P17 treatment in combination with p1073 vaccinia peptide immunization protected mice against challenge with a recombinant vaccinia virus. P17 primed a CTL lytic response that was not seen in immunized mice without P17 treatment. In a murine colon carcinoma model, Chen et al (24) demonstrated direct suppression of antigen specific $CD8^+$ lymphocytes by $CD4^+CD25^+$ T regulatory cells. Injection of tumor specific $CD8^+$ lymphocytes resulted in rejection of the tumor, while co-injection of $CD4^+CD25^+$ lymphocytes prevented tumor rejection. Interestingly, T regulatory cells did not appear to alter $CD8^+$ proliferation or activation phenotype. However, in vitro $CD8^+$ cytotoxicity was

greatly diminished in the presence of $CD4^+CD25^+$ Tregs. Most importantly, $CD8^+$ lymphocytes with TGFβDNR co-injected with $CD4^+CD25^+$ lymphocytes exhibited tumor killing kinetics almost identical to wild type $CD8^+$ controls.

It is likely that T regulatory cells utilize different combinations of the mechanisms that have been described above and are able to suppress T cell responses at different points during the process of effector cell activation. For autoimmune and tumor rejection models, the combination of suppressive mechanisms is probably different than those utilized during parasitic or viral infections. This helps to explain the discrepancy between different models of T regulatory mediated suppression. Therefore, the different models produce snapshots of a working mechanism, while each is unable to reveal this dynamic process in its entirety.

D. T regulatory Cell Ontogeny and Pathogen Induced T regulatory Cells

T regulatory cell ontogeny has not been fully defined; however, there are at least two major populations of T regulatory cells. The first major population, which was the first to be discovered, is known as natural T regulatory cells (nTregs), which originate in the thymus. Natural Tregs are an important link between central and peripheral tolerance to self antigen and are superb in their ability to control autoimmune phenomenon (107). Natural Tregs exit the thymus and reside in peripheral lymphoid tissues to control the response to self-antigen. They are activated via their TCR by self peptide in the context of MHC and are generally activated by much lower quantities of self peptide compared with other T cell subsets (4, 62). Once activated, nTregs then act in a non-specific manner, inhibiting all T cells in the immediate vicinity from responding to the antigenic stimulus.

Later studies demonstrated a second, peripherally induced Treg population, known as adaptive Treg cells (aTregs) that modulate immune responses to microbial pathogens including bacteria, viruses, fungi, and intracellular parasites (10, 57, 63). These pathogen-induced $CD4^+CD25^+$ Treg cells can be derived from either $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells in the periphery (135, 139). Adaptive Tregs are indistinguishable from natural Treg cells phenotypically in that they up-regulate CTLA-4, GITR, certain Toll-like receptors, CD62L, surface TGFβ, and most importantly express Foxp3, which is required for Treg homeostasis and function (25, 44, 139).

A compelling argument for Treg cells as major modulators of immune responses to infectious agents derives from the observation that escape of malaria parasites from the host's protective immune response and establishment of a chronic, ultimately fatal infection requires $CD4^+CD25^+$ Treg cells. Hisaeda et al. (55) demonstrated that antibody-depletion of $CD4^+CD25^+$ Treg cells protects mice from death when challenged with a lethal strain of *Plasmodium yoelii*, and that this protection is associated with an increased T helper-dependent immune response to parasite antigens. Belkaid et al. (10) reported that $CD4^+CD25^+$ Treg cells specific for *Leishmania* antigens accumulate and are activated (pathogen-induced) at the site of infection, where they suppress the protective immune response, thereby allowing persistence of the parasite. Depletion of the $CD4^+CD25^+$ T cells enhanced the protective immune responses, resulting in complete eradication of the parasite. However, complete eradication also made the mice vulnerable to re-infection with the parasite, while a low-level, persistent infection conferred protection from subsequent

Leishmania challenge.

Recent data indicate that both primary and memory immune responses to chronic viral infections such as herpes simplex virus type 1 (HSV-1) and HSV-2 are regulated by $CD4^+CD25^+$ Treg cells. Suvas et al (121) reported that in vivo antibody depletion of $CD25^+$ T cells prior to infection increased both primary and memory $CD8^+$ cytotoxic T cell responses to HSV-1 challenge. These authors observed that IFNγ responses of $CD8^+$ T cells stimulated by an HSV-1 peptide were markedly increased in $CD25^+$-depleted mice as measured by flow cytometry and ELISpot assays. Most significantly, enhanced anti-HSV-1 $CD8^+$ immune responses in the $CD25^+$-depleted mice correlated with a more rapid clearance of virus. Together, these data suggest that immunosuppressive Treg cells are activated during acute HSV-1 infection, and that elimination of Treg cells during the acute state of infection could result in a more robust protective $CD8^+$ immune response and more rapid clearance of the virus. In a follow-up study, Suvas et al. (119) employed a murine HSV-induced stromal keratitis model to demonstrate that depletion of $CD25^+$ T cells prior to challenge with HSV-1 increased the severity of the ocular inflammatory lesions that correlated with an increase in HSV-specific $CD4^+$ T cell responses. Using a similar depletion strategy, Toka et al (127) showed that depletion of $CD25^+$ T cells enhanced primary and memory $CD8^+$ responses to a HSV-1 subunit vaccine. When challenged, memory $CD8^+$ T cells generated in mice depleted of $CD25^+$ cells cleared the virus more effectively than control mice.

Instead of targeting CD25, others have targeted GITR in attempts to modulate Treg

responses and influence HSV-1 pathogenesis. La et al. (76) reported that a single dose of anti-GITR antibody immediately following HSV-1 infection in mice significantly increased the number of $CD4^+$ and $CD8^+$ T cells secreting IFNγ. Similar to anti-CD25 treatment, these results suggest that ligation of GITR on Treg cells may also diminish their ability to suppress $CD4^+$ and $CD8^+$ T cell responses to HSV-1 antigens. Following their earlier anti-CD25 experiments, Suvas et al. (120) again used the murine HSV-1-induced keratitis model to demonstrate that virus specific T cell responses in LN and spleen were enhanced in mice treated with anti-GITR antibodies. However, in contrast to the expected increase in the T cell-mediated inflammatory lesions, anti-GITR treatment resulted in significantly diminished T cell-mediated ocular lesions. The reason for the contradictory results of anti-GITR Treg inactivation leading to enhanced T cell immune responses, but diminished cell-mediated ocular inflammation in the keratitis model is not immediately apparent, but likely has to do with the dual role GITR plays in signaling both T regulatory and activated T helper cells (as mentioned previously). More importantly, these studies highlight the sometimes unpredictable immunological consequences of Treg manipulation in different animal models (80). While the HSV-1 keratitis model may be an exception, the important issue that arises from these studies is that depletion of Treg cells allows for a more robust protective T cell response to pathogens, yet the more aggressive $CD4^+$ and $CD8^+$ anti-viral immune response may incite immunopathology in the target tissue. Thus, $CD4^+CD25^+$ Treg cells appear to be an integral component of the immune response to pathogens, controlling the magnitude and duration of the protective immune response and the collateral immunopathology associated

with unchecked inflammation.

E. TGFβ Control of $CD8^+$ Immune Responses

The TGFβ signaling pathway is one of the major homeostatic mechanisms controlling $CD8^+$ lymphocyte and other T cell responses (137). The TGFβ receptor complex consists of two Type I and two Type II transmembrane serine / threonine complexes, which are displayed on the $CD8^+$ cell surface. TGFβ binds to the Type II homodimer, which then associates with the Type I receptor and phosphorylates it. The Type I phosphorylated receptor recruits and phosphorylates Smad2 and 3 leading to conformational changes, dissociation from the Type I receptor, and formation of a trimeric complex with Smad4. The Smad2-p , 3-p, 4 complex translocates to the nucleus where, in collaboration with other co-transcription factors, activates specific sets of genes including the cell cycle arrest genes such as $p21^{cip1}$ leading to anergy (23). Additionally, Smad3 induces Smad7 expression, which then competes with Smad2 and 3 for binding to the Type I receptor, thus providing an inhibitory feedback loop that shuts down TGFβ signaling (45). Mehal et al (82) demonstrated the importance of TGFβ signaling in experiments utilizing either TGFβ KO or dnTGFβRII mice. They reported that TGFβ KO mice died of severe infiltrative autoimmune disease at 3-4 weeks of age and dnTGFβRII mice had a similar but less rapid course of disease. In both the TGFβ KO or dnTGFβRII mice there was significant expansion of peptide specific $CD8^+$ cells which was likely one of the major mechanisms causing the severe immunopathology in these mice. In adoptive transfer experiments using dnTGFβRII

lymphocytes, Yang and others (150) were able to induce autoimmune cholangitis in recipient mice. Adoptive transfer of $CD8^+$ dnTGFβRII lymphocytes resulted in autoimmune liver disease with histopathology similar to primary biliary cirrhosis in humans. In a murine EL-4 tumor model, Gorelik and Flavell (50) reported that blockade of TGFβ signaling via a TGFβRII dominant negative receptor resulted in tumor specific $CD8^+$ lymphocyte expansion and resistance to tumor growth. In a human melanoma study, Ahmadzadeh and Rosenberg (3) demonstrated that TGFβ suppressed the secretion of inflammatory cytokines such as IFNγ. Specific memory $CD8^+$ lymphocytes isolated from human melanoma patients vaccinated with gp100 melanoma antigen. $CD8^+$ cultures were then activated with the melanoma gp100 peptide and TGFβ was added either simultaneously, or after activation and in both instances, TGFβ inhibited the release of IFNγ and GM-CSF. Although TGFβ significantly suppressed $CD8^+$ function, $CD8^+$ lymphocytes maintained an activated phenotype and the addition of TGFβ to cultures did not affect the expression of markers such as CD45RA, CD45RO and CD62L. Finally, Cumont and others (30) have reported that increased TGFβ expression is associated with reduced effector T cell responses in intestinal lymphoid tissue in a study comparing pathogenic and non-pathogenic SIV infections in rhesus macaques and African green monkeys, respectively. The aim of the study was to explore the relationship between viral replication, lymphocyte activation, lymphocyte apoptosis, and disease outcome during the course of acute SIV infection. Macaques that progressed more rapidly had increased expression of both TGFβ and indoleamine 2,3

dioxygenase in intestinal tissue. Furthermore, they demonstrated that blocking TGFβ in vitro enhanced $CD8^+$ proliferation and reduced $CD8^+$ apoptosis.

F. T regulatory Mediated $CD8^+$ Suppression in Other Types of Viral Infection

T regulatory cells are able to modulate the response to a wide variety of viral pathogens including HSV, Epstein-Barr Virus (EBV), Hepatitis C Virus (HCV), Rotavirus (RV) and Friend virus (FRV). There are some common themes shared by these viral models which include T regulatory cell activation and expansion following infection, possible recruitment of pathogen induced regulatory cells, and T effector inhibition. Using the HSV model, a number of investigators have postulated that Tregs contribute to the establishment of a chronic infection. As discussed previously, Suvas et al (121) demonstrated that $CD4^+CD25^+$ depletion, prior to HSV infection, enhanced $CD8^+$ responses to immunodominant peptides 3-4 fold. However, in a second study, they also reported that $CD4^+CD25^+$ depletion led to increased immunopathology associated with a vigorous T effector response (119). Similarly, $CD4^+CD25^+$ depletion in neonatal mice, prior to HSV infection significantly enhanced in vivo $CD8^+$ activity, including proliferation, granzyme B expression, and IFNγ production (46).

Approximately 20-50% of patients with Hodgkin's Lymphoma (HL) have the oncongenic herpes virus EBV present in the lymphoma cells (27, 143, 148). However, the role of EBV in HL has yet to be fully defined. To further characterize the behavior of EBV in HL, Baumforth and others (9) compared micro arrays from EBV infected and uninfected HL cell lines and discovered that infection with EBV increased the expression of CCL20,

which is involved in T cell chemotaxis. Using PBMCs in transwell chemotaxis assays, they demonstrated that medium conditioned with EBV positive HL cells was enriched with $CD4^+CD25^+FOXP3^+$ cells. Increased migration of $CD4^+CD25^+FOXP3^+$ cells was prevented using anti-CCL20 antibody, suggesting that EBV is able to recruit T regulatory cells to tumor sites using this mechanism, which may contribute to both tumor and viral persistence.

Impaired $CD8^+$ IFNγ production and proliferation can occur in chronic HCV infection. Using both in vitro $CD4^+CD25^+$ depletion and $CD4^+CD25^+$ / $CD8^+$ co-culture experiments, Boettler et al (13) demonstrated that Tregs inhibited $CD8^+$ IFNγ production and proliferation in response to HCV antigen. Further, they reported that chronic HCV infection led to in vivo $CD4^+CD25^+$ expansion and impaired $CD8^+$ responses to other viral antigens such as influenza. These findings were confirmed by Ebinuma et al (38), who also reported that exogenous TGFβ contributed to HCV antigen-specific T regulatory cell expansion in vitro. Although T regulatory expansion was antigen specific, inhibition of $CD8^+$ responses to viral antigen again was both antigen specific and antigen non-specific. Their findings suggest a role for HCV induced T regulatory cell expansion which further contributes to $CD8^+$ suppression in vivo.

In a murine model of RV infection, investigators noted that infection stimulated a modest expansion of Tregs, compared to uninfected controls (69). Depletion of $CD25^+$ lymphocytes from spleen and mesenteric lymph node suspensions resulted in significantly increased IFNγ production in both non-stimulated and RV antigen-stimulated lymphocytes. Furthermore, depletion of $CD25^+$ lymphocytes led to increased IFNγ in both $CD4^+$ and $CD8^+$

lymphocytes following polyclonal RV antigen stimulation.

Friend virus is a murine gamma retrovirus that is associated with the expansion of T regulatory cells and the inhibition of $CD8^+$ antiviral responses (36, 103). Dittmer et al (36) demonstrated that $CD8^+$ T cells are capable of recognizing FRV antigen, however their effector function is suppressed by regulatory T cells. They showed that FRV specific $CD8^+$ lymphocytes adoptively transferred to persistently infected mice failed to produce IFNγ. However, in vivo treatment of mice with anti-GITR antibody, prior to adoptive transfer of $CD8^+$ lymphocytes restored $CD8^+$ effector function and reduced viral load, presumably by inhibition of T regulatory function. In a series of FRV experiments, Robertson and colleagues (103) reported that virus specific $CD8^+$ lymphocytes display an activated phenotype and are able to proliferate in response to viral antigen, however, their antiviral activity is greatly impaired, suggesting a form of $CD8^+$ anergy. The co-stimulatory molecule CD137 enhances $CD8^+$ activity and possibly inhibits T regulatory mediated $CD8^+$ suppression (18, 26). In a follow up study, they reported that treatment of $CD8^+$ lymphocytes with anti-CD137 antibody prior to adoptive transfer into chronically FRV infected mice greatly enhanced $CD8^+$ antiviral proliferation and IFNγ production (104). They also demonstrated that treatment of $CD8^+$ lymphocytes with anti-CD137 prior to in vitro $CD4^+CD25^+$ co-culture rendered the $CD8^+$ cells refractory to $CD4^+CD25^+$ mediated suppression.

G. T regulatory Cell Activation and $CD8^+$ Suppression in HIV / FIV Lentiviral Infection

The previous discussion suggests that failure to eliminate certain pathogens and the resulting chronic antigenemia may be due to the repressive immunomodulatory effects of activated $CD4^+CD25^+$ Treg cells on the protective $CD4^+$ and $CD8^+$ T cell immune responses. The FIV model shares many of the similar themes listed above, including, T regulatory cell activation and expansion following infection, recruitment of pathogen induced regulatory T cells, and T effector cell inhibition. Although there is an abundance of information with other viral infection models, there is much less information regarding the role of T regs during AIDS lentiviral infections. One of the major differences between lentiviral infections and other chronic viral infections is that the balance between protective T cell responses and immunosuppressive Treg cell responses is less clear (15, 67). This laboratory was the first to identify a population of $CD4^+CD25^+$ T cells in the cat and describe their phenotypic and functional activation in the FIV-feline infection model for human AIDS (65-67, 135). Importantly, we were also the first to report that lentivirus infection results in the constitutive activation of Treg cells resulting in immune suppression (135). We found that freshly isolated $CD4^+CD25^+$ T cells from asymptomatic FIV^+ cats suppress IL-2 production and the proliferative response of ConA-stimulated autologous $CD4^+CD25^-$ T cells in a dose-dependent manner, suggesting that the Treg cells are activated in vivo in response to FIV infection. Using the FIV model, we were the first to report that $CD4^+CD25^+$ Treg cells provide a reservoir for productive lentivirus replication thus providing a possible mechanism

for their in vivo activation (66, 67).

These data support the hypothesis that $CD4^+CD25^+$ T regulatory cells are able to suppress T effector responses early during lentiviral infections and throughout the course of infection. Similar to our findings from the FIV lentiviral model, investigators have demonstrated that T regulatory cells suppress $CD4^+$ and $CD8^+$ effector responses in HIV infection. Aandahl et al. (2) reported that depletion of $CD25^+$ T cells from PBMC from HIV^+ patients not on HAART enhanced the frequency of IFNγ and TNFα expressing T cells in response to stimulation with HIV and CMV antigens, indicating that HIV-induced Treg cells suppress T cell responses to specific antigens, as well as unrelated antigens. Eggena et al. (39) similarly reported that in vitro depletion of Treg cells from HIV^+ PBMC increased gag-specific $CD8^+$ responses, as measured by IFNγ ELISpot. Kinter et al. (71) reported that $CD4^+CD25^+$ T cells in the majority of healthy HIV^+ patients significantly suppressed cellular proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells stimulated with HIV peptides in vitro. In a follow up study, Kinter et al (72) reported that Treg cells suppress effector function of HIV specific $CD8^+$ cells in vitro.

Although it is evident that T regulatory cells are able to suppress inflammation during the course of lentiviral infections, it is not clear if suppression is always harmful. Several investigations have indicated that $CD4^+CD25^+$ activation may play a protective role during the course of lentiviral infections. In the study mentioned previously, Kinter (71) et al reported that $CD4^+CD25^+$ levels in the blood correlated inversely with plasma viremia. Findings by Kornfeld et al (73) suggest that early induction of an anti-inflammatory

environment protects African green monkeys from developing a pathogenic SIV infection. Further, they theorize the early development of an anti-inflammatory environment is due to the early activation of $CD4^+CD25^+$ and $CD8^+CD25^+$ T regs. In HIV infection, Eggena et al (39) reported that a decline in $CD4^+CD25^+$ Treg cells in the late stage of disease was associated with increased T cell immune hyperactivation and higher viremia. Conversely, it has been reported that there is a significant expansion of $CD4^+CD25^+$ T cells in the blood of HIV^+ patients on anti-retroviral therapy (142). Using the FIV model we hope to further define the role of T regulatory cells and T effector cell mediated suppression during both the acute and chronic stages of infection. The aims of the experiments that follow are to demonstrate that $CD4^+CD25^+$ T regulatory cells inhibit CD8+ effector responses during acute and chronic FIV infection and to explore the role of membrane TGFβ as a mechanism for Treg mediated $CD8^+$ suppression.

III. T REGULATORY CELLS INHIBIT THE $CD8^+$ ANTIVIRAL RESPONSE

A. Abstract

The action of activated $CD8^+$ lymphocytes is critical to the control and elimination of viral pathogens. Impaired $CD8^+$ immune responses are well recognized in lentiviral infections; however, the mechanisms underlying $CD8^+$ impairment are incompletely understood. Using the FIV model for human AIDS, we reported previously that $CD4^+CD25^+$ Treg cells in both the acute phase and long-term, asymptomatic phase of infection are constitutively activated and suppress $CD4^+CD25^-$ T cell immune responses. Building upon these observations, we tested the hypothesis that $CD4^+CD25^+$ Treg cells suppress $CD8^+$ responses to immune stimulation during both the acute and chronic, asymptomatic stages of FIV infection. SPF cats were infected with $NCSU_1$ FIV. During the acute stage of infection, plasma viremia as well as PBMC and LN lymphocyte phenotype was assessed at regular intervals. Unfractionated lymph node, $CD4^+CD25^+$ depleted lymph node, and $CD8^+$ / $CD4^+CD25^+$ co-cultures were assayed for IFNγ production via a feline specific ELISpot. During the chronic, asymptomatic phase of infection, IFNγ mRNA in $CD8^+$ lymphocytes was assessed using real time RT-PCR following $CD8^+$ co-culture with $CD4^+CD25^+$ lymphocytes. Our results demonstrated that the $CD8^+$ nadir at 14 days corresponds to peak plasma viremia and is followed by an increase in $CD8^+$ number to greater than pre-infection values. Ex-vivo depletion of $CD4^+CD25^+$ lymphocytes from lymph node suspensions significantly enhanced the production of IFNγ during the acute phase of infection. Furthermore, co-culture of $CD8^+$ lymphocytes with $CD4^+CD25^+$ lymphocytes results in suppression of $CD8^+$ IFNγ production

during both the acute and chronic stages of infection. The same observations were not evident in uninfected cats evaluated in an identical manner. These results demonstrate the profound suppressive effect of $CD4^+CD25^+$ T regulatory cells on the $CD8^+$ immune response during the acute and chronic stages of FIV infection.

B. Introduction

In both FIV and HIV infection progressive immune dysfunction is evident during the course of infection (53, 129). Early immune dysfunction is characterized by the reduced capacity to respond to FIV / HIV antigens, poor response to recall antigens, and by the diminished capacity to mount a primary cell mediated immune response to new infections (32, 35). Later in the course of disease, immune dysfunction is characterized by a loss of mitogenic responses, a reduction in $CD4^+$ T cell counts, and the development of opportunistic infections signaling the onset of AIDS (20, 53, 129).

An early $CD8^+$ T cell lymphocytosis is associated with a decline in plasma viremia in both FIV and HIV infection and a strong $CD8^+$ anti-lentiviral response enables the clearance of virus from circulation, while a weaker response is associated with poor or no control of viral replication (16, 17, 34, 105). Likewise, in an SIV model of HIV, $CD8^+$ depletion led to increased viremia and more severe disease (111). These combined results suggest that the $CD8^+$ response plays a major role in the control of viral replication, which ultimately dictates both the viral set point and perhaps the long term outcome of infection (14, 16, 56, 74). More importantly, these results indicate that even in the face of a robust $CD8^+$ response, there appear to be both viral and host factors that allow the virus to escape elimination and

establish and maintain a chronic infection.

In both acute and chronic viral infections, there appear to be immunoregulatory mechanisms in place to control inflammatory responses that might prove detrimental to the host (119, 129, 130). A key component of the virus-host interaction are $CD4^+CD25^+$ T regulatory cells which temper the inflammatory response, but may also be permissive in the development of chronic infections. In a variety of different viral infections such as HSV, HCV, and Friend virus, $CD4^+CD25^+$ T regulatory cells appear to play a pivotal role in the immunopathogenesis of these infections (9, 13, 103, 121). Suvas et al (121) demonstrated that $CD4^+CD25^+$ depletion, prior to HSV infection, enhanced $CD8^+$ responses to immunodominant peptides 3-4 fold. Boettler et al (13) demonstrated that Tregs inhibited $CD8^+$ IFNγ production and proliferation in response to HCV antigen. Furthermore, they reported that chronic HCV infection led to in vivo $CD4^+CD25^+$ expansion and impaired $CD8^+$ responses to other viral antigens such as influenza. The common theme of these models is that depletion of $CD4^+CD25^+$ Tregs leads to enhanced $CD4^+$ and $CD8^+$ effector responses, both against viral antigens and against unrelated recall antigens, and this heightened effector activity may lead to viral clearance. Conversely, depletion of $CD4^+CD25^+$ Tregs may also result in increased immunopathology associated with unchecked inflammation. For example, in a murine HSV keratitis model, Suvas and colleagues reported that $CD4^+CD25^+$ depletion prior to infection led to more severe corneal lesions associated with a vigorous T effector response (119).

It is evident from the HIV literature that the $CD8^+$ response plays an important role in

both the acute and chronic phases of infection and that the ability to escape elimination during the acute $CD8^+$ response is one of the keys to establishing a chronic infection (12, 28). Understandably, much attention has been focused on the mechanisms lentiviruses use to escape the initial $CD8^+$ response and persistently evade elimination during the chronic phase of infection. Based upon evidence from other models of viral infection, it is likely that Tregs play an important role in both the acute and chronic stages of HIV infection. Kinter et al. (71) reported that $CD4^+CD25^+$ T cells in the majority of healthy, chronically infected HIV^+ patients significantly suppressed cellular proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells stimulated with HIV peptides in vitro. Interestingly, the level of Treg suppressor function correlated inversely with plasma viremia. More recently, Kinter and others (72) demonstrated that Tregs from HIV infected individuals suppressed $CD8^+$ antigen-specific HIV Gag responses, expression of IFNγ and TNFα, and antiviral chemokine secretion.

Using the FIV model we have previously shown that FIV infection phenotypically and functionally activates $CD4^+CD25^+$ Tregs during both the acute and chronic stages of infection and these cells are capable of inhibiting $CD4^+$ effector responses. Activated feline Tregs from FIV^+ cats up regulate CTLA4, B7.1 (CD80), B7.2 (CD86), and suppress $CD4^+$ proliferation and IL-2 production (84, 135). Furthermore, we have demonstrated preferential in vitro replication of FIV in the $CD4^+CD25^+$ subset and more recently have observed the same is likely true in vivo (67, 84). These data indicate a unique relationship between lentiviral infections and T regulatory cells, in that $CD4^+CD25^+$ T regulatory cells are

preferentially infected early during the course of infection, are activated early during the course of FIV infection, and are able to effectively suppress $CD4^+$ effector responses in both the acute and chronic stages of FIV. Although there is evidence for Treg suppressive activity in the chronic asymptomatic phase of HIV, there is little data available for T regulatory cell activity during the acute phase of HIV infection. Using the FIV model, we tested the hypothesis that $CD4^+CD25^+$ Tregs are able to inhibit $CD8^+$ responses to mitogenic stimulation in both the acute and chronic stages of FIV infection.

C. Methods

Cats: Specific pathogen free (SPF) cats were obtained from Liberty Research, Inc. (Waverly, NY) at 6 months of age, 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 uninfected control cats. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Infection with FIV: The $NCSU_1$ isolate of FIV was originally obtained from a naturally infected cat at the North Carolina State University College of Veterinary Medicine and has been described in detail elsewhere (32, 43, 128). Virus inoculum was grown as a single tissue culture passage in an IL2-dependent feline $CD4^+$ cell line (FCD4-Ecells) as previously described. Twenty-eight cats were inoculated intravenously with $1x10^5$ $TCID_{50}$ of cell-free virus culture supernatant and seven control cats were sham inoculated with equal volumes of sterile FCD4-E cell culture medium.

Sample collection: Cats were randomized and grouped such that blood was collected from

FIV infected and control cats at 14, 21, 28, 35, 42, 56, and 84 days post inoculation. Blood was collected via jugular venipuncture into EDTA vacutainer tubes. Two ml were retained for a complete blood cell count and lymphocyte subset analysis by multi-colored flow cytometry. Plasma was separated from the remaining blood and frozen for analysis of viral load by RT-PCR. In figure 3, two to five cats from each FIV^+ group had popliteal lymph nodes removed at days 14, 21, 28, 35, 42, 56 and 84. Four sham inoculated cats were used as uninfected controls. In figure 4, popliteal lymph nodes were harvested between days 14-56 of acute FIV infection. Lymph node biopsies were performed as previously described. Briefly, cats were anesthetized with intravenous ketamine and valium and anesthesia maintained with isofluorane gas. Popliteal lymph nodes were excised through a small incision in the caudal aspect of the stifle and the incision sutured with monofilament sutures. Lymph nodes were processed into a single cell suspension as previously described and used for phenotypic analysis by flow cytometry and for purification of lymphocyte subsets (CD^{8+}, $CD4^+CD25^+$ cells). Two lymph nodes were collected from each cat during the course of the study.

Lymphocyte subset analysis: The phenotype of lymphocytes from blood and lymph nodes was determined by multi-colored flow cytometric analysis. At least 5×10^5 LN cells were stained with anti-CD4-Strep-avidin / PerCp, anti-CD25-FITC, and anti-CD8-PE. PBMCs were stained with the same antibodies using an established whole blood lysis protocol (32). For flow cytometric analysis, lymphocytes were gated based upon forward vs. side scatter, and approximately 20,000 gated events were acquired and stored list-mode fashion for

analysis using CellQuest software. Absolute numbers of lymphocytes were calculated using the percentage from FACS analysis, multiplied by the total lymphocyte count from either peripheral blood or LN.

Purification of lymphocyte subsets: Lymphocytes were processed into single cell suspensions from the harvested popliteal LN and were stained as needed with anti-CD4-PE, anti-CD25-FITC, and anti-CD8-biotin / StrepA PercP. For the experiments shown in Fig. 3, LN suspensions were depleted of $CD4^+CD25^+$ lymphocytes using a Moflo high speed cell sorter. Unfractionated LN was also passed through the high speed cell sorter to ensure both groups of cells were subjected to identical processing conditions. As shown in figure 4, lymphocytes were stained as needed with anti-CD4-PE, anti-CD25-FITC, and anti-CD8 biotin / StrepA PercP. The lymphocytes were then sorted into $CD8^+$ and $CD4^+CD25^+$ populations using a high speed cell sorter. Next, 2-4x10^6 $CD8^+$ cells were either untreated, ConA stimulated (5ug/ml) alone, ConA stimulated for 30 minutes followed by TGFβ (10 ng/mL) for 120 minutes, or treated with ConA for 30 minutes followed by co-culture with $CD4^+CD25^+$ cells for 120 mins (1:1 ratio CD8 to $CD4^+CD25^+$). All groups were serum starved for 18 hours prior to treatment. Following $CD8^+$ / $CD4^+CD25^+$ co-cultures, $CD8^+$ cells were positively selected using anti-PE microbeads and MACS LD columns to remove $CD4^+CD25^+$ lymphocytes.

Plasma and cell associated viremia: Evidence of infection was assessed on each plasma sample using a commercially available snap test ELISA's (IDEXX laboratories) to detect antibodies against FIV. In addition, quantitative real time PCR was used to determine viral

gag-mRNA loads in each plasma sample. Briefly, 1ml of plasma was used to extract viral RNA using Qiagen's QIAamp Ultrasense Virus Isolation kits. 10 μl of the isolated viral RNA was reverse transcribed in a separate reaction using Promega's Reverse Transcription System with random primers. This reaction was followed by a real-time PCR step using specific primers for FIV-gag mRNA, universal Taqman PCR Mastermix (Applied Biosystems) and the FIV-specific probe in the relative concentrations specified by the manufacturer. Primers used are listed in Table 1. The reactions were run in duplicates in 96 well plates and incubated at 50˚ C for 2 min, 95˚ C for 10 minutes, followed by 45 cycles of 95˚ C for 15 seconds and 60˚ C for 1 minute, before returning to 25˚ C. A standard curve was run in each reaction using serial dilutions of previously sequenced and quantified FIV-gag-mRNA. The standard curve was used to determine absolute viral mRNA copy numbers per ml of plasma. Sorted populations of 0.5-3x10^6 $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from blood and lymph nodes were maintained in RNA later and evaluated for relative quantities of FIV-gag mRNA using the ΔΔCT method (147). Equal amounts of RNA were used in each test. The calibrator sample consisted of a mixture of RNA samples obtained from all groups of cells. Cells from 2-8 cats were evaluated individually at each time point (84).

ELISpot assays for IFNγ production: Cells were either unstimulated or were stimulated with ConA (5ug/ml) for 18 hours at 37°C, then placed in pre-coated (monoclonal anti-feline IFNγ) 96 well ELISpot plates at a concentration of 3 x 10^5 cells/well. For the experiments in Figure 5, $CD4^+CD25^+$ cells were added to ConA stimulated $CD8^+$ lymphocytes at a ratio of 1:2 ($CD4^+CD25^+$ to $CD8^+$). The plates were incubated for 18-24 hrs, stained with detection

antibody, and developed per the manufacturer's instructions. Once dry, each well was counted with an automated ELISpot reader for quantification of spot forming cells (SFCs) per number of cells plated in each well. Controls included recombinant feline IFNγ (positive control), media only wells (to assess background staining), and wells treated without detection antibody (for non-specific staining).

Reverse transcription real time PCR for IFNγ mRNA: RNA from cell cultures was isolated using the Qiagen® RNeasy plus Mini Kit and reverse transcription was performed using the Promega ® Reverse Transcription System, following the manufacturer's instructions for both. This reaction was followed by a real-time PCR step using the universal Taqman PCR Mastermix® (Applied Biosystems) and the Qiagen Quantitect Sybr Green PCR Kit® using feline specific IFNγ forward and reverse primers (Table 1). The reactions were run in duplicates in 96 well plates. The fold induction was calculated by using the ΔΔCt value, where Fold Induction = $2^{-(\Delta\Delta Ct)}$, as described by Winer et al (147). PBMCs from an FIV negative cat and GAPDH as the internal control were used as the calibrator sample value in the ΔΔCt equation.

Table 1. Feline IFNγ and FIV-gag forward and reverse primers used in PCR reactions.
(*as reported by Leutenegger et al 1999.)

Gene	Forward Primer	Reverse Primer	Probe
IFNγ*	TGG TGG GTC GCT TTT CGT AG	AGA CAT CGG TCT GCT TGA AGG ACT C	Syber Green
FIV-gag	5'-GATTAGGA GGTGAGGAA GTTCAGCT-3'	5'-CTTTCATC CAATATTTCT TTATCTGCA-3'	5'-56-FAM/CATGG CCACATTAATAA TGGCCGCA/36-TAMSp/-3'

Statistical analysis: Statistical analysis was performed for figures 3-5 using a student's T test. P values of less than 0.1 for figure 3 and 0.05 for figures 4-5 were considered significant, using a two-tailed test and assuming two samples of equal variance.

D. Results

Analysis of $CD8^+$ lymphocyte dynamics and plasma viremia during acute FIV infection

The 28 SPF cats inoculated with FIV-$NCSU_1$ followed an infection course similar to that described previously for this isolate. All cats were antibody positive for FIV gag proteins (ELISA) by 5 weeks post infection (data not shown). Plasma, and blood and lymph node $CD4^+$ cells were positive for FIV RNA by RT-PCR as early as 2 weeks post infection (data not shown). Typical of FIV infection, there was an early T cell lymphopenia at day 14 p.i. in both the blood and lymph node followed by a gradual rebound in $CD8^+$ cells (Fig. 1a, b). At approximately week 5, all FIV infected cats exhibited peripheral lymphadenopathy as has been previously described for acute FIV infection (11, 20). Plasma viremia peaked at day 14 p.i. coinciding with the $CD8^+$ nadir and then decreased as the $CD8^+$ cell count increased (Fig. 2). FIV replication in the $CD4^+CD25^+$ subset paralleled the plasma viremia with FIV gag mRNA levels peaking at 14 days p.i. then declined as the $CD8^+$ numbers increased.

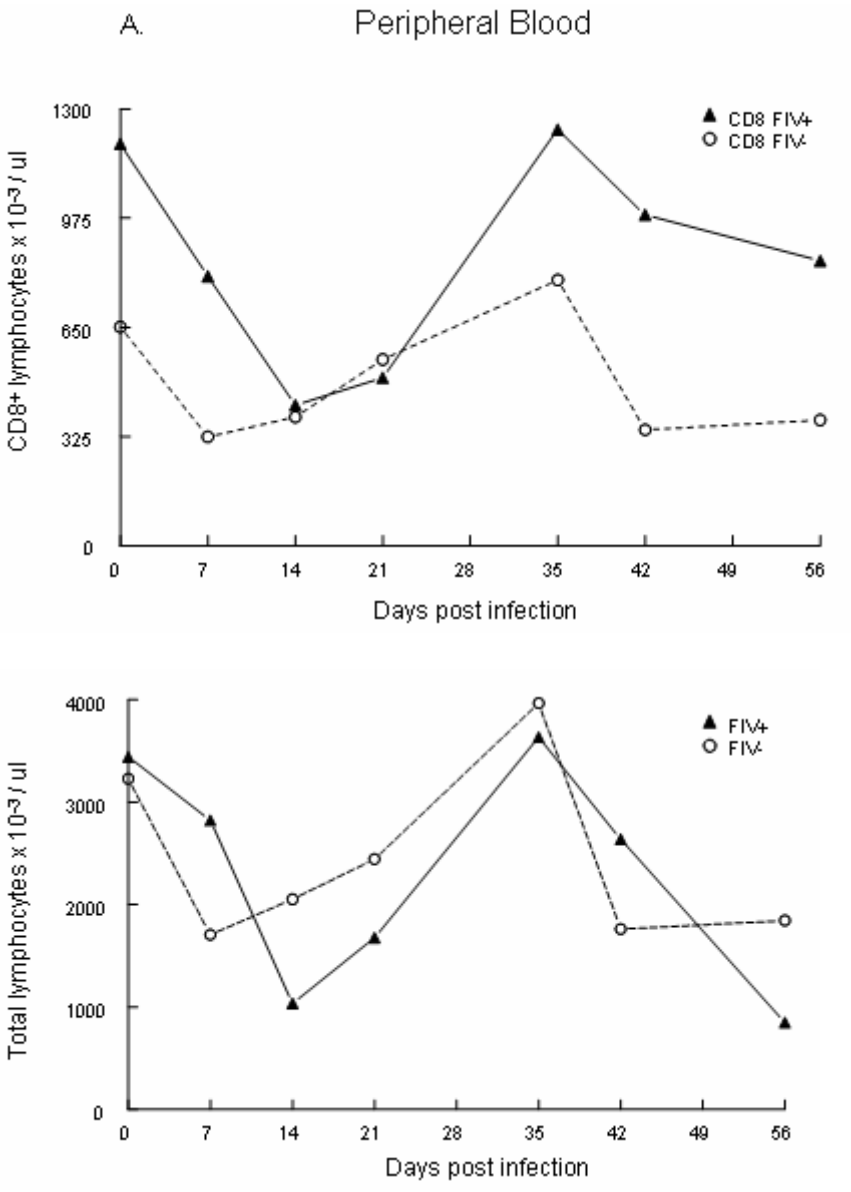

Figure 1a. Peripheral blood $CD8^+$ and total lymphocyte counts during the acute phase of FIV infection. The cats shown above were either sham inoculated (open circles) or inoculated with 10^5 $TCID_{50}$ of FIV-NCSU1(shaded triangles). Peripheral blood was collected via jugular venipuncture at the indicated time points. The lymphocyte count, obtained from the CBC, and multiplied by the percentage $CD8^+$ obtained by FACS analysis, yielded the absolute count for the $CD8^+$ population. The $CD8^+$ nadir for FIV^+ cats occurs at day 14, followed by a peak at day 35. For reference, the total lymphocyte count is shown in the graph below the $CD8^+$ count. Each time point represents the mean cell count (FIV^+ cats n=4, FIV^- cats n=2).

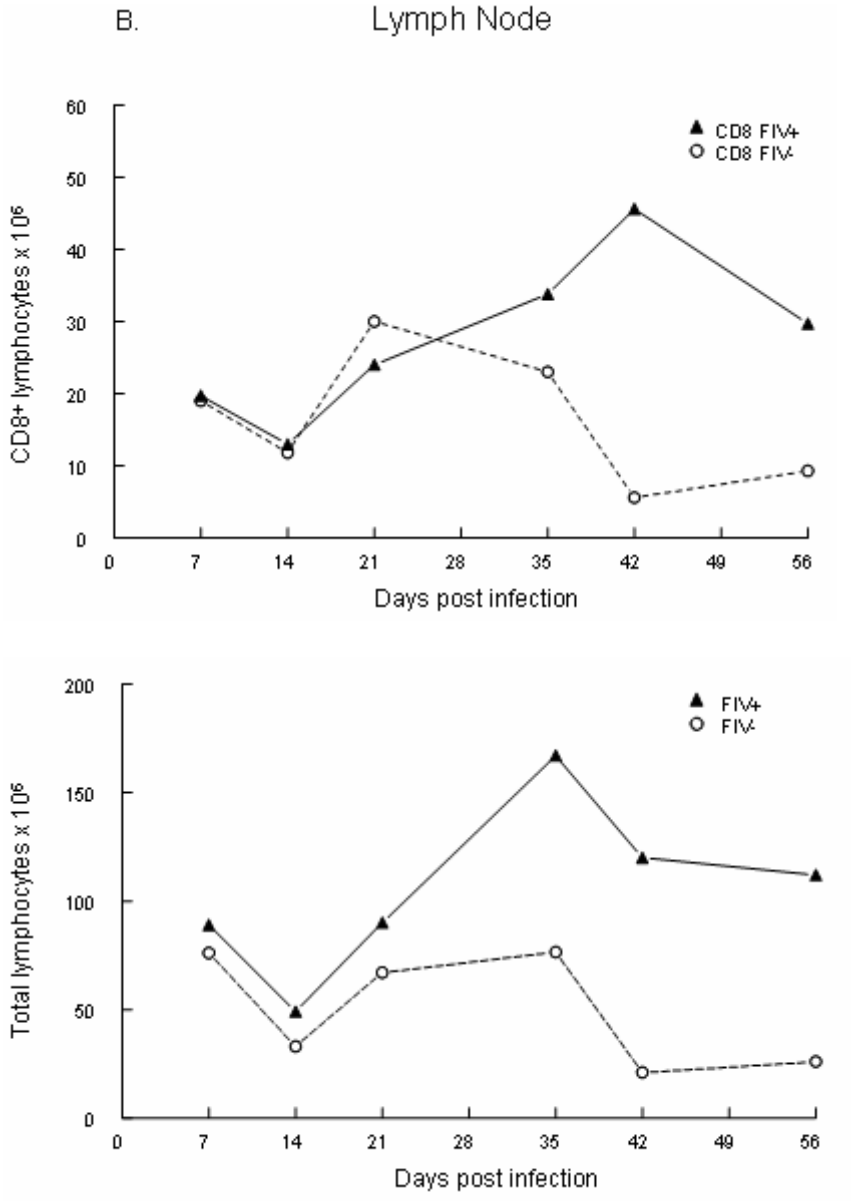

Figure 1b. Lymph Node CD8$^+$ and total lymphocyte counts during the acute phase of FIV infection. A popliteal LN was surgically excised at the indicated time point. A manual lymphocyte count multiplied by the percentage of each population obtained via FACS analysis, yielded the absolute count for the CD8$^+$ population. Similar to the peripheral blood findings, the CD8$^+$ nadir in FIV$^+$ cats occurs at day 14, followed by a peak at 42. For reference, the total lymphocyte count is shown below the CD8$^+$ count. Each time point represents the mean cell count (FIV$^+$ cats n=4, FIV$^-$ cats n=2).

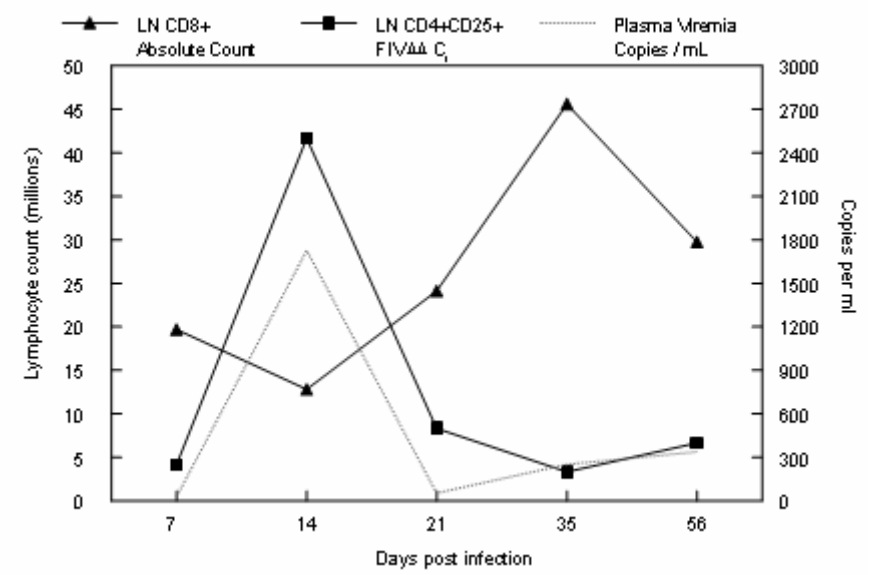

Figure 2. Lymph node CD8$^+$ count with plasma viremia and FIV gag mRNA from CD4$^+$CD25$^+$ lymphocytes superimposed. The CD8$^+$ lymphocyte count (triangle) as shown in Fig. 1 is duplicated here. Quantification of plasma viremia by real time PCR is depicted by the light gray line, with the scale in copies per ml of plasma on the right hand side (n=8). The fold change in FIV gag mRNA assessed by real time PCR (square, relative scale not shown, n=8) in CD4$^+$CD25$^+$ lymphocytes coincides with both the peak in plasma viremia and the CD8$^+$ nadir.

Suppressor activity of CD4$^+$CD25$^+$ cells on CD8+ cells.

As shown above, and reported by others (55), acute FIV infection is associated with a strong CD8$^+$ response that correlates with a decline in plasma viremia. However, despite these responses, virus replication persists, suggesting impaired CD8$^+$ cell function. We have recently reported that CD4$^+$CD25$^+$ Treg cells are able to suppress IL-2 production by CD4$^+$ T helper cells as early as 3 days post FIV infection (84). We asked whether Treg cells also suppressed the CD8$^+$ T cell response during acute FIV infection. CD8$^+$ IFNγ production, as measured by ELISpot, has been correlated to CD8$^+$ CTL function, and we used this method as an indicator of the CD8$^+$ antiviral potential following mitogenic stimulation (59).As

shown in figure 3, popliteal lymph nodes were surgically excised at the indicated time point p.i.. The control group (C) was comprised of sham inoculated cats. Lymphocytes were counted and sorted into two populations as described in the methods section- unfractionated lymph node and $CD4^+CD25^+$ depleted lymph node. After sorting, cells were stimulated with ConA (5ug/ml) for 18 hours at 37°C. Cells were then plated in 96 well ELISpot plates as described in the methods section. For each cat at the given time points, the fold change in IFNγ spot forming cells (SFC) was calculated by dividing the number of $CD4^+CD25^+$ depleted LN SFCs by unfractionated LN SFCs. As shown in Figure 3, IFNγ spot forming cells (SFCs) from FIV^+ cats following $CD4^+CD25^+$ depletion was enhanced at all time points after 21 days p.i. All values greater than 1 reflect an increase in IFNγ SFCs in the $CD4^+CD25^+$ depleted LN. IFNγ production increased in a stepwise fashion, with peak SFCs visible at day 42 p.i. Importantly, once the cats transitioned toward the chronic phase of infection (days 56 and 84 p.i.), a sustained two-fold increase in IFNγ SFCs was evident with $CD4^+CD25^+$ depletion. Two outliers were present at day 56, neither of the cats exhibited increased IFNγ production following $CD4^+CD25^+$ depletion, leading to a very wide range in values, therefore, they were not included in the statistical analysis. When these two cats are included, $p = 0.19$ using a student's t-test, and using a Mann-Whitney test, $p = 0.13$. The results indicate that $CD4^+CD25^+$ depletion from lymph node suspensions during acute FIV infection significantly enhanced IFNγ production at all time points after 21 days p.i..

To characterize this interaction further, we developed a direct Treg suppression assay using $CD8^+$ cells as target cells in an IFNγ ELISpot assay. Lymphocytes were sorted into $CD8^+$and $CD4^+CD25^+$ populations as described in the methods section. After sorting, $CD8^+$ lymphocytes were cultured without ConA (not shown) or with ConA (5ug/ml) for 18 hours at 37°C. Cells were then plated in 96 well ELISpot plates and $CD4^+CD25^+$ lymphocytes were added at a ratio of 0.5 to 1 ($CD4^+CD25^+$ to $CD8^+$ targets). As shown in figure 4, during the first 21 days of infection, there was no evidence of $CD4^+CD25^+$ mediated suppression of $CD8^+$ IFNγ production. However, from days 28 to 56 p.i., there is statistically significant $CD4^+CD25^+$ mediated suppression of $CD8^+$ IFNγ SFCs, as measured by ELISpot.

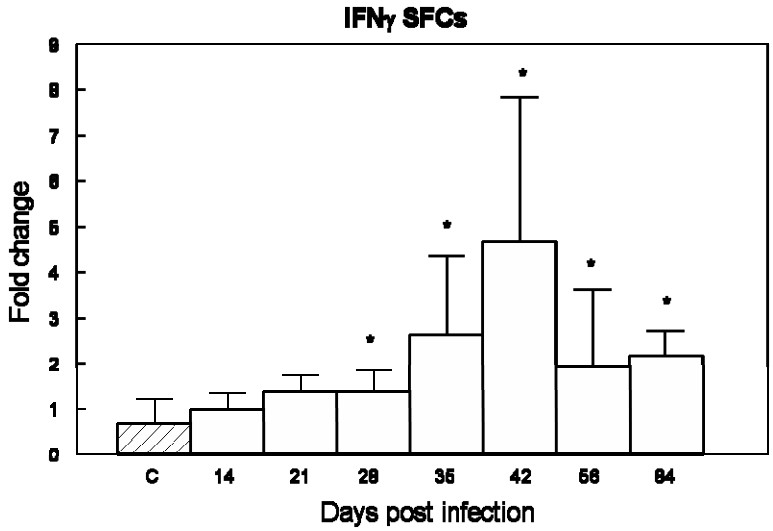

Figure 3. IFNγ production from FIV^+ cats following $CD4^+CD25^+$ Treg depletion is enhanced at all time points after 21 days post infection. Popliteal lymph nodes were surgically excised at the indicated time point. The control group (C) was comprised of sham inoculated cats. (Note: all numbers greater than 1 reflect an increase in IFN-γ SFCs following Treg depletion). A student's t-test was used for all groups to compare the fold change from each group vs. control with p values < 0.1 considered significant (asterisks). Control cats n=4, FIV^+ cats n= 2 -5 for each time point.

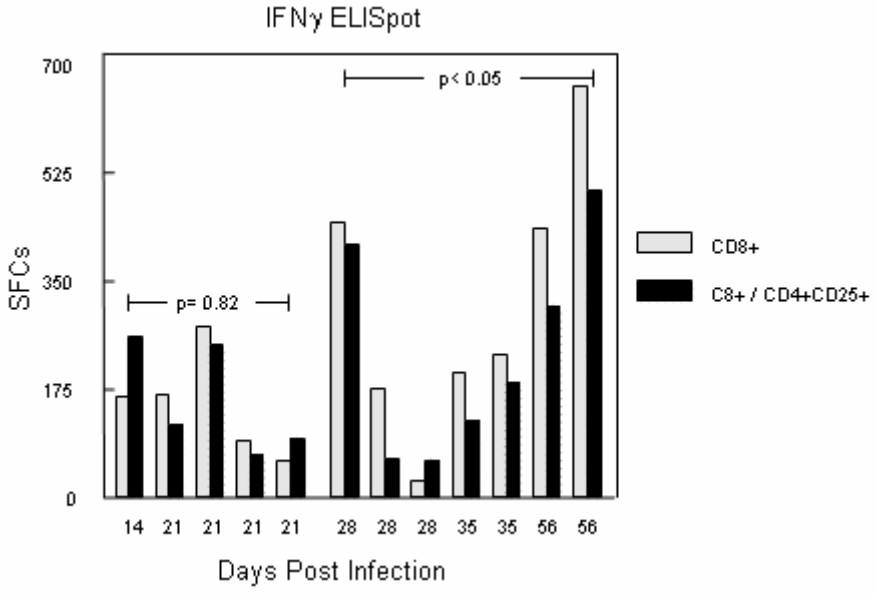

Figure 4. $CD4^+CD25^+$ mediated suppression of $CD8^+$ IFNγ production in acute FIV infection. The bar graph above shows experiments from twelve cats during days 14 through 56 of FIV infection, with the number of SFCs per well shown on the y-axis. The grey shaded bars represent $CD8^+$ cells alone and the black bars represent co-cultures. There was not significant $CD8^+$ suppression during the first 21 days of infection. There is significant $CD4^+CD25^+$ mediated $CD8^+$ suppression at days 28-56 p.i. A paired T test was used to compare IFNγ SFCs from $CD8^+$ alone to $CD8^+$ / CD4+$CD25^+$ co-cultures and a p value of < 0.05 was considered significant.

We have also reported that $CD4^+CD25^+$ T regulatory cells from FIV^+ cats during the chronic, asymptomatic phase of infection are constitutively activated and able to suppress the proliferation and IL-2 production in ConA stimulated $CD4^+CD25^-$ T helper target cells (135). Based upon these past findings and the results from figures 3 and 4, we hypothesized that $CD4^+CD25^+$ T regulatory cells would likewise suppress $CD8^+$ IFNγ production during chronic FIV infection. Figure 5 shows the mean and standard deviation of $CD8^+$ IFNγ mRNA from FIV negative and chronically infected (greater than 1 year) FIV positive cats.

Lymphocytes from FIV^- (gray bars) and FIV^+ (diagonal bars) cats were sorted into $CD8^+$ and $CD4^+CD25^+$ populations using a Moflo high speed cell sorter. Groups of $CD8^+$ lymphocytes were untreated, ConA stimulated (5ug/ml), or ConA stimulated then co-cultured with $CD4^+CD25^+$ Tregs. Following $CD8^+$ / $CD4^+CD25^+$ co-cultures, $CD8^+$ cells were positively selected using anti-PE microbeads and MACS LD columns to remove $CD4^+CD25^+$ lymphocytes. Similar to previous data reported by our lab and others (78, 95), untreated $CD8^+$ lymphocytes from FIV^+ cats displayed over a 50-fold increase in mean IFNγ mRNA when compared with FIV^- control cats. Most importantly, following co-culture with $CD4^+CD25^+$ Tregs, there was a marked reduction in IFNγ mRNA in ConA stimulated $CD8^+$ lymphocytes from FIV^+ cats. These results indicate that $CD4^+CD25^+$ Tregs from chronically infected FIV cats exhibit potent $CD8^+$ immunosuppressive capability.

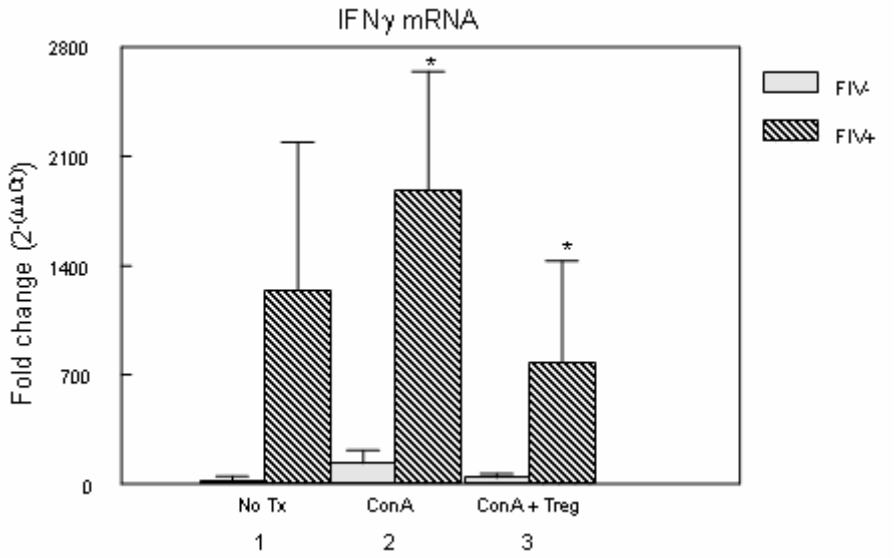

Figure 5. CD8$^+$ IFNγ mRNA is markedly reduced in chronically infected FIV$^+$ cats by TGFβ treatment and following co-culture with CD4$^+$CD25$^+$ T regulatory cells. 2-4x10^6 CD8$^+$ lymphocytes from FIV$^-$ (gray bars) and FIV$^+$ (diagonal bars) were untreated (1), ConA stimulated (2), or treated with ConA for 30 minutes followed by co-culture with CD4$^+$CD25$^+$ cells for 120 minutes (3). In untreated CD8$^+$ lymphocytes (1), there is a 50x increase in IFNγ mRNA in FIV$^+$ cats when compared to FIV$^-$ cats. In FIV$^+$ cats, there was a statistically significant reduction in CD8$^+$ IFNγ mRNA following co-culture with CD4$^+$CD25$^+$ Tregs (4 and 2, asterisks). Each bar represents the mean and standard deviation from 5 experiments.

D. Discussion

Figure 1 demonstrates CD8$^+$ lymphocyte dynamics during the course of acute FIV infection. It has previously been reported that cats experience transient lymphadenopathy during the acute phase infection, and this was noted in the FIV infected cats in our experiment at about week five p.i., corresponding to the increased lymphocyte count in both blood and LN. Prior to the lymphadenopathy, at weeks 2-3, the popliteal LN was small and friable in every cat that had popliteal LN excision, and in several cats the LNs were so shrunken, they could not be located for excision. These observations correspond to the lymphocyte count in both the blood and peripheral LNs and it is likely that acute viremia

causes massive lympholysis. In support of these observations, similar findings were recently reported during acute SIV infection of rhesus macaques (29).

In both FIV and HIV infection an early $CD8^+$ T cell lymphocytosis is noted several weeks P.I. and is important to controlling the initial viremia (16, 17, 34, 105). Using the FIV model, Bucci et al (16) reported that cats with poor $CD8^+$ anti-FIV activity did not have a subsequent reduction in cell associated viremia. Conversely, cats with robust $CD8^+$ anti-FIV activity demonstrated a progressive reduction in cell associated virus, with several cats appearing to eliminate the virus entirely. In a follow up study, Bucci et al (17) identified a $CD8\alpha^+\beta^{lo}$ subpopulation that was responsible for anti-FIV activity. This unique subset of $CD8^+$ lymphocytes displays an activated phenotype (L-selectin^{lo}, $CD44^+$) and expands during the course of FIV infection. Figure 2 shows that peak plasma viremia occurs 2 weeks P.I., as has previously been reported, and the decline in plasma viremia is associated with an increase in $CD8\alpha^+$ lymphocytes.

We recently reported that the peak in $CD4^+CD25^+$ FIVgag mRNA also occurs at week two post infection (84). The fold change in $CD4^+CD25^+$ FIV gag mRNA is shown in Figure 2 in relation to $CD8^+$ cell counts and plasma viremia. Although cause and effect are difficult to establish, it is tempting to speculate that they represent a series of key events involved in early virus specific $CD8^+$ immunosuppression which include, massive lympholysis associated with the high viral load, concurrent $CD4^+CD25^+$ regulatory T cell activation, and a spike in FIV infection of $CD4^+CD25^+$ Tregs (66, 84).

We have also shown that following a peak in FIV gag mRNA at 14 days p.i., there is an increase in the Treg transcription factor FOXP3 starting at 21 days p.i. (84). Any surviving virus specific $CD8^+$ lymphocytes within the node attempting to combat infection are in close proximity to the activated Tregs, thus allowing ample opportunity for Treg mediated $CD8^+$ suppression.

The role of $CD4^+CD25^+$ responses during acute viral infections has been examined using the HSV infection model in mice. Investigation has shown that $CD4^+CD25^+$ depletion, prior to HSV infection, enhances $CD8^+$ responses to immunodominant peptides 3-4 fold and enhances in vivo $CD8^+$ cytotoxic activity, including proliferation, granzyme B and IFNγ production (46, 121). Work from our laboratory suggests $CD4^+CD25^+$ T regulatory cells are activated early during the course of FIV infection and that $CD8^+$ lymphocytes are critical in controlling acute viremia (16, 84). Although T regulatory cell activation appears to be antigen specific, once activated Tregs initiate both antigen specific and antigen non-specific lymphocyte suppression (32, 35). We therefore sought to demonstrate that ex vivo depletion of $CD4^+CD25^+$ regulatory cells, during the acute phase of FIV infection, enhanced $CD8^+$ T cell responses.

As such, IFNγ production was used as an indicator of potential antiviral activity. We previously reported that acute FIV infection leads to increased intracellular FOXP3 expression in $CD4^+CD25^+$ lymphocytes at days 21, 28 and 35 P.I. (84). FOXP3 is a forkhead transcription factor which binds DNA adjacent to NFAT regulatory sites and is one of the key determinants of $CD4^+CD25^+$ T regulatory cell function (112). Depletion of $CD4^+CD25^+$

lymphocytes during acute FIV^+ infection resulted in a progressive increase in IFNγ production, at days 28, 35 and peaked at approximately day 42 P.I.. More importantly $CD4^+CD25^+$ co-culture with $CD8^+$ targets significantly suppressed $CD8^+$ IFNγ production after 21 days P.I.. These data suggest that the overall $CD8^+$ response during acute FIV is diminished by $CD4^+CD25^+$ Tregs, and that the magnitude of $CD8^+$ inhibition follows the course of FOXP3 up-regulation in the $CD4^+CD25^+$ subset.

In studies of chronic HCV infection, Boettler et al (13) and Ebinuma et al (38) demonstrated that Tregs inhibited $CD8^+$ effector function in response to HCV antigen. Furthermore, they reported that chronic HCV infection led to in vivo $CD4^+CD25^+$ expansion and impaired $CD8^+$ responses to other viral antigens such as influenza. We have shown that $CD4^+CD25^+$ T regulatory lymphocytes from FIV^+ cats are constitutively activated and up-regulate FOXP3 during the course of FIV infection (84, 135). As shown in Figure 5, $CD8^+$ lymphocytes from FIV^+ cats demonstrate far greater capacity for mitogen induced IFNγ production when compared to FIV^- control cats, but this response is substantially reduced by a two hour co-culture with $CD4^+CD25^+$ T regulatory cells.

There is an intricate interplay between activated $CD4^+CD25^+$ lymphocytes, $CD8^+$ function, and viral replication during the chronic phase of certain viral infections, including lentiviral infections. Schmitz and others (111) showed that in vivo depletion of $CD8^+$ lymphocytes during chronic SIV infection led to increased viremia, which resolved with the reappearance of virus specific $CD8^+$ lymphocytes. Previously, our laboratory reported that in vitro depletion of $CD8^+$ lymphocytes led to increased FIV replication in PBMC cultures and

that $CD8^+$ lymphocytes that expanded during the course of FIV displayed an activated phenotype (17). Recently, Kinter et al (72) reported that T regulatory cells suppressed anti-HIV effector function of $CD8^+$ lymphocytes in vitro. Once activated, T regulatory cells appear to suppress T cell effector responses in an antigen non-specific manner (108). Taken together, these data indicate that $CD4^+CD25^+$ T regulatory cells are activated during the course of FIV infection and once activated, mediate potent $CD8^+$ suppression in an antigen non-specific fashion.

The FIV model demonstrates the paradox evident in lentiviral infections- chronic immune activation paired with chronic immunosuppression. There is a vigorous $CD8^+$ response during the acute phase of infection, and during the course of infection, $CD8^+$ lymphocytes are progressively activated. During both the acute and chronic stages of infection, the $CD8^+$ response is partially successful because it controls viremia, but ultimately fails because the virus is not eliminated. As the infection progresses, $CD8^+$ responses to unrelated antigens also become diminished, contributing to the development of opportunistic infections. The immune system is chronically activated by the persistent viral infection with lymphocytes experiencing constant activation and turnover- and despite this activated status, the $CD8^+$ immune response is rendered ineffective by the presence of simultaneously activated T regulatory cells.

IV. T REGS UTILIZE MEMBRANE TGFβ TO INHIBIT $CD8^+$ LYMPHOCYTES

A. Abstract

Impaired $CD8^+$ immune responses are well recognized in HIV infection; however, the mechanisms underlying $CD8^+$ impairment are not completely understood. Using the FIV lentiviral model, we reported previously that $CD4^+CD25^+$ Treg cells in both the acute phase and long-term, asymptomatic phase of infection are constitutively activated and suppress both $CD4^+CD25^-$ and $CD8^+$ T cell immune responses. Although the mechanism of $CD4^+CD25^+$ T cell-mediated suppression is controversial, there is strong evidence to suggest that, at least in some models, it occurs via a TGFβ / TGFβRII signaling pathway. We hypothesize that during the early acute stage of FIV lentiviral infection, TGFβ is up-regulated on the plasma membrane of Treg cells (mTGFβ), which engages TGFβRII on the surface of antigen activated $CD8^+$ cells thus transducing a signal through the Smad pathway for G1 cell cycle arrest (anergy) and effectively aborting $CD8^+$ T cell expansion and a sustained $CD8^+$ immune response. The experiments that follow demonstrate up-regulation of mTGFβ in the $CD4^+CD25^+$ subset and up-regulation of TGFβRII in the $CD8^+$ subset of FIV^+ cats as assessed by FACS analysis. Furthermore, we demonstrate Smad 2 phosphorylation in $CD8^+$ targets following $CD4^+CD25^+$ / $CD8^+$ co-culture.

B. Introduction

Using the FIV model for AIDS lentiviral infection, we have previously shown that FIV infection phenotypically and functionally activates $CD4^+CD25^+$ Tregs during both the

acute and chronic stages of infection and these cells are capable of inhibiting $CD4^+$ and $CD8^+$ effector responses in a contact dependent fashion. Activated feline Tregs from FIV^+ cats up regulate CTLA-4, B7.1 (CD80), B7.2 (CD86), and suppress $CD4^+$ proliferation and IL-2 production and $CD8^+$ IFNγ production (84, 135).

The mechanism(s) of $CD4^+CD25^+$ T regulatory cell mediated suppression remain elusive. Different mechanisms of contact-mediated suppression have been proposed such as granzyme-dependent, CTLA-4 dependent, and TGFβ dependent suppression (49, 137). Granzymes are serine proteases whose main action is to activate intracellular caspases, initiating programmed cell death and they function more efficiently when released in conjunction with perforin, which polymerizes in target cell membranes to form open channels (64). Grossman and others (52) reported that after CD3/CD46 stimulation, $CD4^+CD25^+FOXP3^+$ lymphocytes isolated from human blood showed increased granularity caused by accumulation of cytoplasmic Granzyme A. In co-culture experiments, these stimulated T regulatory cells were able to kill autologous $CD4^+$, $CD8^+$ and $CD14^+$ target cells in a Granzyme A – perforin- dependent fashion, as perforin inhibitors blocked target cell killing. Interestingly, activated $CD4^+$ and $CD8^+$ target cells were more susceptible to T regulatory killing than non-activated controls. In a mouse model, Gondek et al (49) reported that treatment of activated Tregs with anti-GITR antibody caused down-regulation of Granzyme B in vitro. Following these observations, they also demonstrated that Treg function was reduced in Granzyme B knockout mice, suggesting that one mechanism of T regulatory suppression was contact mediated, Granzyme B secretion with apoptosis of the

target cell. Granzyme mediated killing of activated $CD4^+$ and $CD8^+$ targets is probably more relevant to autoimmune and transplantation models, because it appears that in many infectious disease models, effector cells are anergized and not destroyed by Treg interactions (49, 52).

Different mechanisms for CTLA-4 mediated suppression have been proposed. The first and simplest mechanism is that an APC activates a T regulatory cell and a T effector cell via antigen presentation. Being in close proximity to the target, CTLA-4 on the T regulatory cell surface is able to ligate CD80/86 on the T effector surface and shut down activation. In a more complex mechanism, CTLA-4 on the Treg surface ligates CD80/86 on the APC, increasing indoleamine 2,3 dioxygenase within the APC, therefore reducing available levels of tryptophan necessary for T effector activation (137). In vitro and in vivo studies have suggested that CD80/CD86 ligation by CTLA-4 accounts for at least partial T effector suppression and it is probable that this is a secondary pathway whose purpose is to strengthen the primary suppressive signal (96, 122).

Several laboratories, including ours, have demonstrated surface bound TGFβ (mTGFβ) on activated Treg cells (5, 88, 93). However, the role of TGFβ in Treg-mediated suppression is controversial. Nakamura et al. (88) demonstrated that activated Treg cells expressed mTGFβ and that their contact-dependent suppressor function was abrogated by treatment with anti-TGFβ neutralizing antibody. Others have demonstrated that suppressor function of both mouse and human $CD4^+CD25^+$ thymocytes are at least partially inhibited by neutralization of TGFβ (5, 48). In contrast, Piccirillo et al. (100) reported that anti-TGFβ

had no effect on in vitro Treg cell-mediated suppression. Additionally, studies have shown that Treg cells from TGFβ deficient mice were able to suppress as well as those from normal mice (75, 100).

TGFβ functions by binding to its receptor (TGFβRII) and transmitting a signal via activation of a Smad pathway and, in the case of Treg cells, downstream activation of FOXP3, transcriptional suppression of IL-2, and induction of anergy (138). Thus, if Treg cell function occurs via the membrane bound TGFβ, then the responder cell must express TGFβRII. In support of this, there is a body of evidence to suggest this pathway is essential in controlling $CD8^+$ responses. Mice expressing a dominant negative TGFβRII (dnTGFβRII) specific to their T cells develop a profound $CD8^+$ hyper proliferation resulting in massive expansion of their peripheral lymphoid tissue by 2-9 months of age (79). Interestingly, $CD8^+$ T cell development in the thymus is normal, suggesting that the homeostatic control occurs in the periphery. With regards to Treg function, in a tumor rejection model, introduction of a dnTGFβRII into antigen specific $CD8^+$ cells rendered these cells resistant to Treg cell mediated suppression (24). Furthermore, TGFβ inhibits $CD8^+$ anti-tumor effector function while suppressed $CD8^+$ lymphocytes maintain an activated phenotype (3). Similarly, in an autoimmune diabetes model, delayed progression of diabetes was associated with an increased percentage of mTGFβ positive, $CD4^+CD25^+$ lymphocytes within the peripheral nodes and pancreas. However, adoptive transfer of anti-islet cell $CD8^+$ cells expressing a dnTGFβRII resulted in the recipients rapidly developing diabetes (51).

It is evident from this discussion that the role of the TGFβ / TGFβRII signaling pathway in Treg cell-mediated suppression is controversial and likely depends on the model system used. Using the FIV model, we have data to support mTGFβ-mediated suppression, as we have been able to block the suppressor function of Treg cells from FIV-infected cats by either treatment of the Treg cells with anti-TGFβ or treatment of $CD4^+CD25^-$ target cells with anti-TGFβRII antibodies (98). The experiments that follow support the hypothesis that Treg mediated $CD8^+$ suppression likely occurs via the interaction between Tregs displaying mTGFβ and TGFβRII on target $CD8^+$ cell surfaces.

C. Methods

Cats: Specific pathogen free (SPF) cats were obtained from Liberty Research, Inc. (Waverly, NY) at 6 months of age, 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 uninfected control cats. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Infection with FIV: The $NCSU_1$ isolate of FIV was originally obtained from a naturally infected cat at the North Carolina State University College of Veterinary Medicine and has been described in detail elsewhere. Virus inoculum was grown as a single tissue culture passage in an IL2-dependent feline $CD4^+$ cell line (FCD4-Ecells) as previously described. The cats were inoculated intravenously with $1x10^5$ $TCID_{50}$ of cell-free virus culture and control cats were sham inoculated with equal volumes of sterile FCD4-E cell culture medium. The cats were originally used for the FIV acute phase infection study described in

section III and at the time of these experiments had been infected with FIV for approximately one year.

Lymphocyte subset analysis: The phenotype of lymphocytes from blood and lymph nodes was determined by multi-colored flow cytometric analysis. LN cells were harvested either by surgical excision of a peripheral lymph node, or following euthanasia. At least 5×10^5 LN cells were used for FACS evaluation. As shown in figure 6, PBMC or LN from FIV-infected and control cats were stained with anti-CD4-PE, anti-CD25-FITC, and anti-TGFβ-APC. As shown in Figure 7, PBMC or LN from FIV-infected and control cats were stained with anti-CD8 FITC and anti-TGFβRII PE. PBMCs were stained with the same antibodies using an established whole blood lysis protocol (32). For FACS analysis, lymphocytes were gated based upon forward vs. side scatter, and approximately 20,000 gated events were acquired and stored list-mode fashion for analysis using CellQuest software.

Smad 2 Western Blots: $CD8^+$ and $CD4^+CD25^+$ lymphocytes were collected by high speed cell sorting as previously described. 4.0 x 10^6 $CD8^+$ cells per treatment group were serum starved for 18 hours. TGFβ (10 ng/mL) was added 2 hours prior to cell lysis. $CD8^+$ and $CD4^+CD25^+$ cells were combined at a 1:1 ratio 2 hours prior to cell lysis. After 2 hours, $CD4^+CD25^+$ lymphocytes were depleted from $CD8^+$ cultures using anti-PE microbeads as previously described. All groups were lysed with NP-40 and separated by SDS-Page. The blots were analyzed using anti-P-SMAD 2, followed by HRP-conjugated goat anti-mouse IgG1 and detected by chemiluminescence. The blots were then stripped and re-probed with anti-actin and HRP-conjugated goat anti-mouse antibody. For each treatment group, actin

and P-SMAD 2 were evaluated by photodensitometry and normalized, with unstimulated $CD8^+$ controls assigned a value of 1.

Statistical analysis: Statistical analysis using a student's T test was performed for figures 6 and 7. P values of less than 0.05 were considered significant, using a two-tailed test and assuming two samples of equal variance.

D. Results

$CD4^+CD25^+$ lymphocytes exhibit increased expression of mTGFβ in FIV^+ cats

We have demonstrated that CD4+CD25+ Treg cells from FIV+ cats suppress both $CD4^+$ and $CD8^+$ T cell function. However, the mechanism of $CD4^+CD25^+$ Treg mediated suppression during lentiviral infections has been incompletely defined. Suppression appears to be contact dependent, and work from our laboratory and from others suggests that mTGFβ may play a key role (87, 88, 98, 99). We therefore examined the expression of mTGFβ on $CD4^+CD25^+$ cells from peripheral blood and lymph node in FIV^- cats and FIV^+ cats, one year post infection. Cells were stained with anti-feline CD4 and anti-human TGFβ and analyzed by FACS. Membrane TGF-beta positive cells ($mTGF\beta^+$) were identified by first gating on $CD4^+CD25^+$ lymphocytes, and then examining the %mTGFβ+ vs. forward scatter. As shown in figure 6, there was a significant difference in the percentage of lymph node $mTGF\beta^+CD4^+CD25^+$ lymphocytes, with the mean from FIV^+ cats (56%) being almost twice that from FIV^- cats (27%). There was no difference in the percentage of $mTGF\beta^+CD4^+CD25^+$ lymphocytes from PBMCs of FIV^+ and FIV^- cats.

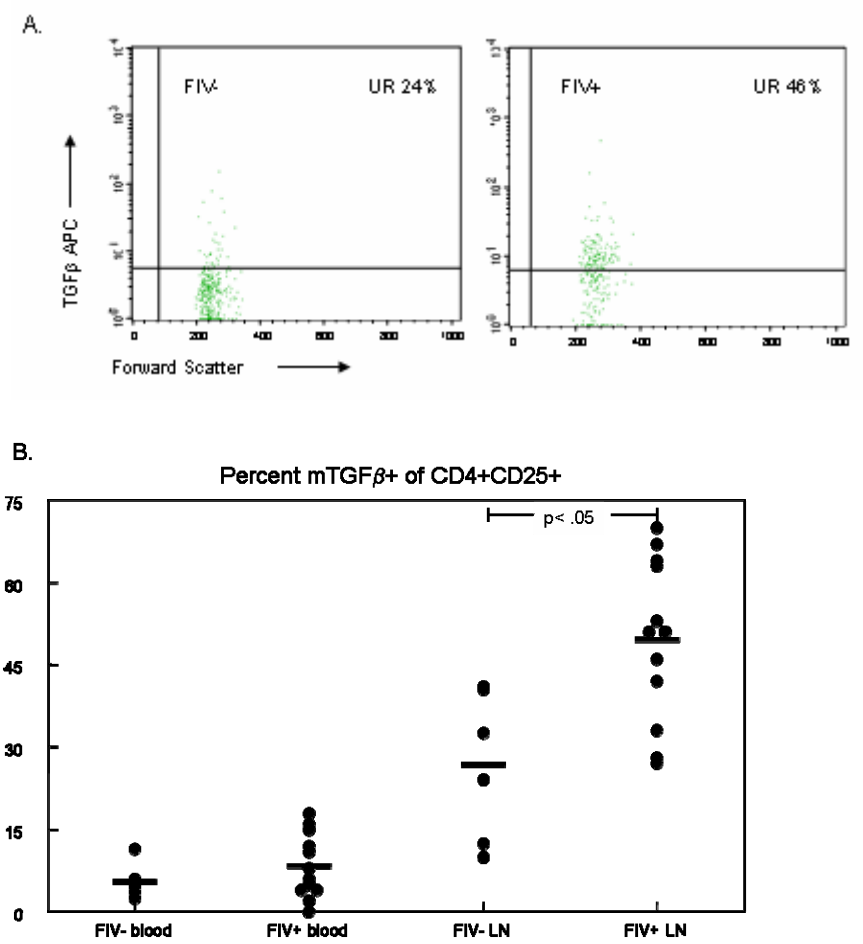

Figure 6. $CD4^+CD25^+$ lymphocytes exhibit increased expression of mTGFβ in FIV^+ cats when compared to FIV^- control cats. (a). Dot plots demonstrating the percentage of mTGFβ positive cells within the $CD4^+CD25^+$ population, from the LN of an uninfected and a chronically infected, FIV^+ cat. The upper right quadrant represents the % $mTGF\beta^+$, 24% and 46%, respectively. (b). The horizontal line represents the mean of percent of $mTGF\beta^+$ from the $CD4^+CD25^+$ population and dots represent individual cats. There was no statistical difference between the FIV^- (6%, n=7) and FIV^+ (8%, n=15) blood. The mean $mTGF\beta^+$ was considerably lower in the FIV^- LN (27%, n=6) when compared to FIV^+LN (50%, n=15). A two tailed student's T test assuming samples of equal variance was performed and p values of less than .05 were considered significant.

$CD8^+$ lymphocytes exhibit increased expression of TGFβRII in FIV^+ cats

TGFβ is important to controlling $CD8^+$ responses and the sensitivity of lymphocytes to TGFβ correlates with the levels of TGFβRII expression (61). Because there is ample evidence for reduced $CD8^+$ lymphocyte function during the course of lentiviral infection, we hypothesized that chronic FIV infection likely increased TGFβRII expression on $CD8^+$ lymphocytes, making them much more sensitive to TGFβ inhibition. TGFβ functions by binding to its receptor (TGFβRII) and transmitting a signal via activation of a Smad pathway and induction of anergy (138). Thus, if Treg cell function occurs via membrane bound TGFβ, then the responder cell must express TGFβRII. PBMC and LN $CD8^+$ lymphocytes were examined by FACS for the percentage of $TGF\beta RII^+$ cells. In both PBMC and LN, the mean percentage of $TGF\beta RII^+CD8^+$ lymphocytes was more than five times greater in FIV^+ cats when compared to FIV^- cats (Figure 7). We have previously reported that FIV is capable of infecting $CD8^+$ lymphocytes in vitro and in vivo during acute and asymptomatic FIV infections (42). It is unclear during the course of FIV infection whether $CD8^+$ lymphocytes up-regulate TGFβRII as a generalized response to chronic viremia or a more specific response to virus infection of the individual $CD8^+$ cell. It is probable that both of these mechanisms contribute to $CD8^+$ lymphocytes exhibiting increased expression of TGFβRII in FIV^+ cats.

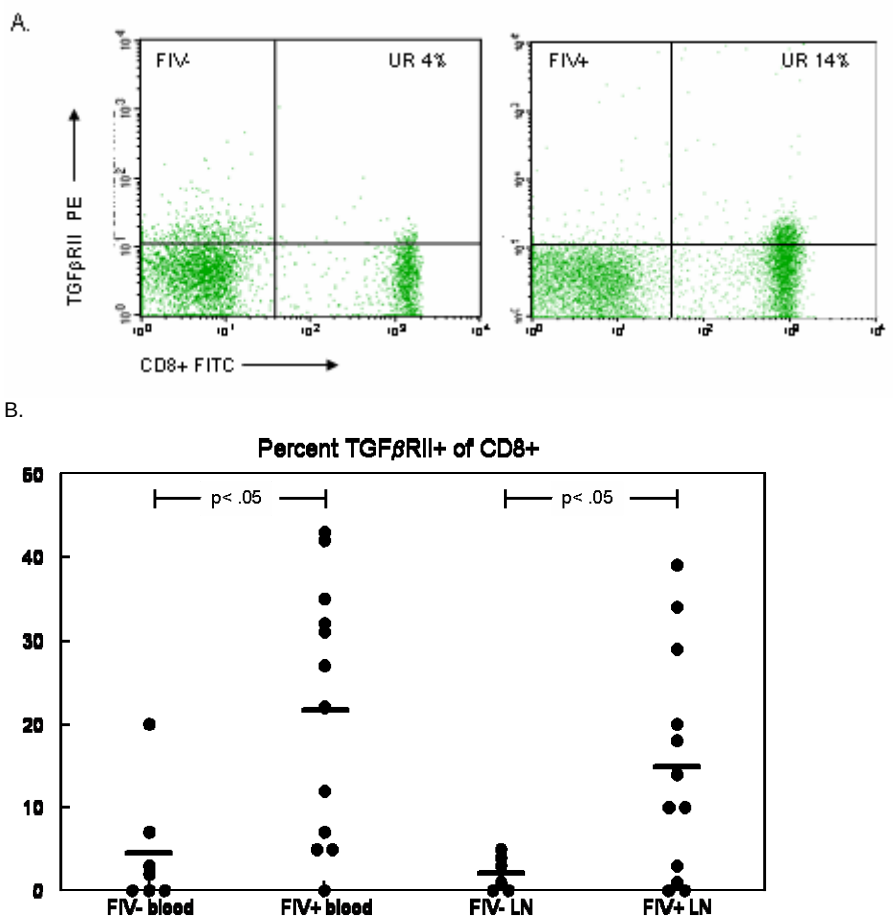

Figure 7. CD8$^+$ lymphocytes exhibit increased expression of TGFβRII in FIV$^+$ cats when compared to FIV$^-$ control cats. PBMC and LN CD8$^+$ lymphocytes were evaluated by FACS for TGFβRII expression. (a). Dot plots demonstrating the percentage of TGFβRII positive cells within the CD8$^+$ population from the LN of an uninfected and a chronically infected, FIV$^+$ cat (upper right quadrant, 4% and 14%, respectively). (b). There is a marked difference in the mean from FIV$^-$ (5%, n=7) and FIV$^+$ (22%, n=15) blood and between FIV$^-$ (n=6, 2%) and FIV$^+$ LN (n=15, 15%). The horizontal line represents the mean % TGFβRII$^+$ of CD8$^+$ lymphocytes from the blood and LN of FIV$^-$ and FIV$^+$ cats, dots represent individual cats. A two tailed student's T test assuming samples of equal variance was performed and p values of less than .05 were considered significant.

T regulatory cell mediated Smad 2 phosphorylation in $CD8^+$ lymphocytes.

Smad 2 phosphorylation is specific to the TGFβ signaling pathway (45). Upon ligation of TGFβ receptor II by TGFβ, the receptor dimerizes with TGFβ receptor I (Figure 8). The TGFβRI - TGFβRII heterodimer phosphorylates Smad 2, initiating the intracellular TGFβ signaling cascade.

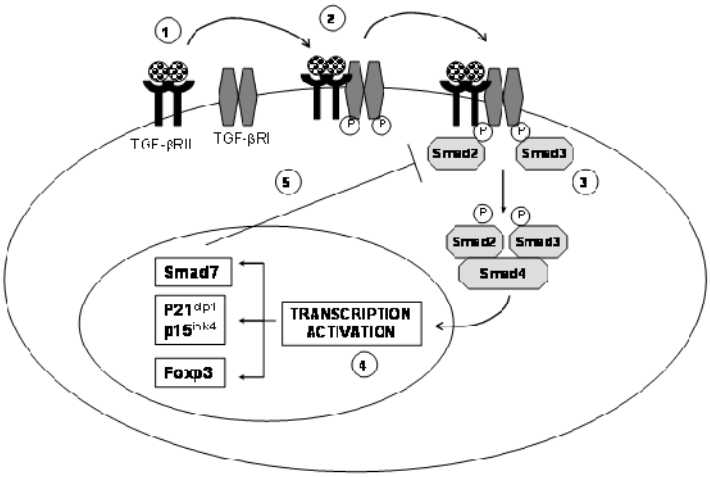

Figure 8. The TGFβ signaling pathway. The TGFβ receptor complex consists of two Type I and two Type II complexes. **(1)** TGFβ binds to the Type II receptor. **(2)** It then associates with the Type I receptor and phosphorylates it. **(3)** The phosphorylated receptor complex then recruits and phosphorylates Smad2 and 3, leading to dissociation of the Smads from the receptor followed by their complexing with Smad4. **(4)** The Smad2-p,3-p,4 complex translocates to the nucleus where it activates, along with other co-transcription factors, transcription of a variety of genes including those for $p21^{cip1}$, Smad7, and Foxp3. **(5)** Smad7 competes with Smad2 and 3 for binding to the Type I receptor, thus providing a feedback loop to shut down the TGFβ signal.

As was stated earlier, TGFβ is one of the major sources of $CD8^+$ inhibition. In the experiments shown in Figure 9, TGFβ is used as a positive control to demonstrate Smad 2 signaling. Figure 9a shows that after a two hour co-culture with $CD4^+CD25^+$ lymphocytes,

$CD8^+$ lymphocytes from an FIV^+ cat exhibit Smad 2 phosphorylation exceeding the TGFβ positive control. As shown in Figure 9(b), Smad 2 phosphorylation in both FIV^- and FIV^+ cats exceeds the TGFβ positive control following $CD4^+CD25^+$ co-culture. Figure 10 shows IFNγ mRNA in $CD8^+$ lymphocytes as assessed by real time RT-PCR. Lymphocytes from chronically infected FIV^+ cats were sorted into $CD8^+$ and $CD4^+CD25^+$ populations using a Moflo high speed cell sorter. 2-4x10^6 $CD8^+$ cells were untreated, ConA stimulated, ConA stimulated for 30 minutes followed by TGFβ for 120 minutes, or treated with ConA for 30 minutes followed by co-culture with $CD4^+CD25^+$ cells for 120 minutes. Following $CD8^+$ / $CD4^+CD25^+$ co-cultures, $CD8^+$ cells were positively selected using anti-PE microbeads and MACS LD columns to remove $CD4^+CD25^+$ lymphocytes. There was a statistically significant reduction in $CD8^+$ IFNγ mRNA following treatment with TGFβ ($p = 0.10$) and following co-culture with $CD4^+CD25^+$ Tregs ($p < 0.05$). The same results were not evident in FIV^- cats treated in an identical manner (data not shown). These results suggest that $CD4^+CD25^+$ Tregs utilize mTGFβ to inhibit $CD8^+$ lymphocyte responses.

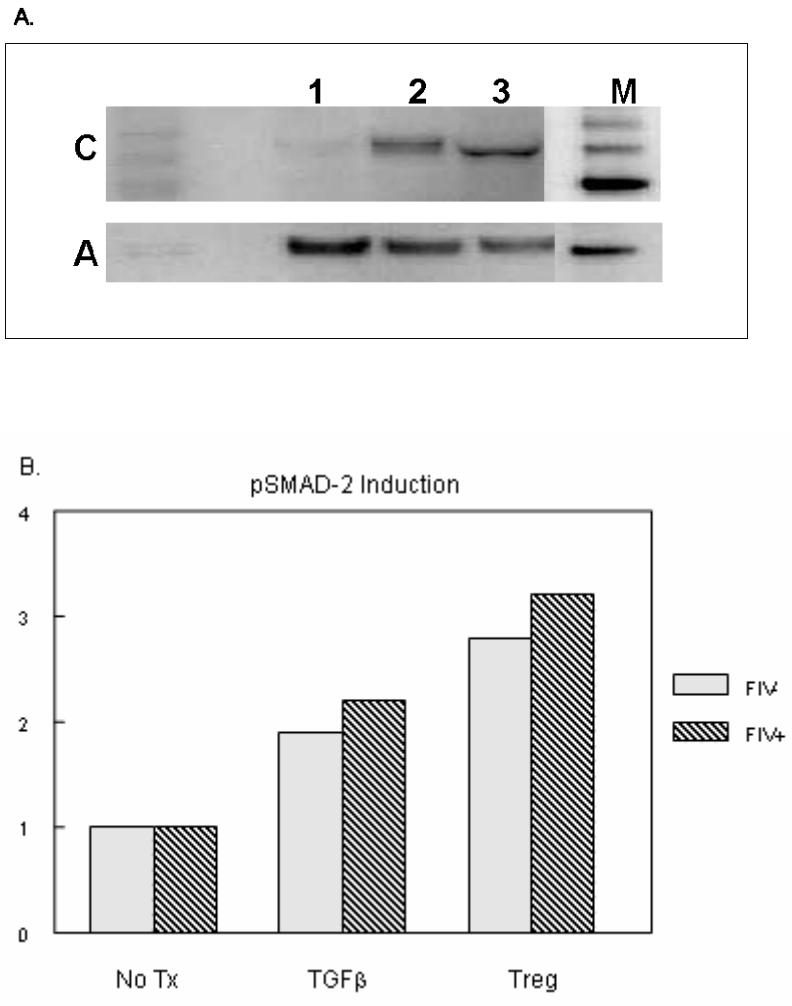

Figure 9. T regulatory cell mediated Smad2 phosphorylation in CD8$^+$ lymphocytes. (a). CD8$^+$ and CD4$^+$CD25$^+$ lymphocytes from an FIV$^+$ cat were isolated by Moflo cell sorting as described in the methods section. Only a trace amount of Smad 2 phosphorylation is visible in untreated CD8$^+$ cells (Lane 1). Treatment of CD8$^+$ lymphocytes with soluble TGFβ (10ng/ml) for 120 minutes results in Smad 2 phosphorylation (Lane 2). Following co-culture with CD4$^+$CD25$^+$ T reg cells for 120 minutes at a ratio of 1:1, CD8$^+$ targets exhibit robust Smad2 phosphorylation (Lane 3). The lower row, labeled A, represents actin and the column labeled M represents the kDa marker (phosphorylated Smad 2 is found between 50-60 kDa). The faint band to the right of the letter C represents the MvLu lysate control. (b). The bar graph represents the mean of Smad2 phosphorylation from 3 FIV$^+$ cats and 2 FIV$^-$ control cats that were treated as described in 4a. Untreated groups were given a value of 1, and the fold Smad 2 phosphorylation was then calculated for each treatment group.

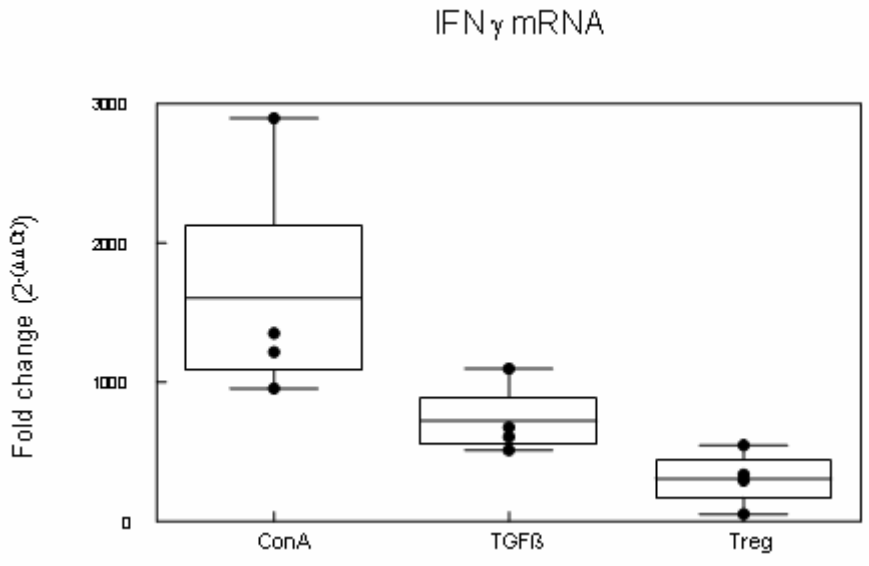

Figure 10. CD8$^+$ IFNγ mRNA is markedly reduced in FIV$^+$ cats by TGFβ treatment and following co-culture with CD4$^+$CD25$^+$ T regulatory cells. 2-4x10^6 CD8$^+$ cells were untreated (1), ConA stimulated (2), ConA stimulated for 30 minutes followed by TGFβ for 120 minutes (3), or treated with ConA for 30 minutes followed by co-culture with CD4$^+$CD25$^+$ cells for 120 minutes (4). The box-whisker plots represent 5th and 95th percentiles (whisker), 25th and 75th percentiles (box) and mean of IFNγ induction from CD8+ lymphocytes, each dot represents an individual cat (n=4). There was a statistically significant reduction in CD8$^+$ IFNγ mRNA following treatment with TGFβ ($p=0.1$) and following co-culture with CD4$^+$CD25$^+$ Tregs ($p < 0.05$).

Discussion

It is well recognized that T regulatory cells are relatively anergic and are resistant to apoptosis. We have demonstrated that T regulatory cells from FIV infected cats inhibit $CD4^+CD25^-$ cell proliferation and IL-2 production and $CD8^+$ IFNγ production during both acute and chronic FIV infection (84, 135). The exact mechanism of T regulatory mediated suppression is incompletely understood and it is likely that Tregs are capable of suppression by different means and that the mechanisms of suppression vary between the experimental models used. Previously, we reported that $CD4^+CD25^+$ lymphocytes are functionally activated and up-regulate mTGFβ during the course of FIV infection (84, 135). More importantly, we have shown that blocking the mTGFβ / TGFβRII interaction between $mTGFβ^+$ $CD4^+CD25^+$ Tregs and in vitro stimulated $CD4^+CD25^-$ targets, blocks their conversion to $CD4^+CD25^+FOXP3^+$ T regulatory cells (99). However, the link between TGFβ, T regulatory cells, and $CD8^+$ inhibition has not been clearly elucidated.

In 2001, Nakamura and colleagues (88) reported that $CD4^+CD25^+$ mediated suppression of $CD4^+CD25^-$ proliferation was inhibited by the presence of anti-TGFβ antibody. More importantly, they showed that $CD4^+CD25^+$ regulatory T cells stimulated with anti-CD3 and IL-2 expressed high levels of membrane TGFβ, as assessed by FACS and western blot. In a follow-up study, Nakamura et al (87) reported that treatment of $CD4^+CD25^+$ T cells with recombinant latency associated peptide of TGFβ (rLAP) also inhibited $CD4^+CD25^+$ mediated suppression. They also described experiments in which

$CD4^+CD25^+$ cells from TGFβ deficient mice were capable of suppressing $CD4^+CD25^-$ proliferation in vitro, but did not protect recipient mice from the development of colitis in an in vivo SCID transfer model. In contrast to these findings, Picirillo and others (100) demonstrated that neutralization of TGFβ by treatment with anti-TGFβ antibody or soluble anti-TGFβRII-Fc did not inhibit the in vitro suppressive capacity of $CD4^+CD25^+$ T regulatory cells. Furthermore, they demonstrated that $CD4^+CD25^-$ target cells from Smad3 deficient or dnTGFβRII mice were susceptible to Treg mediated suppression; and $CD4^+CD25^+$ T regulatory cells from TGFβ deficient mice were capable of suppressing $CD4^+CD25^-$ targets.

In concert with the findings from Nakamura et al, we have reported that $CD4^+CD25^+$ T regulatory cells from FIV infected cats exhibit increased expression of mTGFβ (40, 84). During acute FIV infection, mTGFβ is transiently up-regulated on $CD4^+CD25^+$ lymphocytes within the lymph node, but not in the blood (84). Similar to these findings, Figure 6 shows a significantly increased percentage of $mTGF\beta^+CD4^+CD25^+$ lymphocytes within the lymph node but not in the blood of FIV^+ cats, approximately 1 year post-infection. We have also observed in long term FIV-infected cats (greater than 2 years) that mTGFβ is up-regulated on $CD4^+CD25^+$ lymphocytes from peripheral blood (40). Taken together these observations indicate that mTGFβ is progressively up-regulated on $CD4^+CD25^+$ T regulatory cells during the progression of FIV infection.

TGFβ appears to be essential in regulating $CD8^+$ immune responses. Mice expressing a dnTGFβRII specific to their T cells develop a profound $CD8^+$ hyper

proliferation (77, 79). In murine models, adoptive transfer experiments utilizing dnTGFβRII $CD8^+$ lymphocytes have demonstrated the development of different autoimmune diseases (51, 150). The role of TGFβ in controlling $CD8^+$ responses to infectious agents is more complex. Plasmodium sp organisms stimulate dendritic cells to produce TGFβ and can activate endogenous, latent TGFβ to its active form (92, 94). Ocana-Morgner and colleagues (92) reported that blocking TGFβ restored in vitro $CD8^+$ T cell IFNγ production, and more importantly restored protective immunity against plasmodium in vivo. In a murine model of Trypanosma cruzi infection, dnTGFβRII $CD8^+$ lymphocytes exhibited increased proliferation but did not exhibit restored effector function (81). In certain viral models of infectious disease, profound $CD8^+$ proliferation with an IFNγ dominated response is evident during the acute stage of infection and it is likely that TGFβ is one of the major negative regulators of this response (79, 133). In a human melanoma vaccine study, TGFβ was shown to inhibit $CD8^+$ effector function in vitro, while the suppressed $CD8^+$ lymphocytes maintained an activated phenotype (3). There is little information regarding the role of TGFβ in controlling $CD8^+$ immune responses during chronic lentiviral infections, but there is ample evidence for $CD8^+$ immune dysfunction during the course of AIDS lentiviral infections (6, 20, 53, 113, 129). In considering possible mechanisms for $CD8^+$ dysfunction, we hypothesized that chronic FIV infection may promote TGFβRII expression on $CD8^+$ lymphocytes, therefore making them much more sensitive to TGFβ inhibition. Figure 7 demonstrates that $CD8^+$ lymphocytes from FIV^+ cats have increased surface expression of TGFβRII when compared

to FIV$^-$ cats. Activation of the TGFβ pathway in CD8$^+$ lymphocytes may help to explain one of the apparent paradoxes of chronic lentiviral infections, in that CD8$^+$ T cells display an activated phenotype but exhibit reduced effector function (17, 129).

In CD8$^+$ lymphocytes, TGFβ ligation of TGFβRII leads to TGFβRI / TGFβRII dimerization followed by Smad 2/3 phosphorylation for intracellular propagation of the TGFβ signal (Figure 8). The phosphorylated Smad complex translocates to the nucleus and binds transcription factors, which promote cell cycle arrest, inhibit cytolytic gene products, leading to CD8$^+$ anergy and reduction in CD8$^+$ function (86, 123). Studies of CD8$^+$ anti-tumor activity have highlighted the importance of the TGFβ signaling pathway as a mechanism for tumor escape. TGFβ has been shown to reduce antigen specific effector and memory responses in CD8$^+$ lymphocytes harvested from melanoma patients vaccinated with the gp100 melanoma vaccine (3). The murine EL-4 thymoma tumor line produces TGFβ and may induce host tissue TGFβ production (50, 123). Gorelik and Flavell (50) reported that neutralizing TGFβ or utilization of dnTGFβRII CD8$^+$ lymphocytes enabled eradication of the tumor. Using the murine EL-4 thymoma model, Tomas and Massague′ (123) showed in a series of experiments that TGFβ inhibits the expression of CD8$^+$ IFNγ through Smad-ATF1 mediated binding of the IFNγ promoter. Similar to these findings, we have previously demonstrated (Figures 3-5) that CD8$^+$ treatment with TGFβ, or co-culture with activated CD4$^+$CD25$^+$ T regulatory cells inhibits IFNγ production. Furthermore, co-culture of CD8$^+$ lymphocytes with CD4$^+$CD25$^+$ T regulatory cells induces Smad 2 phosphorylation equivalent

to that of the TGFβ positive control (Figure 9). Smad 2 induction was observed in both FIV^- and FIV^+ cats following $CD4^+CD25^+$ co-culture and we believe this is a result of the relatively high (1:1) $CD4^+CD25^+$ to $CD8^+$ ratio used in this in vitro assay.

During the course of FIV infection, mTGFβ is up-regulated on $CD4^+CD25^+$ Treg cells with these cells being found in the lymph node during the acute and early chronic stage of infection, and later in the peripheral blood. $CD8^+$ lymphocytes exhibit an increased percentage TGFβRII in both the blood and lymph node when compared to FIV^- cats. It is likely that once in the lymph node; $CD8^+$ lymphocytes interact with $CD4^+CD25^+$ lymphocytes in a TGFβ-dependent fashion. In support of this, Smad2 phosphorylation in $CD8^+$ lymphocytes following $CD4^+CD25^+$co-culture is equivalent to that of the TGFβ positive control. We have also shown that $CD8^+$ IFNγ production is inhibited following $CD4^+CD25^+$ co-culture in FIV^+ cats but not FIV^- controls. Taken together, these results strongly suggest that in FIV lentiviral infection, $CD4^+CD25^+$ T regulatory cells utilize a membrane-TGFβ dependent mechanism to suppress $CD8^+$ lymphocyte responses.

V. In Vivo $CD4^+CD25^+$ Depletion Using Anti-CD25 Antibody

A. Abstract

The regulatory function of $CD4^+CD25^+$ lymphocytes was first discovered in mice by depleting $CD25^+$ cells from $CD4^+$ lymphocyte suspensions in adoptive transfer models. Since this discovery, different researchers have demonstrated that it is possible to administer anti-CD25 monoclonal antibody to mice in order to achieve in vivo $CD4^+CD25^+$ depletion. Intraperitoneal administration of anti-CD25 antibody has been utilized in a diversity of murine models to study the role of $CD4^+CD25^+$ T regulatory cells in autoimmunity, cancer and infectious disease. Using the FIV model for AIDS lentiviral infections, our laboratory has reported that $CD4^+CD25^+$ T regulatory cells are activated by FIV infection and suppress $CD4^+$ and $CD8^+$ effector responses during the acute and chronic stages of infection. In an effort to study $CD4^+CD25^+$ suppression in vivo, we have sought to develop a CD25 depletion strategy similar to murine models. First, in a pilot study, we show that the murine monoclonal anti-feline CD25 antibody, 9F23, can be safely administered to cats. In a larger, follow up study we demonstrate that 9F23 effectively depletes $CD4^+CD25^+$ lymphocytes from the blood following intravenous and intraperitoneal administration. Furthermore, following the $CD4^+CD25^+$ nadir, the cats did not demonstrate any physical or laboratory parameters consistent with autoimmunity as has been reported in different murine $CD4^+CD25^+$ depletion models.

B. Introduction

$CD4^+CD25^+$ T regulatory cells have been shown to suppress effector T cell responses to self antigen, tumors, transplanted tissue and many different infectious agents (54, 108, 118). T regulatory cells were first identified by the systematic ex vivo depletion of various T cell subsets. In similar experiments by Sakaguchi et al (108) and Suri-Payer et al(118), $CD4^+CD25^+$ T regulatory cells were first identified by depleting $CD25^+$ cells from lymphocyte suspensions. When the $CD25^+$ depleted suspensions were injected into nude or thymectomized mice, the mice developed a constellation of autoimmune diseases which were abrogated by re-constitution with $CD4^+CD25^+$ cells. Subsequent to this discovery, many different in vivo $CD25^+$ depletion models have been developed, mostly in the mouse. In vivo $CD4^+CD25^+$ depletion studies have illustrated the pivotal role T regulatory cells play in response to antigen. Inhibition of the inflammatory response can be beneficial by preventing autoimmunity or transplant rejection, but can be detrimental by preventing the elimination of tumors or infectious agents (54, 85, 89, 140, 141).

While in vivo depletion of T regulatory cells may enhance effector T cell responses, there is also evidence that depletion may result in collateral immunopathology. For example, in vivo depletion of $CD4^+CD25^+$ regulatory T cells in a mouse lymphoma model led to a reduction in tumor burden and prolonged survival when compared to non- $CD4^+CD25^+$ depleted controls (54). However, approximately 40% of the $CD4^+CD25^+$ depleted mice also developed clinical signs consistent with autoimmunity, such as wasting disease and colitis. In another example, Suvas and colleagues (121) reported that enhanced anti-HSV-1 $CD8^+$

immune responses in $CD25^+$-depleted mice correlated with a more rapid clearance of virus. Then, in a follow-up study using an HSV keratitis model, Suvas et al. (119) demonstrated that depletion of $CD25^+$ T cells prior to challenge with HSV-1 increased the severity of the ocular inflammatory lesions that correlated with an increase in HSV-specific $CD4^+$ T cell responses.

We previously reported that $CD4^+CD25^+$ T regulatory cells from FIV^+ cats during the acute and chronic, asymptomatic phase of infection are constitutively activated and able to suppress the proliferation and IL-2 production in ConA stimulated $CD4^+CD25^-$ T helper target cells (84, 135). Previous experiments outlined in this manuscript demonstrate that $CD4^+CD25^+$ T regulatory cells inhibit $CD8^+$ immune responses in both acute and chronic FIV infection. We have shown that ex-vivo depletion of $CD4^+CD25^+$ lymphocytes from FIV^+ but not FIV^- cats enhances mitogen induced $CD4^+$ and $CD8^+$ IFNγ production.

In an effort to assess the role of $CD4^+CD25^+$ lymphocytes in vivo during acute and chronic FIV infection, we developed an in vivo $CD25^+$ depletion model, based upon the murine models described above. For in vivo depletion, we utilized the feline specific anti-CD25 monoclonal antibody, 9F23, that was previously described for use in the ex vivo depletion experiments. This antibody is an IgG2a subtype which supports both antibody dependent cytotoxicity and complement-dependent cytotoxicity. Both intravenous and intraperitoneal antibody injection were evaluated as routes of administration. Our results demonstrate that the monoclonal antibody 9F23 depletes $CD25^+$ lymphocytes in vivo in FIV negative cats. Furthermore, we report that the cats did not develop clinical or laboratory

signs of autoimmunity following $CD4^+CD25^+$ depletion.

C. Methods

Cats: The initial pilot study consisted of three SPF cats, then twenty 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. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. The cats were divided into four groups consisting of five cats each. Groups were untreated control cats, cats which received an IV isotype control antibody, cats that were treated with 9F23 IV, and cats that were treated with 9F23 IP.

9F23 anti-CD25 antibody depletion: The murine monoclonal IgG2a antibody 9F23 is directed against the feline IL-2 alpha chain, also known as CD25 and was provided to the Tompkins laboratory by M. Honda (National Institute of Health, Tokyo, Japan). The isotype control antibody was a murine IgG2a monoclonal antibody directed against yellow fever antigen (CRL-1689, ATCC, Manassas, VA). Control cats were administered PBS IV on days 0, 3, and 7. Two groups of cats received either isotype control or 9F23 antibody IV at 3 mg/kg on days 0, 3, and 7. The fourth group of cats received a single IP 9F23 dose of 9 mg/kg on day 0.

Sample collection: Blood was collected via jugular venipuncture into EDTA vacutainer tubes. Two ml were retained for a complete blood cell count and lymphocyte subset analysis by multi-colored flow cytometry. Lymph node biopsies were performed as previously

described. Briefly, cats were anesthetized with intravenous ketamine and valium and anesthesia maintained with isofluorane gas. Popliteal lymph nodes were excised through a small incision in the caudal aspect of the stifle and the incision sutured with monofilament sutures. Lymph nodes were processed into a single cell suspension as previously described and used for phenotypic analysis by flow cytometry and for purification of lymphocyte subsets ($CD8^+$, $CD4^+CD25^+$ cells).

Lymphocyte subset analysis: The phenotype of lymphocytes from blood and lymph nodes was determined by multi-colored flow cytometric analysis. At least 5×10^5 LN cells or PBMCs were stained using a combination of the antibodies listed in Table 2. PBMCs were stained using an established whole blood lysis protocol (32). To determine the amount of 9F23 in vivo binding, PBMCs and LN cells were incubated with goat anti-mouse IgG FITC only. For flow cytometric analysis, lymphocytes were gated based upon forward vs. side scatter, and approximately 20,000 gated events were acquired and stored list-mode fashion for analysis using CellQuest software. Absolute numbers of lymphocytes were calculated using the percentage from FACS analysis, multiplied by the total lymphocyte count from either peripheral blood or LN.

Table 2: Antibody Sources

ANTIBODY SPECIFICITY	CONJUGATES	SOURCE
PAN T (anti-cat)	FITC	Tompkins Laboratory
CD4 (anti-cat)	FITC, PE, Biotin (SA-PerCP or APC)	Tompkins Laboratory
CD8 (anti-cat)	FITC, PE	Tompkins Laboratory
CD25 (anti-cat)	FITC	Tompkins Laboratory
CD21 (anti-canine)	PE	Serotec
B7.1 (anti-cat)	FITC, PE, Biotin	Tompkins Laboratory
CD62L (anti-human)	PE	BD Biosciences
TGFβ1 (anti-human)	APC (conjugated in Tompkins Lab)	R&D Systems

Antinuclear antibody (ANA) immunofluoresence assay: Day 21 and 28 serum samples from the five cats treated by i.p. anti-CD25 administration were evaluated for anti-nuclear antibody activity. Positive control serum was obtained from the NCSU-CVM clinical immunology laboratory. The control and experimental serum samples were stored frozen as described previously. Serial dilutions (1:20 to 1:200) of control serum and each sample were prepared using PBS as a diluent. Ten microliters of 1:20 dilution from the control and from each sample was placed on ANA Hep-2 substrate slides® (Immuno Concepts, Sacramento, CA). Slides were incubated at 37° C for fifteen minutes. The slides were then washed three times with PBS. FITC conjugated anti-feline IgG was then applied to each sample and incubated at 37° C for fifteen minutes. The slides were again washed three times with PBS. Slides were mounted with a coverslip and evaluated on a fluorescence microscope. All ten samples were negative (less than 1:20) for ANA activity.

D. Results

A pilot study to evaluate the safety and feasibility of 9F23 administration

Evaluation of 9F23 mediated depletion was separated into two separate studies. The first pilot study involved 3 cats and the objective was to assess the safety of administration and to indicate if $CD25^+$ depletion appeared feasible. Three cats were given 9F23 antibody IV as described in the methods section, and no adverse reactions were noted following each administration. Figure 11 shows flow cytometry results from 2 cats administered 9F23 and there appears to be $CD4^+CD25^+$ depletion in these cats. Figure 12 shows the peripheral blood $CD4^+CD25^+$ count and the anti-mouse IgG titer from days 0-36. Figures 11 and 12 demonstrate that the $CD4^+CD25^+$ nadir was at 7 and 9 days for the two cats. The third cat exhibited a high anti-mouse IgG titer starting at day 0 and did not exhibit appreciable $CD4^+CD25^+$ depletion (data not shown). These results suggest that administration of 9F23 murine anti-feline CD25 is safe and likely caused peripheral blood $CD25^+$ depletion. The results also suggest that the magnitude of $CD25^+$ depletion was dictated by the individual cat's immune response to the murine monoclonal antibody. Based upon this preliminary data, we proceeded with a more extensive study of $CD25^+$ depletion using 9F23 antibody.

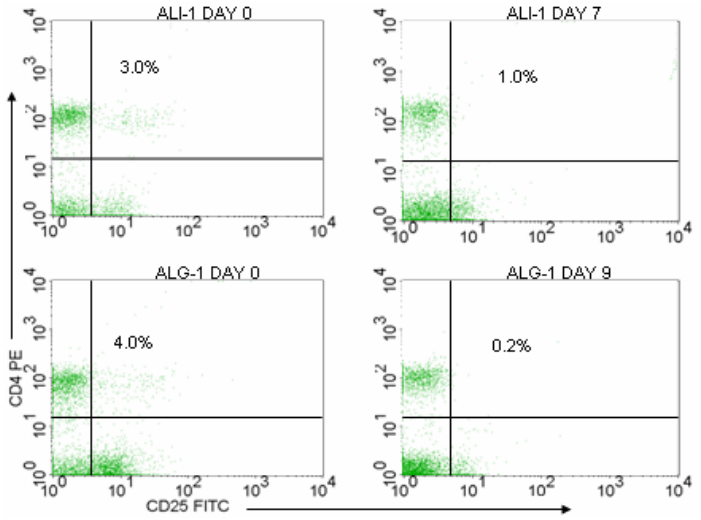

Figure 11. **Flow cytometry analysis from the pilot CD25$^+$ depletion study**. Day 0 shows CD4$^+$CD25$^+$ lymphocytes as 3-4% of the gated events (upper right quadrant). Day 7 and 9 represent the CD4$^+$CD25$^+$ nadir post 9F23 anti-CD25 injection (upper right quadrant). There is a reduction in the percent CD4$^+$CD25$^+$ lymphocytes in both cats.

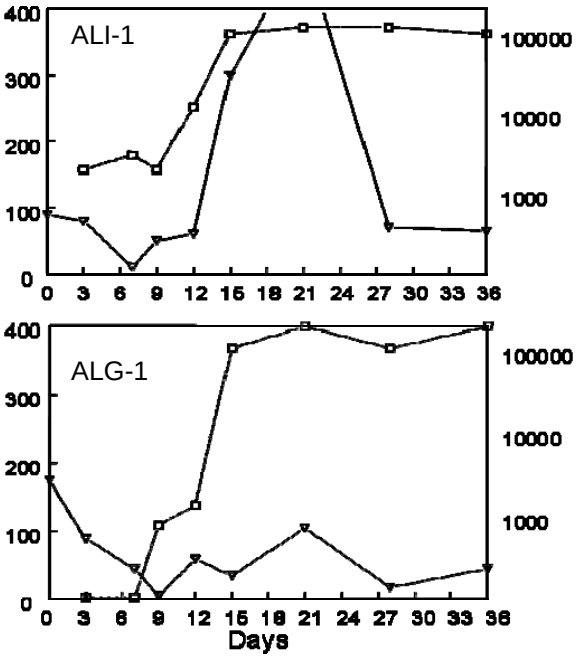

Figure 12. Cat ALI-1 and ALG-1 CD4$^+$CD25$^+$ cell count and anti- mouse IgG titer over time. The numbers on the left side of the figure represent the peripheral blood CD4$^+$CD25$^+$ count, the numbers on the right represent the anti-mouse IgG titer. SPF cats were injected with 9F23 (3 mg/kg IV), on days 0, 3, and 7. The approximate CD4$^+$CD25$^+$ cell count (solid line with triangles) demonstrates greater than 90% depletion in both cats. Serum anti-mouse IgG titer (dashed line with squares) rises and then plateaus by day 21.

9F23 anti-CD25 antibody administration results in peripheral blood $CD25^+$ depletion

Mouse studies have shown that IP administration of a single intraperitoneal anti-CD25 dose depletes $CD25^+$ cells as early as three days post-injection (121). We examined $CD25^+$ kinetics following both intravenous and intraperitoneal administration of 9F23 starting at 2-3 days post-injection. Figure 13(a) shows the $CD4^+CD25^+$ depletion nadir at days 2-3 for IV treated cats and at days 6-7 for IP treated cats, followed by a second nadir at day 35. Figure 13(b) shows that IP administration of 9F23 resulted in significant $CD4^+CD25^+$ depletion from days 6-7 until day 21. Although there is a second $CD4^+CD25^+$ nadir at day 35, there were not enough control animals at that time point for valid statistical comparison. The marker CD25 is the alpha chain of the IL-2 receptor and is displayed by $CD4^+$ and $CD8^+$ T lymphocytes as well as $CD21^+$ B lymphocytes (64). Administration of 9F23 also resulted in peripheral blood depletion kinetics in $CD8^+$ and $CD21^+$ lymphocytes, similar to those seen in $CD4^+$ lymphocytes (Figure 14). Based upon the results from figure 12 we conclude that 9F23 intraperitoneal administration is safe and effectively depletes peripheral blood $CD4^+CD25^+$ lymphocytes from peripheral blood from approximately day 6 that persists until day 21 and possibly further.

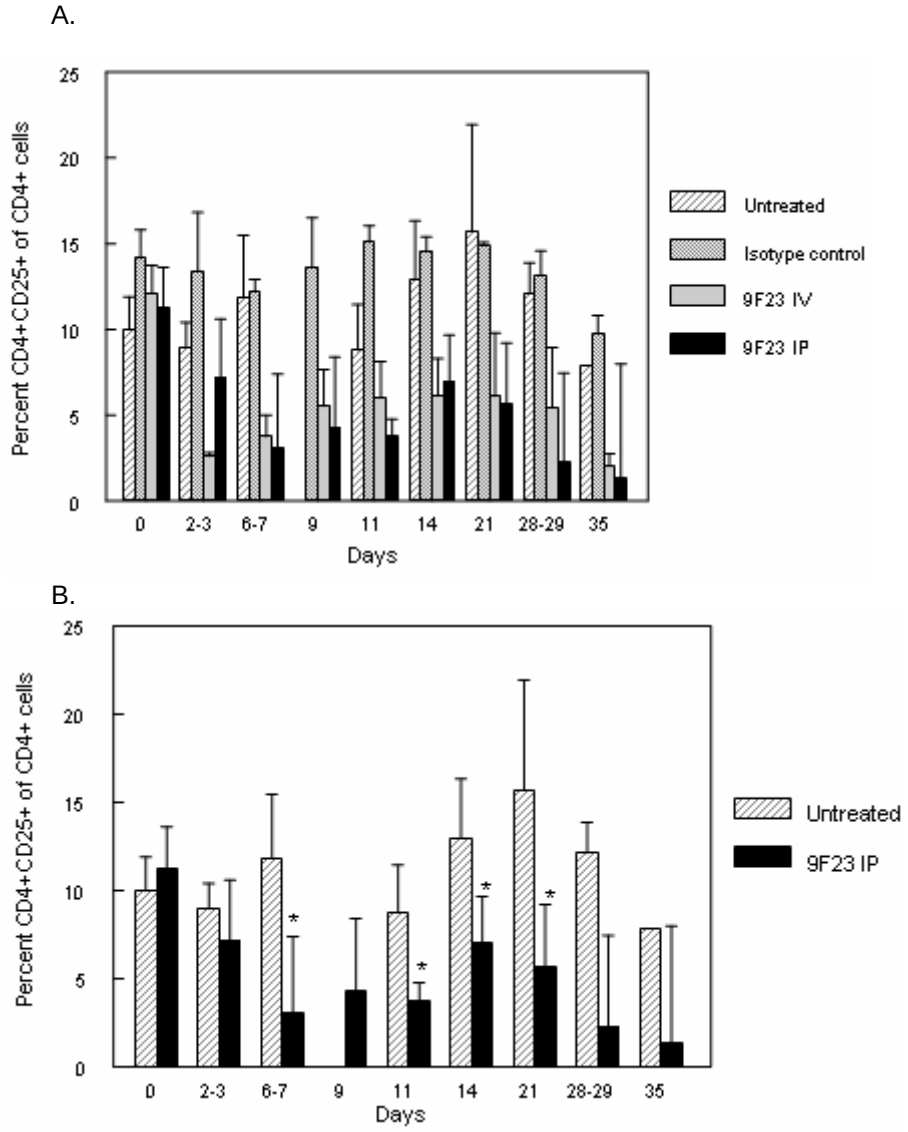

Figure 13. Administration of 9F23 anti-CD25 antibody results in $CD4^+CD25^+$ depletion from the $CD4^+$ population (a). The percentage of peripheral blood $CD4^+CD25^+$ lymphocytes within the $CD4^+$ population from untreated, isotype control, and 9F23 treated cats. Intravenous (IV) 9F23 treatment results in the $CD4^+CD25^+$ nadir at day 2-3 (gray bar), while intraperitoneal (IP) treatment results in the $CD4^+CD25^+$ nadir at day 6-7 (black bar). Each bar represents the mean and standard deviation for each treatment group at the indicated days post-treatment. SPF cats were injected with 9F23 (3 mg/kg IV), on days 0, 3, and 7 or with 9F23 (9 mg/kg IP) on day 0. **(b).** IP administration of 9F23 results in significant $CD4^+CD25^+$ depletion (asterisks) at day 6-7 through day 21 as compared to untreated control animals.

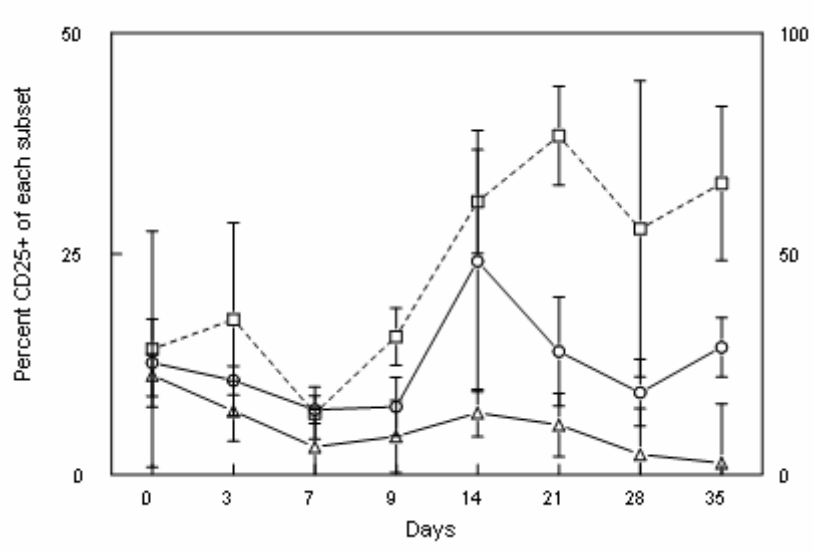

Figure 14. Intraperitoneal administration of 9F23 results in depletion of all $CD25^+$ lymphocyte subsets. Depletion kinetics for the $CD8^+CD25^+$ (circles) and $CD21^+CD25^+$ (squares) follows a similar pattern to $CD4^+CD25^+$ depletion (triangles). The percentage of $CD4^+CD25^+$ and $CD8^+CD25^+$ of total $CD4^+$ and $CD8^+$ lymphocytes is depicted on the primary y axis and the percentage of $CD21^+CD25^+$ of total $CD21^+$ lymphocytes is depicted on the secondary y axis. The mean and standard deviation for each group is shown for each time point evaluated post 9F23 administration.

Analysis of lymph node $CD4^+CD25^+$ cells following 9F23 administration

Lymph nodes from 9F23 treated cats (IV, n=2 and IP, n=3) and control cats (n=2) were evaluated at days 9 and 21. Lymph node cells were phenotyped by flow cytometry as described in the methods section. As shown in figure 15, the number of $CD4^+CD25^+$ cells did not appear to differ in treated versus untreated cats, although the sample size was very small. Subsequent investigation, however, has demonstrated that 9F23 does deplete $CD4^+CD25^+$ cells in the lymphoid compartment (117).

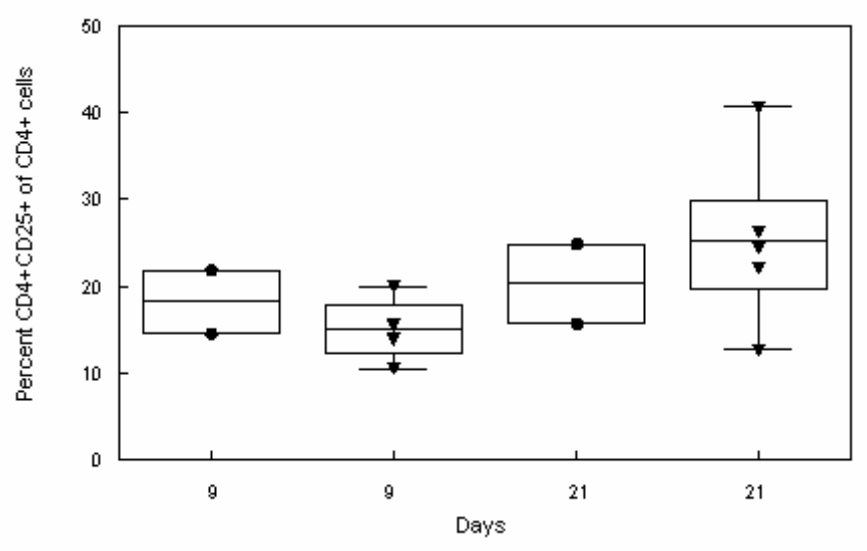

Figure 15. $CD4^+CD25^+$ percentage in LN from control and 9F23 treated cats. SPF cats were injected with 9F23 (3 mg/kg IV), on days 0, 3, and 7 or with 9F23 (9 mg/kg IP) on day 0. Lymph nodes from control cats (n=2, black circles) and 9F23 treated cats (black triangles) were evaluated at days 9 (n=2 IV, n=2 IP) and 21 (n=2 IV, n=3 IP) by FACS. $CD4^+CD25^+$ values are expressed as the percentage of $CD4^+$ cells ($CD4^+CD25^+/CD4^+$ x 100). There is not enough data for statistical analysis at either time point.

Evaluation of anti-nuclear antibody in 9F23 treated cats

Murine models have demonstrated exacerbation of inflammatory lesions and the development of autoimmune symptoms following $CD4^+CD25^+$ depletion (54, 119). During the course of investigation, the cats receiving 9F23 treatment did not exhibit any abnormal physical examination findings. As a marker for the development of autoimmunity, we evaluated serum from all IP treated cats (n=5) three and four weeks post 9F23 treatment for the presence of anti-nuclear antibodies (ANA). As shown in table 2, all ten samples were ANA negative. The lack of ANA production was unlikely due to depletion of the B cell compartment by 9F23, because anti-mouse IgG production was evident in all three cats from the pilot study, two of which are shown in figure 12. The lack of abnormal physical

examination findings combined with the normal ANA suggest that administration of a single 9 mg/kg dose of 9F23 does not result in the development of autoimmunity.

Table 3: Antinuclear antibody titer (ANA) approximately three and four weeks following 9F23 administration. Monoclonal anti-CD25 was delivered by intraperitoneal injection. Antinuclear antibody was assessed by IFA. All cats were negative for antinuclear antibody.

Cat Number	ANA titer Week 3	ANA titer Week 4
AUJ 3	< 1:20	< 1:20
QVH 4	< 1:20	< 1:20
AUI 3	< 1:20	< 1:20
AUI 2	< 1:20	< 1:20
QVH 2	< 1:20	< 1:20

Discussion

The first objective of this study was to ensure that 9F23 administration to cats was safe. Because the monoclonal antibody 9F23 is a murine IgG2a monoclonal antibody it is recognized by the cats as a foreign protein. Our first concern was that the cats may develop an immediate hypersensitivity reaction following administration. No cats in the study developed symptoms consistent with an acute hypersensitivity such as a fever, tachypnea, or tachycardia. Furthermore, no cats developed symptoms consistent "serum sickness" such as sore joints, anorexia or malaise. Because 9F23 is a murine protein, $CD25^+$ depletion is limited by the development of each cat's anti-mouse antibody response as shown in figure 12.

In murine systems, $CD25^+$ depletion has been utilized to study the role of $CD4^+CD25^+$ mediated suppression of effector T cell responses to self-antigen, tumors and

infectious agents. Most murine models utilize a single IP injection with maximal depletion following within 1 to 3 days post treatment (55, 89, 121). Our pilot study indicated that IV administration of 9F23 anti-CD25 caused maximal depletion between days 7 and 9 post treatment. Intraperitoneal administration also resulted in depletion and was a much easier route of administration. In this feline model, intraperitoneal injection of 9F23 caused maximal depletion at 7 days post treatment and 9F23 caused sustained $CD4^+CD25^+$ depletion out to 21 days, and likely even further (Figure 13).

The marker CD25 is the high affinity IL-2 receptor and is displayed by activated $CD4^+$ T helper cells, $CD8^+$ lymphocytes and B lymphocytes. Therefore, $CD25^+$ depletion not only depletes $CD4^+CD25^+$ $FOXP3^+$ T regulatory cells, but also depletes these other lymphocyte subsets. Figure 13 indicates that 9F23 resulted in depletion of all $CD25^+$ subsets. However, murine models have demonstrated that $CD25^+$ depletion does not result in immunosuppression caused by depletion of other lymphocyte subsets (54, 108, 118). The results from the pilot indicate that the humoral response was intact following 9F23 administration because the cats effectively mounted an anti-mouse antibody response. Further study by Smithberg et al (117) using this 9F23 cat model has confirmed that the humoral response is intact and has also shown that the T effector response is likewise intact.

Data from mouse studies suggests that in vivo administration of anti-CD25 antibody depletes $CD25^+$ cells from the lymphoid compartment as well as peripheral blood (55, 141). In this study, evaluation of lymph node depletion was limited to two control cats and five 9F23 treated cats (n=2 intravenous, n=3 intraperitoneal) at days 9 and 21 after 9F23

administration (Figure 15). The number of control and experimental cats were insufficient to determine $CD25^+$ depletion. Subsequent to this study, 9F23 antibody treatment has been shown to deplete $CD4^+CD25^+$ lymphocytes from lymphoid tissue and more importantly, reduce the overall expression of the T regulatory cell marker FOXP3 in lymphoid tissue (117).

One concern following $CD4^+CD25^+$ depletion is the development of autoimmunity. This is understandable considering that T regulatory cells were first discovered in adoptive transfer experiments with ex-vivo $CD4^+CD25^+$ depletion from $CD4^+$ lymphocyte suspensions and the subsequent development of autoimmunity (108, 118). Since this discovery, other studies have noted the development of clinical signs consistent with autoimmunity following $CD25^+$ depletion (54). Furthermore, in a murine model of SLE, adoptive transfer of apoptotic cell- pulsed dendritic cells, combined with $CD25^+$ depletion enhanced anti- DNA antibody production (60). The cats in our study did not develop any physical manifestations consistent with autoimmunity. We also looked for the development of anti-nuclear antibody as a laboratory marker for autoimmunity. Maximal $CD4^+CD25^+$ depletion was noted in IP injected cats 7 days post 9F23 treatment. The cats in Figure 12 developed a rapid anti-mouse antibody response which was evident at 9 days and peak at 21 days post 9F23 treatment, indicating that the humoral response was intact. Based upon these findings, we theorized that if present, autoantibody production would be evident 2-3 weeks following the $CD4^+CD25^+$ nadir. Samples were evaluated from days 21 and days 28 post 9F23 treatment, which correspond to 2 and 3 weeks after the $CD25^+$ nadir shown in Figure 12. As shown in Table

2, none of the IP treated cats exhibited autoantibody production following $CD4^+CD25^+$ depletion. It is likely that in an outbred species such as the cat, transient $CD4^+CD25^+$ depletion alone is not enough to break self tolerance.

This laboratory was the first to identify a population of $CD4^+CD25^+$ T cells in the cat and describe their phenotypic and functional activation in the FIV-feline infection model for human AIDS (65-67, 135). Similar to human and murine Treg cells, feline Treg cells are anergic and fail to produce IL2 and proliferate in response to immune stimulation. We reported that lentivirus infection results in the constitutive activation of Treg cells resulting in immune suppression (135). We found that freshly isolated $CD4^+CD25^+$ T cells from asymptomatic FIV^+ cats suppress IL2 production and the proliferative response of ConA-stimulated autologous $CD4^+CD25^-$ T cells in a dose-dependent manner, suggesting that the Treg cells are activated in vivo in response to FIV infection. Using the FIV model, we were the first to report that $CD4^+CD25^+$ Treg cells provide a reservoir for productive lentivirus replication (66, 67). More recently we have reported that $CD4^+CD25^+$ T regulatory cells suppress $CD8^+$ responses in both the acute and chronic stages of FIV infection. We have also shown that ex-vivo $CD4^+CD25^+$ depletion enhances IFNγ production in lymph node suspensions, following mitogenic stimulation. In related studies, Aandahl et al. (2) reported that depletion of $CD25^+$ T cells from PBMC from HIV^+ patients not on HAART enhanced the frequency of IFNγ and TNFα expressing T cells in response to stimulation with HIV and CMV antigens, indicating that HIV-induced Treg cells suppress T cell responses to specific antigens, as well as unrelated antigens. Eggena et al. (39) similarly reported that in vitro

depletion of Treg cells from HIV^+ PBMC increased gag-specific $CD8^+$ responses, as measured by IFNγ ELISpot. The results from this study and subsequent investigation has demonstrated that 9F23 effectively depletes $CD4^+CD25^+$ lymphocytes from both the peripheral blood and lymphoid tissues of normal, healthy cats (117). Taken together, these data indicate that in vivo $CD4^+CD25^+$ depletion in FIV infected cats may indeed enhance both $CD4^+$ and $CD8^+$ immune responses.

$CD4^+CD25^+$ depletion in FIV infected cats may just as likely result in severe immunopathology resulting from poorly controlled inflammation. Suvas et al (119) clearly showed that administration of anti-$CD25^+$ antibody prior to HSV infection resulted in greater immunopathology associated with expansion of effector T cell populations. Furthermore, lower infectious doses of virus were able to induce pathology, compared with undepleted, HSV infected controls. Kinter et al. (71) reported that $CD4^+CD25^+$ T cells in the majority of healthy HIV^+ patients significantly suppressed cellular proliferation and cytokine production by $CD4^+$ and $CD8^+$ T cells stimulated with HIV peptides in vitro. Intriguingly, the level of Treg suppressor function correlated inversely with plasma viremia in these patients. Eggena et al. (39) reported a decline in $CD4^+CD25^+$ Treg cells in late stage HIV infection that was associated with increased T cell immune hyperactivation and higher viremia. In another study, Weiss et al. (142) reported a significant expansion of $CD4^+CD25^+$ T cells in the blood of HIV^+ patients on anti-retroviral therapy. So the involvement of T regulatory cells in lentiviral pathogenesis may be a double-edged sword- the very cell that contributes to viral persistence and immunosuppression may also be preventing overwhelming systemic

inflammation. Further in vitro and in vivo investigations, using the FIV model, may help to more clearly define the role of T regulatory cells in AIDS lentiviral infection.

VI. SUMMARY

In 2004, this laboratory was the first to report that FIV infection activates $CD4^+CD25^+$ T regulatory cells and that $CD4^+CD25^+$ lymphocytes from chronically infected cats were able to suppress $CD4^+CD25^-$ proliferation. Since that time, we have been able to demonstrate that FIV preferentially replicates in $CD4^+CD25^+$ cells in vitro and in vivo; and that infection of Tregs results in up-regulation of the transcription factor FOXP3. Data also suggest that T regulatory cells express surface TGFβ and that this may be one mechanism for T regulatory mediated suppression.

The aims of the experiments in section III were to demonstrate that $CD4^+CD25^+$ T regulatory cells inhibited $CD8^+$ responses during the acute and chronic stage of FIV infection. To study the acute stage of infection, cats were infected intravenously with FIV. Blood was sampled for lymphocyte phenotype and for plasma viremia. Peripheral lymph nodes were harvested periodically during the acute phase of infection to assess lymphocyte phenotype and function. Depletion of $CD4^+CD25^+$ cells from lymphocyte suspensions resulted in increased IFNγ production, as measured by ELISpot, starting at 21 days p.i. Co-culture of $CD4^+CD25^+$ lymphocytes with $CD8^+$ lymphocytes resulted in suppression of IFNγ starting at approximately 28 days p.i. We previously identified that FOXP3 is up-regulated in $CD4^+CD25^+$ lymphocytes in the lymph node of FIV^+ cats starting at approximately 21 days p.i. and this corresponds to the suppression of IFNγ production during the acute phase of FIV infection. During the chronic stage of FIV infection, we also demonstrated by real time RT-PCR that $CD4^+CD25^+$ T regulatory cells from FIV^+ cats suppressed IFNγ mRNA in

ConA stimulated $CD8^+$ lymphocytes following a 2 hour co-culture. Taken together, these results suggest that $CD4^+CD25^+$ T regulatory cells from FIV^+ cats inhibit $CD8^+$ responses during both the acute and chronic stages of FIV.

The series of experiments in section IV were designed to explore the mechanism of T regulatory mediated $CD8^+$ suppression. Previous work from our laboratory had suggested that mTGFβ was up-regulated on $CD4^+CD25^+$ T regulatory cells during chronic FIV infection. We postulated that if mTGFβ was the mechanism of suppression, then the responder cell, in this case $CD8^+$ lymphocytes, must also display TGFβRII. Peripheral blood and LN $CD4^+CD25^+$ and $CD8^+$ lymphocytes from chronically infected (1 year) FIV^+ and FIV^- control cats were examined by flow cytometry for the expression of mTGFβ and TGFβRII, respectively. There was a significantly higher mean percentage of $CD4^+CD25^+mTGF\beta^+$ cells in the lymph node of FIV^+ (50%) cats when compared to FIV^- cats (27%). There was a marked difference in the mean percentage $CD8^+TGF\beta RII^+$ lymphocytes in FIV^+ cats when compared to FIV^- cats in both the blood (22% vs. 5%) and lymph node (15% vs. 2%). Expanding on these observations, we next examined the TGFβ signaling pathway in $CD8^+$ lymphocytes, following $CD4^+CD25^+$ co-culture. Activation of the TGFβ receptor complex by TGFβ leads to a series of intracellular phosphorylation events to propagate the signal and one of the first events specific to this pathway is the phosphorylation of the Smad2 protein. Following a 2 hour co-culture with $CD4^+CD25^+$ lymphocytes, $CD8^+$ target cells exhibited Smad2 phosphorylation in excess of the TGFβ

positive control. Finally, we demonstrated that $CD4^+CD25^+$ lymphocytes from FIV^+ cats markedly suppressed IFNγ mRNA expression in ConA stimulated $CD8^+$ target cells with greater suppression than the TGFβ control. These results indicate that utilization of mTGFβ by $CD4^+CD25^+$ lymphocytes is one of the mechanisms of $CD8^+$ suppression during the course of FIV lentiviral infection.

Section V outlines two experiments which explore the utility of in vivo T regulatory cell depletion using a mouse monoclonal anti-feline CD25 antibody, 9F23. The first experiment was a pilot study using three FIV negative cats. The aims were to ensure that 9F23 could be safely administered to cats and to show that 9F23 may cause $CD25^+$ depletion. The 9F23 antibody was administered to all three cats intravenously without complication. Two of three cats exhibited $CD4^+CD25^+$ depletion in the peripheral blood approximately one week after 9F23 administration. All three cats mounted an anti-mouse antibody response suggesting that lymphocyte depletion did not cause immunosuppression.

The second experiment examined 9F23 administration in a larger number of FIV negative, specific pathogen free cats. Cats were given 9F23 by intravenous and intraperitoneal routes and a group of control cats that was administered an isotype control antibody. There was also concern that the cats may develop autoimmunity, because mice have developed autoimmunity in murine models of $CD4^+CD25^+$ depletion. Peripheral blood depletion of the $CD4^+CD25^+$ subset was noted in both groups administered 9F23. The intraperitoneal route of administration proved easier and resulted in consistent $CD25^+$ depletion. As a marker for autoimmunity, the cats in this study were evaluated for anti-

nuclear antibodies (ANA) following 9F23 administration at two different time points and none of the cats developed ANAs. The results of these experiments demonstrate that 9F23 anti-CD25 antibody can be safely administered to cats intraperitoneally and results in peripheral blood $CD4^+CD25^+$ depletion.

VII. FUTURE INVESTIGATIONS

The experiments outlined in section III will be repeated using FIV viral antigen to stimulate $CD8^+$ lymphocytes, instead of ConA. Also, we have not answered the question of T regulatory suppression of $CD8^+$ proliferation during FIV infection. Using CFSE labeling, we will perform co-culture experiments with acutely and chronically infected cats to assess $CD8^+$ proliferation.

The experiments from section IV explored the role of mTGFβ as a possible mechanism for T regulatory mediated $CD8^+$ suppression. We have demonstrated that Tregs up-regulate mTGFβ, that $CD8^+$ lymphocytes up-regulate TGFβRII, and that co-culture of Tregs and $CD8^+$ lymphocytes results in activation of the TGFβ signaling pathway. We are currently trying to block this interaction by different means- including antibody directed against both TGFβ and TGFβRII, drugs that block the Smad signaling pathway, and possibly a TGFβ peptide inhibitor called P17. Finally, we will explore the intracellular signaling events in $CD8^+$ lymphocytes following T regulatory co-culture in an effort to further characterize events leading to $CD8^+$ anergy and / or apoptosis.

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