ABSTRACT

NAG, MUKTA. Utilization of Lentiviral Models to Assess Epigenetic Changes in $CD8^+$ T cells. (Under the direction of Dr. Jonathan E. Fogle and Dr. Kristina De Paris)

$CD8^+$ T cells are critical for controlling viremia during Human Immunodeficiency Virus (HIV) infection. These cells produce cytolytic factors and antiviral cytokines that eliminate virally-infected cells. During the chronic phase of HIV infection, $CD8^+$ T cells progressively lose their proliferative capacity and antiviral functions. These dysfunctional cells are unable clear the productively infected and reactivated cells, representing a roadblock in HIV cure. Therefore, mechanisms to understand $CD8^+$ T cell dysfunction and strategies to boost $CD8^+$ T cell function need to be investigated. T regulatory cells are known to mediate $CD8^+$ T cell dysfunction during HIV infection. Here, using the feline and the simian models of HIV-infection, we explore the mechanism of Treg-mediated suppression of $CD8^+$ T cells. Using the FIV model, we demonstrate that Foxp3 induced by Treg cells, binds to the IL-2, IFN-γ and TNF-α promoters in virus-specific $CD8^+$ T cells resulting in their suppression. We aim to further test the translational relevance of the Treg-induced Foxp3-mediated mechanism of $CD8^+$ T cell suppression in the SIV model. As a first step, here we demonstrate the binding of Foxp3 to the antiviral cytokine promoters in stimulated rhesus macaque $CD8^+$ T cells *in vitro*. Next, we elucidate the molecular events enabling the binding of Foxp3 to the cytokine promoters. Specifically, we demonstrate that histone acetylation plays an important role in allowing Foxp3 binding at the IL-2 promoter, and that by blocking histone acetylation, Foxp3 binding is reduced. Therefore, we propose that epigenetically controlled Foxp3-mediated suppression of $CD8^+$ T cells can be alleviated by modulating these cells. Epigenetic modulation of dysfunctional $CD8^+$ T cells holds promise in

augmenting the function of these cells upon subsequent stimulation. These functionally competent $CD8^+$ T cells used in conjunction with ART and Latency reversal agents may open up new avenues for HIV cure strategies.

Utilization of Lentiviral Models to Assess Epigenetic Changes in $CD8^+$ T cells.

by
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DEDICATION

Dedicated to my grandfather- Dr. Krishna K. Sharma … miss you always!

BIOGRAPHY

Mukta Nag is an Immunology Doctoral candidate in the Comparative Biomedical Sciences Program. Originally from New Delhi, India, she received her undergraduate degree in Biotechnology Engineering from Manipal, India. She then moved to Raleigh, North Carolina for a Master's program from NC State. Through her Master's program, she got interested in the technology commercialization aspect of biologics. Upon working with various start-ups during her Master's, she decided to eventually pursue vaccine development and commercialization. She graduated from the Microbial Biotechnology program, North Carolina State University in 2014 and joined the CBS program. She was trained in Dr. Jonathan Fogle's lab at NC State and in Dr. Kristina De Paris' lab at UNC, Chapel Hill. She gained exposure in molecular biology and epigenetic techniques using the feline and the Simian Immunodeficiency Virus models of HIV infection. Next, she will be working on new HIV vaccine strategies in the AIDS and Cancer Virus Program, National Cancer Institute, MD. She plans to get an MBA to act upon her plan to work in vaccine technology commercialization.

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CHAPTER 1

Introduction

Epigenetic modulation of $CD8^+$ T cell function in lentivirus infections: a review

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Acquired immune deficiency syndrome (AIDS) epidemic was first described by the cases of *Pneumocystis carnii* pneumonia (PCP) infection and later by the cases of Kaposi's Sarcoma reported in 1981 by the U.S. Centers for Disease Control and Prevention (CDC). In September 1982, the CDC released the first definition of AIDS for the first time. Luc Antoine Montagnier and members of his group including Françoise Barré-Sinoussi at the Pasteur Institute found the virus that was later called Human immunodeficiency virus (HIV) and named it "lymphadenopathy –associated virus" (LAV) in 1983. Robert Gallo's group at the U.S. National Institutes of Health (NIH) also published similar findings in 1983 where they called the virus "human T-lymphotropic virus type III" (HTLV-III). The controversy was settled in 2008 when the Nobel Prize was awarded to Montagnier and Françoise Barré-Sinoussi for the discovery of HIV (1). A steep increase in the number of HIV infected patients was recorded between 1981-early 2000's as the first commercial diagnostic blood test for HIV was licensed in 1985 and the various routes of infection/ transmission were extensively studied. With the widespread adoption of Highly Active Antiretroviral therapy (HAART) worldwide since its first approval in 1995, the mortality associated with HIV infection has reduced from approximately 2 million deaths in 2005- 2006 to 1 million in 2016 globally (2). However, despite reduced mortality and morbidity associated with HIV infection approximately 1.1 million people continue to live with HIV

infection within the U.S. in 2018 (3). Therefore, effective cure strategies need to be developed to eradicate/ better control the disease.

$CD8^+$ T lymphocytes (CTLs) play a critical role in the control of HIV infection (4, 5). The HIV-specific $CD8^+$ T cell response peaks just after peak viremia during the acute phase of infection, and then reaches a lower level steady state in the chronic phase of infection (6). Efficient control of HIV replication by HIV-specific $CD8^+$ T cells is attributed to both the cytotoxic function and the polyfunctional cytokine response (7). Despite the robust antiviral cytolytic CTL response in the acute phase, lentiviruses are able to escape immune recognition and persist in the host. Long-term viral persistence results in continued immune activation, and this in turn impairs the $CD8^+$ T cell response. $CD8^+$ T cells become progressively dysfunctional as the lentivirus infection proceeds. Dysfunctional $CD8^+$ T cells exhibit reduced proliferative capacity and defective effector function, including decreased production of the antiviral cytokines IL-2, IFN-γ and TNF-α (8, 9). Figure 1.1 summarizes the $CD8^+$ T cell response during HIV infection.

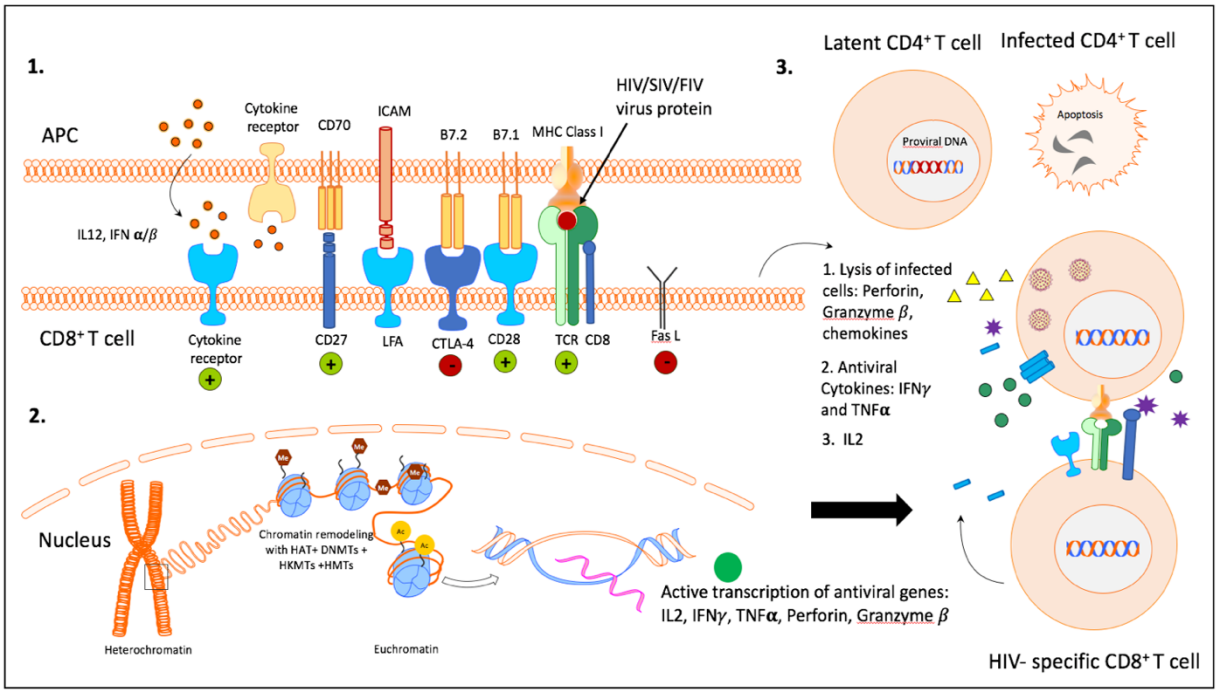
The introduction of HAART and combination ART (cART) have transformed HIV/AIDS from a fatal disease to a chronic disease with almost normal life expectancy. The new challenge in our fight against the HIV/ AIDS epidemic is now finding a cure. The biggest obstacle in cure research is developing an effective strategy that can reactivate latently infected cells and at the same time eliminate the purged virus and virally infected cells (10-12). $CD8^+$ T cells are vital in the process of eliminating infected cells (13-15). However, chronically activated $CD8^+$ T cells that are functionally impaired are unable to clear the infection (16, 17). Therefore, there is a critical need to identify novel methods to boost and restore $CD8^+$ T cell function.

To achieve this goal, we need to understand the molecular mechanisms leading to $CD8^+$ T cell dysfunction. Exhausted $CD8^+$ T cells can be phenotypically characterized by the expression of inhibitory molecules, such as PD-1, CTLA-4, LAG-3, TIM-3, and TIGIT, that interfere with effective T cell activation (18, 19). In HIV infection and the rhesus macaque model of simian immunodeficiency virus (SIV) infection, checkpoint inhibitors targeting these molecules can partially restore $CD8^+$ T cell function (20-22). These studies provide proof-of-concept for the feasibility of developing effective immune strategies to enhance $CD8^+$ T cell function *in vivo*.

In this dissertation, we will discuss additional mechanisms that may be exploited to enhance $CD8^+$ T cell function. Specifically, we will focus on the role of regulatory $CD4^+$ T cells (Treg cells) and epigenetic modifications in the regulation of $CD8^+$ T cell function. Figure 1.2A illustrates some of the main mechanisms leading to the generation of dysfunctional $CD8^+$ T cells.

Figure 1.1: $CD8^+$ T cell response to Human Immunodeficiency Virus (HIV)/ Simian Immunodeficiency Virus (SIV)/ Feline Immunodeficiency Virus (FIV) infection.

Part 1: Interaction of a professional antigen presenting cell (APC), e.g. dendritic cells, with a $CD8^+$ T cell during lentivirus infection. The APCs present the viral antigens on the surface via major histocompatibility complex (MHC) Class I molecules for recognition by the T cell receptor (TCR) on $CD8^+$ T cells. Three signals are needed for $CD8^+$ T cell activation. The first signal is provided by the engagement of the $CD8^+$ TCR with MHC Class I on the APC cell surface presenting the viral peptide. The second signal is provided by the engagement of co-stimulatory molecules, here CD28, on the $CD8^+$ T cells with CD80 (B7.1) on the APCs. Cytokines provide the third signal required for T cell proliferation. As the $CD8^+$ T cells become activated, they also begin upregulating CTLA-4. CTLA-4 binding with CD86 (B7.2) on APCs delivers an inhibitory signal to maintain immune homeostasis. Part 2. Upon $CD8^+$ T cell activation, the chromatin within the nucleus "relaxes" into euchromatin conformation to allow the binding of various factors of the transcriptional machinery. Chromatin remodeling enzymes such as Histone Acetyltransferases (HAT), Histone methyltransferases (HMTs), DNA methyltransferases (DNMTs) and Histone lysine methyltransferases (HKMTs) alter the accessibility of chromatin at specific sites based on the signals provided to direct the specific response. During HIV infection, "relaxed" chromatin in gene promoter regions allows for the active transcription of antiviral genes such IL-2, IFN-γ, TNF-$\boldsymbol{\alpha}$, Perforin, Granzyme β. Part 3 illustrates the interaction of activated HIV- specific $CD8^+$ T cells with productively HIV-infected $CD4^+$ T cells. Upon recognition, the infected $CD4^+$ T cells undergo lysis and apoptosis due to the action of perforin, Granzyme β, chemokines, IFN-γ and TNF-$\boldsymbol{\alpha}$. IL-2 expressed by activated $CD8^+$ T cells aids in T cell proliferation. Despite a robust HIV – specific $CD8^+$ T cell response in the acute phase, latently infected $CD4^+$ T cells escape immune recognition and may become reactivated at a later during the infection. Effective reactivation and elimination of these latently infected cells is one of the major obstacles to HIV cure.



Symbol	Key
	IL12
	Perforin
	IL2, IFNγ and TNFα
	Granzyme β
	HIV virus
	Chemokine
	DNA

Animal models of HIV infection to study $CD8^+$ T cell dysfunction

Animal models provide a useful tool to study HIV infection, its pathogenesis and cure strategies. The three most commonly used animal models in HIV research are the humanized mouse model, the feline immunodeficiency virus (FIV) model, and the simian immunodeficiency virus (SIV) model in non-human primates (NHP). Humanized mice represent a relatively simple, rapid and small-sized animal model used in HIV research. These mice are immunocompromised animals that are reconstituted, in part, with human immune cells. Humanized mice, specifically SCID-hu mice have been instrumental to documenting the existence of viral latency and understanding latently infected cells (23, 24). Initial investigations using LRAs demonstrating the activation of HIV-1 and one of the earliest "shock and kill" experiments was shown in SCID-hu mice (24). The humanized- mouse model and its utilization in HIV research have been reviewed in Policicchio et al., 2016 (25). In this chapter, we will be discussing the FIV and SIV models of HIV infection.

The feline immunodeficiency virus (FIV) infection model represents the only animal model of a naturally occurring immunodeficiency infection caused by a lentivirus. This outbred model is representative of HIV disease progression in humans (26-28). FIV, akin to HIV, is transmitted by blood transfer, mucosal contact and vertically via prenatal and postnatal routes (29, 30). During FIV infection, there is a short acute phase lasting a few weeks, followed by a protracted asymptomatic phase of chronic infection spanning several months to years. The type of infecting FIV strain and the genotype of the cat significantly influence the rate of disease progression. Infection eventually leads to the development of immunodeficiency symptoms consistent with the disease progression in HIV-infected patients (reviewed in Elder et al., 2010 (27)). FIV-

infected cats exhibit high viremia during the acute phase, and, as the disease progresses, the drop in $CD4^+$ T cells causes a sharp increase in viral RNA levels ultimately leading to immune dysfunction (27). The compromised immune system increases the risk of muscle wasting, opportunistic infections and neoplasia in cats, analogous to humans (31). This phase is defined as Feline Acquired Immunodeficiency Syndrome (FAIDS) (26). Certain strains of FIV are neuro-virulent and have degenerative effects on the central nervous system similar to the neurological impairment seen during HIV-1 infection (32-34). A commercially available prophylactic vaccine against FIV (Fel-O-Vax FIV by Boehringer Ingelheim) was released in the U.S. in 2002 and informed the identification of conserved vaccine epitopes for HIV vaccine development (35). The FIV model has been extensively used for the development and efficacy studies of antiretroviral drugs for HIV treatment. Studies determining the *in vivo* efficacy of acyclic nucleoside phosphonate analogues, which led to the development of tenofovir, one of the most commonly used drugs in the ART regime was demonstrated first in the FIV model (36, 37). FIV is sensitive to Nucleoside- analogue reverse-transcriptase inhibitors (NRTIs) and HIV-1 integrase inhibitors, enabling efficacy studies of these classes of drugs (38-41). Transgenic cats, genetically engineered to express HIV –specific restriction factors such as TRIMCyp, have improved the translational relevance of the FIV model to study HIV infection and pathogenesis (42). FIV has also been studied as a model for lentivirus latency (reviewed in McDonnel et al., 2013(28)). The FIV promoter in latent *in-vivo* $CD4^+$ T cells exhibits de-acetylated histones suggesting a repressive transcriptional state consistent with the findings in HAART treated HIV patients (43-45). Therefore, the FIV model was applied to study the effects of the latency reversal agent (LRA) suberoylanilide hydroxamic acid (SAHA) in reactivating latent viral reservoirs (46). In summary, the FIV model has enabled the testing of multiple treatment and

lentivirus control strategies that are not feasible to perform in HIV – infected patients. This model has, however, some limitations. There are differences between the FIV and HIV-1 viral genome. FIV lacks the *vpr, vpu* and *nef* equivalent genes, and does not encode the transactivating protein Tat or the gene for the transactivating responsive element Tar that are present in the HIV-1 genome. Instead, FIV expresses OrfA, an accessory protein enabling enhanced viral transcription and viral release from infected cells, which is not encoded by the HIV genome (27, 47). FIV uses CD134 as the main binding receptor instead of CD4, enabling the virus to infect B cells and $CD8^+$ T cells along with $CD4^+$ T cells and macrophages, whereas $CD4^+$ T cells and macrophages serve as the main target cells in HIV infection (48, 49). CD134 also known as the OX-40 receptor belongs to the TNFR superfamily, member 4 and is required for T cell co-stimulation. CD134 is expressed 24-72 hrs post activation on both $CD4^+$ and $CD8^+$ T cells and APCs such as DCs, macrophages and B cells, therefore enabling FIV to infect all cell types (49-51). Due to the difference in FIV and HIV targets, the reservoir dynamics differ between the two infections (reviewed in Policicchio et al., 2016 (25)). However, in a recent study $CD4^+$ and $CD21^+$ leukocytes were identified as cellular reservoirs for FIV replication which is similar to the subsets identified in HIV infection. The same study identified FIV reservoirs in tissues such as the spleen, the intestine and mesenteric lymph node with B cell follicles as the foci of viral replication in chronically infected cats exhibiting viral latency (52). This demonstrates that the FIV model offers the advantage of performing longitudinal infection studies which can be beneficial in understanding tissue viral reservoir dynamics (52). Despite the great promise in expanding our understanding of HIV infection and treatment by utilizing this feline model, more translationally relevant models are needed to more accurately reflect HIV pathogenesis.

The most widely used *in vivo* animal model for HIV is the non-human primate (NHP) model of SIV infection. NHPs and humans share several important aspects of genomic structure, physiology, and complexity of the immune system (53). These shared features enable the widespread use of the NHP SIV model to study HIV pathogenesis, prevention, treatment and cure strategies. There is a wide range of NHP models that are used for HIV studies, each with its respective limitations. African Green monkeys and apes are the natural hosts for SIV, but generally the infection does not manifest into a disease-state. Asian macaques, which are not natural hosts for SIV, can be infected with certain strains of SIV leading to high viremia, $CD4^+$ T cell depletion and an immunocompromised state characterized by tumors and opportunistic infections. The main features of SIV infection in natural and non-natural hosts have been contrasted and reviewed in Chahroudi et al., 2012 and Evans et al., 2012 (54, 55). Rhesus macaques of Indian origin and pig-tailed macaques are the most commonly used NHP model for AIDS research (55). All modes of HIV transmission can be recapitulated in these NHP models. Many antiretroviral (ARV) drugs, such as tenofovir, zidovudine, nevirapine and emtricitabine, which are now used to treat HIV- infected patients, were first tested for efficacy in the macaque models (56-58) Due to the similarities in metabolism and physiology, macaque models have been invaluable in toxicity and dose testing studies of ART drugs. These drugs have informed pre-exposure prophylaxis and post-exposure prophylaxis treatment regimes in humans. Macaque studies also established that early treatment with ART reduces HIV –viremia, delays disease progression, enhances antiviral responses and lowers the risk of transmission (6, 57). Analogous to ARV testing in the NHP model, NHP models have been instrumental in the testing of first-generation (mostly surfactants and poly anionic compounds) and second-generation (antiretrovirals) microbicides that confer local mucosal antiviral protection against HIV infection

(reviewed by Van Rompay, 2012 (56)). The high translational impact of NHP research is best represented by the success of the CAPRISA trial in which 1% tenofovir gel demonstrated partial efficacy in protecting women against HIV infection (59).

The NHP model has been widely used to test HIV vaccine strategies. In fact, the outcomes of the 3-major phase III HIV vaccine trials, i.e. Vax003, Vax004 and RV144 trials, were predictable by prior NHP studies (60-62). A new HIV phase 2b vaccine trial was launched in November of 2017 that will test an adenovirus 26 vector (Ad26) expressing 4 mosaic HIV proteins (Ad26.Mos4HIV) in combination with clade C gp140 protein boosting. This vaccine had shown protective efficacy against SHIV infection in adult rhesus macaques (63).

In recent years, NHP models have been extensively used to study the formation and maintenance of the viral reservoir, and to evaluate cure strategies. SIV integrates into the host genome at preferred integration sites (64, 65). Similar to HIV, SIV undergoes histone acetylation in long terminal repeats (LTRs) and these changes contribute to latency (66). CTLs are unable to clear latently SIV-infected cells (67). As the distribution patterns of HIV and SIV DNA and RNA in peripheral blood, lymph nodes and mucosal tissues are also very similar, NHP models likely accurately reflect reservoir dynamics of HIV-infected humans (68-70). A potential limitation of the SIV infection model is the use and efficacy of specific ARVs in this model. However, with the development of novel chimeric simian-human immunodeficiency viruses (SHIVs), clinically relevant NHP models can be established. However, the NHP model, despite its widespread application in HIV cure research, remains greatly unexplored for epigenetic studies defining lentivirus infections.

$CD8^+$ T cell suppression by $CD4^+$ $CD25^+$ T regulatory cells (Treg) cells

Treg cells are immunomodulatory cells that control exaggerated immune activation upon infection, thereby mitigating the extent of tissue damage. These cells express high levels of the repressive transcription factor forkhead box P3 (Foxp3), express the IL-2 receptor alpha chain CD25 and low levels of the IL-7 receptor (CD127) (71-74). Two types of Treg cells have been described. Natural Treg (nTreg) cells are derived from the differentiation of $CD4^+CD8^+$ precursors into $Foxp3^+$ $CD4^+$ $CD25^+$ T cells in the thymus. Whereas $CD4^+$ T cells that are induced and mature into $Foxp3^+CD4^+$ $CD25^+$ T cells in the periphery are called induced Treg (iTreg) cells (75-77). iTreg cells are comparable to thymus-derived nTreg cells in suppressor function and express similar transcription factors and surface markers (76, 78, 79). $Foxp3^+$ Treg cells can also be induced *in vitro* by TCR-mediated activation of naïve $CD4^+CD25^-$ T cells in the presence of TGF-β and IL-2 (80, 81).

$CD4^+$ $CD25^+$ Treg cells modulate immune response via Foxp3. IL-2 is required for the differentiation and survival of activated T cells. Upon activation, the CD28 co-stimulatory signal increases the production of AP-1 and NF-*κ*B within the T cells. TCR engagement with antigen increases the NFAT levels in the cells. Increased production of NFAT, AP-1 and NF-*K*B enables increased binding of these transcription factors to the IL-2 promoter in activated T cells resulting in higher IL-2 mRNA levels compared to resting T cells (82, 83). The binding and co-operation of NFAT: AP1 is required for IL-2 transcription and T cell activation. Foxp3 interferes with the co-operation of NFAT and AP-1 at the IL-2 promoter which suppresses IL-2 production resulting in diminished proliferative capacity of activated T cells (84, 85). They also regulate the immune response by inhibiting T cell proliferation by competing for the growth factor IL-2, by

CTLA-4 binding to DCs, through the secretion of inhibitory cytokines such as IL-10 and TGF-β, and utilizing cytolytic molecules such as granzyme B and perforin (86). While Treg cells play a critical role in maintaining self-tolerance, they also suppress antigen-specific immune responses that enable the pathogen to persist (87, 88).

In HIV infection, Treg cells can have positive or detrimental effects depending on the stage of disease, viral load, tissue and cell type. Upon infection, Foxp3 has been reported to inhibit HIV transcription through inhibition of NFAT and NF-*κ*B activation *in vitro* (89), whereas other studies suggest that Foxp3 enhances HIV gene expression via the NF-κB signaling pathway (90). Chronic HIV infection leads to an expansion of Treg cells in peripheral blood and lymphoid tissues; preferentially in regions with active HIV replication, such as lymphoid and mucosal tissues (91, 92). This expansion has been attributed to multiple reasons, including persistent immune activation, increased survival of Treg cells, and increased generation of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells in the thymus of HIV –infected patients (93). An increased frequency of Treg cells correlates with lower $CD8^+$ T cell activation in HIV-1 infection (20). An early induction of $Foxp3^+$ Treg cells in the blood and an early accumulation of Treg cells in mucosal tissues and peripheral lymph nodes is demonstrated in the nonpathogenic and pathogenic models of SIV infection i.e. African green monkeys and rhesus macaques respectively (94, 95). There is a rapid depletion of Treg cells in the pathogenic model of pigtailed macaques infected with SIV (96, 97). In the FIV model, Treg cells are phenotypically and functionally activated during the acute phase and remain activated through the chronic phase of infection (98). These combined findings indicate that Treg frequency, longevity and accumulation dynamics are influenced by multiple factors.

Treg cells are susceptible to HIV infection because they also express the HIV co-receptors CCR5 and CXCR4. Indeed, both human and animal studies demonstrate that Treg cells support HIV-1, FIV and SIV replication *in vitro* and *in vivo* (99-101). A very low percentage of peripheral Treg cells are infected by HIV-1 *in vivo* (<0.7% peripheral Treg cells) (102). SIV infected $Foxp3^+$ T cells are found in multiple tissues, including mucosal tissues such as gut- associated lymphoid tissue (94, 99, 103). FIV+ cats harbor productively infected Treg cells that are phenotypically and functionally activated (104). Treg cells in HIV-infected humanized mice also support high levels of HIV-1 which are depleted upon infection with HIV-1 (105).

This dissertation will focus on the interaction of Treg cells with $CD8^+$ T cells during lentivirus infections and the resulting suppression of antiviral $CD8^+$ T cell function. There are conflicting reports on the role and the functional capacity of Treg cells in HIV infection (106). For example, Treg cells in HIV-1 infected patients support HIV infection which results in the downregulation of Foxp3 and impairment of their suppressive capacity when assessing individual cells (107, 108). In contrast to these studies, *in vitro* and other *in vivo* studies suggest that bulk Treg cells retain, or in some cases enhance their immunosuppressive function during the course of HIV-1 and FIV infection (90, 102, 104, 108, 109).

Treg cells isolated from HIV-infected patients suppress cytolytic function of HIV-specific $CD8^+$ T cells (92). Similarly, increased Treg frequencies during acute SIV infection correlate with suppressed SIV- specific $CD8^+$ T cell responses (99). The effector T cells also enhance their sensitivity towards Treg –mediated suppression in HIV-1 infected patients (110). Enhanced HIV/SIV/FIV-specific T cell responses upon *ex vivo* depletion of Treg cells from PBMCs or

lymphoid cell suspensions support the immunosuppressive role of Treg cells (111, 112). $CD8^+$ T cells from HIV-infected patients with the protective MHC HLA B*27 and HLA B*57 alleles evade Treg suppression, further suggesting that the mechanism of Treg suppression of $CD8^+$ T cell functions is important for disease progression (113). Nikolova et al. recently reported that Treg cells signal via PD-1/PD-L1 pathway to suppress HIV-specific $CD8^+$ T cells contributing to $CD8^+$ T cell dysfunction during the chronic infection phase (114). Our group has previously reported that, in the FIV model, lentivirus activated $CD4^+$ $CD25^+$ Treg cells signal via membrane bound TGF-β/ Smad signaling to induce Foxp3 in $CD8^+$ T cell targets (115, 116). We have also demonstrated that Foxp3 mediates antiviral cytokine suppression by directly binding to IL-2, TNF-α and IFN-γ promoter region in the acute and chronic phase of FIV infection (109, 117, 118). Figure 1.2B shows a summary of our findings demonstrating Treg-mediated $CD8^+$ T cell suppression in the FIV model. Based on our findings, this Chapter will focus on the immunosuppressive function of Treg cells, but we acknowledge that Treg can also have beneficial effects on HIV infection and control, as reported by other investigators (reviewed in Moreno et al., 2012(112)).

Epigenetic mechanisms regulating gene transcription: a general overview

Epigenetic modifications are alterations in DNA and nucleosomes that occur in response to changes in the cellular environment. These changes are independent of changes in the primary DNA sequence and are reversible. Epigenetic modifications provide a second layer of transcriptional control by altering chromatin accessibility for the transcriptional machinery to bind and enable gene expression. Following cellular activation, the epigenetic patterns within the

gene and its regulatory regions change to enable transcription factors to bind and initiate transcription of required genes.

There are different types of epigenetic modifications, and we will briefly review them here. Histone modifications and changes in DNA methylation are two of the better understood epigenetic mechanisms that regulate gene transcription. Table 1.1 summarizes the most well characterized histone modifications and their effects upon gene expression.

DNA wraps around histones forming the main structural unit of nucleosomes. The N-terminus of the histone is susceptible to a variety of post-translational modifications such as acetylation, methylation, phosphorylation, ubiquitination and sumoylation that alter the affinity of nucleosome for DNA (119). Histone Methyltransferases (HMTs) and demethylases work to modify the methylation status of histone tails, thereby promoting an active or repressive chromatin conformation. The extent of histone lysine and serine methylation determines the activation or repression of specific genes (see Table 1.1). Acetylation of histone lysine residues is generally associated with a non-repressed state. Histone acetyltransferases (HATs) and Histone deacetylases (HDACs) mediate histone acetylation and deacetylation respectively, changing the affinity of the nucleosome for DNA.

Promoters of “active” genes have acetylated histones and possess other activating histone marks such as H3K4me3 (histone H3 trimethylated on lysine 4) (120) (Table 1.1). Silencing of genes is a complex process which involves a wide range of proteins. Methyl-cytosine binding proteins (MBP) recruit deacetylases resulting in de-acetylated histones- H3 and H4. Other repressive

modifications such as H3K9me3 (histone H3 trimethylated on lysine 9) and H3 K27me3 are also found in silenced genes/ promoters (Table 1.1) (121).

In addition to histones, direct methylation of DNA represents another epigenetic program that can regulate chromatin accessibility and hence gene transcription. In mammals, DNA methylation occurs at the C5- position(5-mc) of cytosine residues in the context of CpG dinucleotides where a guanine follows a cytosine nucleotide in a DNA sequence (122-124). The frequency of CpG dinucleotides is 10 times greater than average in CpG islands (CGI) (125). Generally, CpGs outside the CGIs are mostly methylated, whereas the CpGs within these islands are prone to de-methylation making them important for gene regulation (121, 125, 126). Methylation of CpG residues is usually associated with a "closed" chromatin (heterochromatin) structure resulting in gene suppression (127, 128). CpG methylation patterns are maintained or added *de novo* by DNA Methyltransferases 1, 3a and 3b respectively (DNMT 1, 3a and 3b). Active promoters are usually un-methylated and result in an "open" chromatin conformation (euchromatin).

Both histone modifications and DNA methylation alter chromatin accessibility. These changes enable the transcriptional machinery to bind to promoters or other regulatory regions of genes, thereby controlling their level of expression. A single promoter can have a combination of these modifications simultaneously, that instruct a gene for active transcription or suppression. As the chromatin "opens" for transcription, it also allows repressive transcription factors to bind to gene regulatory and/or promoter regions resulting in gene suppression. Therefore, epigenetic

modulation of gene expression is a complex process that provides a secondary level of gene regulation.

Figure 1.2: Persistent antigenic stimulation results in CD8$^+$ T cell dysfunction during chronic HIV infection.

Figure 1.2A illustrates three mechanisms of CD8$^+$ T cell dysfunction during chronic HIV infection. Part 1: Upregulation of inhibitory surface markers such as PD-1, CTLA-4, TIM-3, TIGIT and Fas-L that inhibit effective CD8$^+$ T cell activation. Part 2: Treg-mediated suppression. Treg-mediated suppression has been observed in HIV-patients, SIV- infected macaques and in FIV-infected cats. Our group, using the FIV model of HIV infection, has demonstrated that lentivirus-activated Treg cells upregulate membrane-bound TGF-β which ligates the TGF-βRII on activated CD8$^+$ T cells. This ligation leads to downstream phosphorylation of the Smad complex, resulting in its nuclear translocation. In addition, CD8$^+$ T cell activation and signaling via the TCR and CD28 co-stimulatory molecule promotes the co-operation of NFAT: AP1 within the nucleus, an interaction required for gene activation in T cells. Smad complexes coupled with NFAT: AP1 binding at the Foxp3 promoter results in the induction of the repressive transcription factor *Foxp3*. Foxp3 induction in CD8$^+$ T cells can also be induced by exogenous addition of TGF-β1 if simultaneous TCR activation via anti-CD3/anti-CD28 occurs. Part 3: Epigenetic modulation of antiviral cytokine genes and their regulatory regions. As a result of CD8$^+$ T cell activation (see Figure 1.1, Part 2), antiviral cytokine promoters are "relaxed". This open conformation allows repressive transcription factors such as Foxp3 to bind and thereby suppress the transcription of IL-2, IFN-γ and TNF-$\boldsymbol{\alpha}$ as shown in Figure 1.2B.

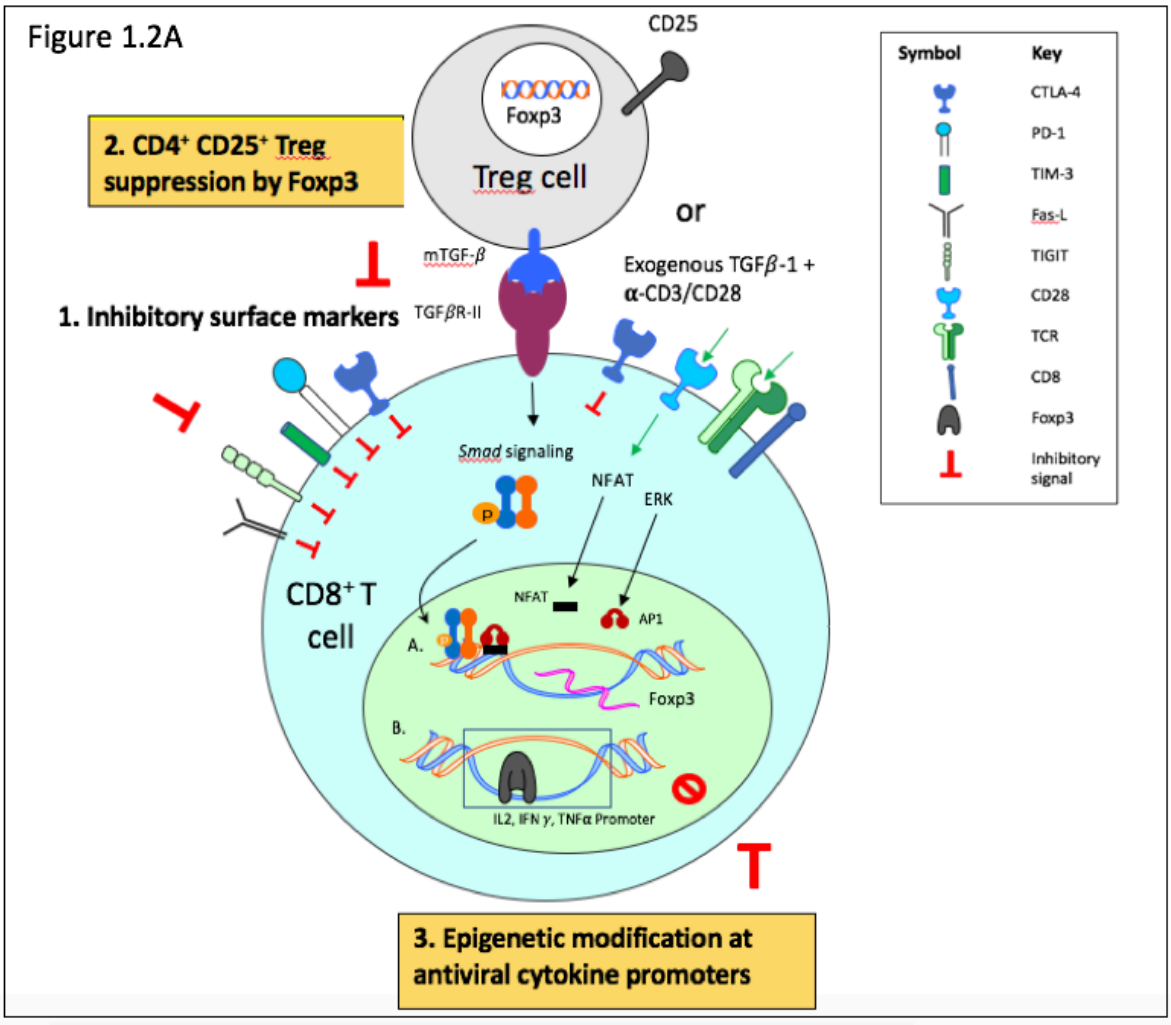


Figure 1.2A
CD25
Foxp3
Treg cell
2. CD4+ CD25+ Treg suppression by Foxp3
or
mTGF-β
TGFβR-II
Exogenous TGFβ-1 + α-CD3/CD28
1. Inhibitory surface markers
Smad signaling
NFAT
ERK
P
CD8+ T cell
NFAT
AP1
A.
Foxp3
B.
IL2, IFN γ, TNFα Promoter
3. Epigenetic modification at antiviral cytokine promoters
Symbol
Key
CTLA-4
PD-1
TIM-3
Fas-L
TIGIT
CD28
TCR
CD8
Foxp3
Inhibitory signal

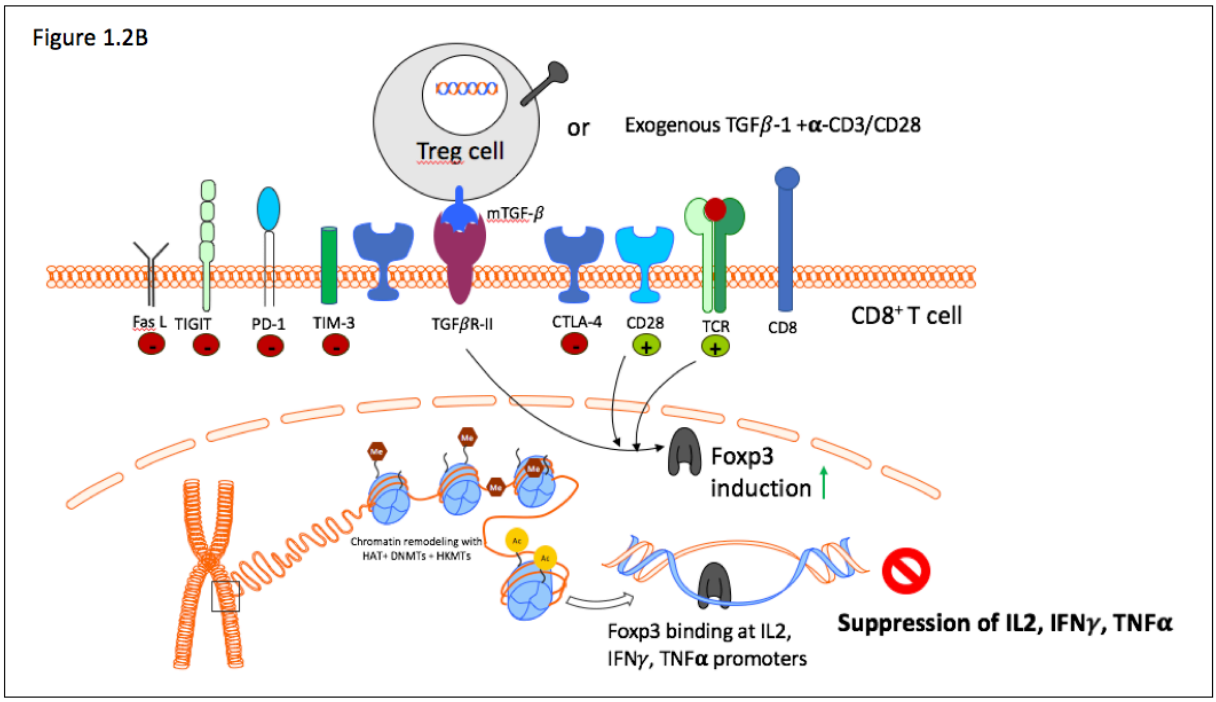


Figure 1.2B
Treg cell
or
Exogenous TGFβ-1 +α-CD3/CD28
mTGF-β
Fas L
TIGIT
PD-1
TIM-3
TGFβR-II
CTLA-4
CD28
TCR
CD8
CD8+ T cell
Foxp3
induction
Chromatin remodeling with
HAT+ DNMTs + HKMTs
Foxp3 binding at IL2,
IFNγ, TNFα promoters
Suppression of IL2, IFNγ, TNFα

Table 1.1: Summary of epigenetic histone modifications and their effect on gene expression (129-131)

Histone/Position/Modification	Location	Effect	Enzyme
H3K4me2		Gene activation	Set1, MLL, Set7/9, SMYD3, LSD1, JAR1D1A
H3K4me3	5' end of transcriptionally active genes	Gene activation	Set1, MLL, Set7/9, SMYD3, JAR1D1A
H3K9me	euchromatin	Gene silencing	G9a; Suv91, StB1, PRD14, CLL8, GLP, Suv39h1, Suv39h2
H3K9me2	euchromatin	Gene silencing	G9a; Suv91, StB1, PRD14, CLL8, GLP, Suv39h1, Suv39h2, JMJD2A
H3K9me3	Promoters and heterochromatin Gene coding region	Gene silencing Gene activation	G9a; Suv91, StB1, PRD14, CLL8, GLP, Suv39h1, Suv39h2, JMJD2A
H3K27me	heterochromatin	Gene silencing	EZH2
H3K27me3	Inactive-X chromosome, homeotic genes	Gene silencing	
H3K36me	promoter	Not well characterized	JHDM1A
H3K36me2	Near double strand breaks- for repair	Gene silencing	NSD1, JMJD2A, JHDM1A
H3K36me3	3' end of active genes. Marks exons.	Gene activation	JMJD2A

Table 1.1 (continued)

H3K79me2		Gene activation	Dot1L
H3K79me3		Gene activation	Dot1L
H4K20me2	heterochromatin	Gene silencing	NSD1, Suv4-20h1, Suv4-20H2, Set8/PR-SET7
H4K20me3	heterochromatin	Gene silencing	NSD1, Suv4-20h1, Suv4-20H2, Set8/PR-SET7

Epigenetics in HIV

There are multiple reports describing epigenetic modifications during chronic viral infections such as HIV, Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Papillomavirus (HPV) and Epstein-Barr virus (EBV) infections in humans (132-136). For example, HBV and HCV induce DNA methylation in mice with humanized livers and the HPV viral genome is subjected to DNA methylation-mediated control during infection (136, 137). The epigenetic alterations in these chronic viral infections and the use of epigenetic therapy for their treatment has been reviewed in Moos et al., 2017 (138).

Epigenetic modifications in HIV infection been extensively studied in the context of HIV latency. HIV proviral latency is maintained by histone deacetylases that inhibit transcription from the HIV LTR (139). Deacetylation and methylation of histones during HIV-1 infection interfere with the binding of DNA polymerase and restrict the production of active HIV-1 transcripts from proviral DNA. The 5’ long terminal repeat (5’ LTR) region of HIV DNA is

highly susceptible to inhibitory epigenetic modification (43). Silencing epigenetic modifications of other viral proteins are a major contributor to viral latency. The genes of several HIV accessory proteins, such as *Nef, Vpr* and *Tat,* are hyper-methylated and thereby promote viral latency (140, 141). As these epigenetic modifications are reversible, efforts have been made to identify epigenetic modulators that can reactivate these silenced viral genes, and thereby reactivate latently infected cells. The "Shock and Kill" strategy aims to reverse latency by manipulating histone post- translational modifications to enable virus replication; once virus is activated and released, multiple different strategies are being pursued to eliminate virally infected cells or free virus. The latter include suppression of virus replication by interfering with the virus life cycle through antiretroviral drugs or the boosting of antiviral immune responses (14). HDAC inhibitors such as Valproic acid, SAHA (Vorinostat) and Panobinostat have been reported to increase HIV-1 transcription from proviral DNA in latently infected cells *in vitro* and in clinical trials (142-144). Similarly, treatment with the Histone Lysine Methyl Transferase inhibitor, HKMT G9a antagonist BIX01294, has resulted in increased HIV RNA levels, and combination therapy approaches utilizing different HDAC inhibitors and HKMT inhibitors are being explored (145, 146). Other studies have tested the manipulation of epigenetic moieties at the DNA level by using modulators such as 5-aza-2′- deoxycytidine (aza-CdR) (147). Apart from epigenetic modifiers, several small molecules, such as JQ1 and PKC agonists can reactivate the latent reservoirs as well.

An important question that needs to be pursued in parallel is whether the patient's immune response will be robust enough to clear the infection upon successful purging of the reservoir. As described above, virus-specific $CD8^+$ T cells exhibit progressive dysfunction during the course

of infection due to a number of factors including persistent immune activation, Treg-mediated suppression, and lentivirus induced epigenetic modifications. Thus, to successfully cure HIV infection, we need to address this problem from multiple angles. Along with latency reactivation, it is important to identify strategies that can boost $CD8^+$ T cell function to eliminate reactivated cells.

Epigenetic modulation of immune cells as a result of HIV infection

In order to develop strategies to boost $CD8^+$ T cell function, we need a better understanding of the epigenetic changes in immune cells infected with and responding to HIV infection, including $CD8^+$ T cells, and this will be the focus here. Recent studies indicate that epigenetic modifications are induced very early after infection (148). *In vitro*, epigenetic modifications in PBMC or primary $CD4^+$ T cells can be detected as early as 36 hours after HIV-1 infection (148). Enrichment of H3K9me3 and H3K27me3 marks result in transcriptional repression, including the downregulation of important antiviral cytokine genes, such as IL-2 and IFNγ (148). Similarly, Mikovitz et al., reported that increased methylation of the IFN-γ promoter along with increased DNMT expression results in the suppression of IFN-γ in HIV-infected $CD4^+$ T cell *in vitro* (149). In another study, Hosoya et al., demonstrated that DNA methylation during chronic HIV infection regulates loss of IL-2 in senescent $CD4^+$ T cells(150). They also reported that the CD28 co-stimulatory signaling pathway plays an important role in de-methylation/re-methylation of the *Il2* gene (150). Both IL-2 and IFN-γ are necessary to support CTL and Natural Killer (NK) cell activity and thus, the suppression of these cytokines in $CD4^+$ T cells might negatively impact virus control during HIV infection (151).

In addition, $CD8^+$ T cells themselves are epigenetically modified during HIV infection (152, 153). For example, the expression of the inhibitory PD-1 molecule is regulated by methylation of the PD-1 promoter. $CD8^+$ T cells from HIV-infected subjects classified as LTNP or patients with fully suppressed plasma viremia by ART have an unmethylated PD-1 promoter (153). Zhang et al., reported that the inability of exhausted $CD8^+$ T cells to produce IFN-γ and IL-2 positively correlates with low levels of diAcH3 in the regulatory regions of the *Ifng* and *Il2* genes. They further demonstrated that exhausted $CD8^+$ T cells have overall lower levels of histone acetylation (154). These data imply that $CD8^+$ T cell function is epigenetically controlled during HIV infection. Therefore, reversing repressive epigenetic signatures using epigenetic modulators could potentially recover $CD8^+$ T cell function.

Our research group has studied the molecular events responsible for $CD8^+$ T cell dysfunction in the FIV model. Data by our group and others suggest that FIV provirus and infected $CD4^+$ T cells are epigenetically modulated during infection and that epigenetic modulators can be used to reverse these events (28, 117). We have further demonstrated that dysfunctional $CD8^+$ T cells, that evolve during the chronic phase of FIV infection, are epigenetically modulated (117). Specifically, lentivirus activated $CD4^+$ $CD25^+$ Treg cells induce Foxp3 expression in $CD8^+$ T cells, and Foxp3 mediates antiviral cytokine suppression by directly binding to the IL-2, TNF-α and IFN-γ promoter regions (109, 117, 118). We have also demonstrated that blocking DNA de-methylation and histone acetylation reduces Foxp3 binding to the IL-2 promoter in the FIV model ((117), unpublished data, 2018). These data reveal a novel Foxp3- mediated mechanism contributing to $CD8^+$ T cell dysfunction in lentivirus infections.

In addition, Treg cells themselves can be epigenetically modulated by HIV-1 infection (107). HIV-1 infection results in downregulation of Foxp3 expression in Treg cells followed by loss of suppressive activity and alterations in cytokine expression profile. The same group found the CpG sites in the Foxp3 locus to be hyper-methylated due to increased expression of DNMT3b (107). Another report, however, showed increased frequency of Treg cells in the gut mucosa of HIV-1 infected patients due to decreased methylation of Foxp3 promoters in the gut associated T cells, causing the induction of iTreg cells (155). They also reported that the Foxp3 promoter was significantly de-methylated possibly due to the downregulation of key DNMT enzymes in HIV patients when compared to uninfected control subjects (155). These conflicting results regarding the methylation status of the Foxp3 promoter after HIV-1 infection indicate the lack of literature describing the epigenetic changes induced by lentivirus infections in Treg cells which necessitates further studies.

Current strategies to boost $CD8^+$ T cell function

Several immune strategies have been tested to improve $CD8^+$ T cell function during chronic HIV infection. Exhausted $CD8^+$ T cells upregulate inhibitory receptors such as PD-1, CTLA-4, TIM-3, CD160, 2B4 and LAG-3, and *in vitro* and *in vivo* blockade of these inhibitory receptors has shown promising results in enhancing the cytotoxic function of $CD8^+$ T cells (156, 157). Toll-like receptor 2 (TLR 2) agonists and agonistic antibodies against 4-1BB or CD40 have also yielded positive results in reversing $CD8^+$ T cell exhaustion *in vivo* (158). Additionally, recombinant cytokines alone or in combination with checkpoint inhibitors can increase $CD8^+$ T cell function. For example, due to the success of recombinant IL-15 in enhancing $CD8^+$ T cell function in animal models, the superagonist IL-15 ALT-803 is being tested in a clinical trial with

ART- treated HIV patients (159, 160). In the past, adoptive transfer studies using functionally competent $CD8^+$ T cells that were expanded *ex-vivo* were not very successful, in part due to the absence of standardized protocols for *ex-vivo* expansion of T cells and for a lack of knowledge on how to avoid non-specific immune activation (161, 162). However, with the recent advancements and success of the chimeric antigen receptor (CAR) technology in cancer therapy, CAR-T cells hold great promise in HIV- treatment. Recent reports described that T cells engineered to express anti-HIV CAR (HIVCAR) can specifically target HIV-infected T cells *ex-vivo* (163). Epigenetic modulations using HDACi have been explored for their ability to boost $CD8^+$ T cell function. Agarwal et al., demonstrated that the cytolytic response of stimulated $CD8^+$ T cells can be increased by increasing the expression of IFN-γ, MIP-1α and MIP-1β using HDACi (164). Similarly, *in vitro* treatment of exhausted $CD8^+$ T cells with HDAC inhibitors could restore diAcH3 levels, improving $CD8^+$ T cell function, and adoptive transfer of these cells resulted in long term persistence of these $CD8^+$ T cells and development into functional memory cells in mice (154). These studies support the rationale to further explore epigenetic modulators to reverse detrimental HIV-induced modifications and enhance antiviral host immunity.

Conclusion

$CD8^+$ T cell function is essential to fight chronic lentivirus infection and to eliminate reactivated latently infected cells. However, often these $CD8^+$ T cells have impaired proliferation and functional capacity during the chronic phase of lentiviral infections. We need to identify novel methods to boost these dysfunctional $CD8^+$ T cells to improve current HIV cure strategies. Epigenetic manipulation of dysfunctional $CD8^+$ T cells holds great promise to solve this

problem. Figure 5.1 illustrates the mechanism of boosting $CD8^+$ T cell function by epigenetically modulating dysfunctional cells.

In the FIV model, we have previously reported that Treg cells suppress IL-2 function of $CD8^+$ T cell by inducing stable Foxp3 in $CD8^+$ T cells, with Foxp3 directly binding to the IL-2 promoter (117). In Chapter 2, we further demonstrate that Treg cells progressively suppress IL-2, TNF-α and IFN-γ by inducing Foxp3 which binds to the antiviral cytokine promoters in FIV-specific $CD8^+$ T cells (118). Our group reported that Foxp3 binding at the IL-2 promoter is epigenetically modulated and by blocking DNA de-methylation we could reduce Foxp3 binding to the IL-2 promoter region (117). In Chapter 3, we demonstrate the role of another epigenetic medication, i.e. histone acetylation in mediating Foxp3-binding to the IL-2 promoter (Chapter 3). These data suggest that therapeutic interventions targeted at specific epigenetic modifications in dysfunctional $CD8^+$ T cells can reverse Foxp3-mediated suppression. Currently, we are attempting to validate these findings in the rhesus macaque model of SIV infection. As a first step, in Chapter 4, we report increased Foxp3 binding at the antiviral cytokine promoters in $CD8^+$ T cells induced to express Foxp3 by TGF-β stimulation *in vitro*. Next, we aim to identify differences in the epigenetic signatures of SIV- infected and -uninfected macaques and whether epigenetic changes differ in distinct anatomic locations, such as mucosal tissues, secondary lymphoid tissues and peripheral blood. As epigenetic remodeling events can alter gene expression, we are specifically focusing on identifying epigenetic signatures associated with $CD8^+$ T cell dysregulation. Utilizing the FIV and SIV animal models, our ultimate goal is to identify epigenetic targets that can be modulated for therapeutic use in HIV cure.

CHAPTER 2

T regulatory cell induced Foxp3 binds the IL-2, IFN-γ, and TNF-α promoters in virus-specific CD8+ T cells from feline immunodeficiency virus infected cats.

Wang Y, Nag M, Tuohy JL, De Paris K, Fogle JE. 2017.

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Introduction

A rapid rise in $CD8^+$ T lymphocytes displaying an activated phenotype is observed during early HIV infection and the quality of the $CD8^+$ T cell response is associated with a decrease in plasma viremia (165, 166). A small subset of HIV-infected individuals called elite controllers (ECs) exhibit a low viral set point, low viral load, and appear to control virus naturally, in the absence of anti-retroviral therapy (reviewed in O'Connell (167)). Compared to HIV progressors, $CD8^+$ T cells from ECs exhibit polyfunctional responses to HIV antigen, potent suppression of HIV replication, and enhanced proliferation, indicating that robust $CD8^+$ T cell function is important in controlling HIV (5, 168, 169). However, for most individuals (HIV progressors) despite this early, vigorous $CD8^+$ T cell antiviral response, the virus is not eliminated, and it establishes a persistent infection with a relatively high viral set point and viral load (5, 168, 169). Establishment of persistent infection relies upon a complex series of virus and host factors. One important factor is the early and progressive loss of antigen-specific T cell responses during the course of AIDS lentiviral infection (170). In addition to HIV infection, the phenomenon of $CD8^+$ T cell hypo-responsiveness to viral antigens is well documented for several viral infections including Hepatitis C virus, and LCMV in mice (171, 172). Investigations using the LCMV model have offered insights into the mechanisms underlying progressive antigen-specific $CD8^+$

T cell dysfunction. These studies suggest that $CD8^+$ T cell immune dysfunction is not an all or none phenomenon, but represents progressive loss of function (173, 174) . The loss of antigen-stimulated $CD8^+$ T cell cytokine secretion is first manifested by the depression and then loss of IL-2, followed by loss of TNF-α and finally loss of IFN-γ, resulting in complete dysfunction as manifested by non-responsiveness to viral antigens (174). Collectively, these findings suggest that an understanding of the molecular events contributing to $CD8^+$ T cell dysfunction during the progression of AIDS lentiviral infection may be key to enhanced vaccination and reservoir elimination strategies.

Using the feline immunodeficiency virus model (FIV) for AIDS lentiviral persistence, our group investigates Treg cell, $CD4^+$, and $CD8^+$ T cell interactions from the very early stage of infection through long-term infection. Treg cells from FIV+ cats are activated during the course of FIV infection, meaning that they potently suppress autologous T cell function when compared to Treg cells from uninfected control cats. Treg cells from FIV+ cats suppress IL-2 and IFN-γ production in $CD8^+$ T cells early and progressively during the course of infection (104, 109, 115, 175). The mechanism of suppression is TGF-β-dependent, with a membrane bound form of TGF-β displayed upon FIV-activated Treg cell surfaces ligating TGF-βRII upon target effector cells (116). Following co-culture with activated Treg cells, the TGF-β signaling cascade leads to the induction of the repressive transcription factor forkhead box P3 (Foxp3), in the target effector cells. The induction of Foxp3 in $CD4^+$ T helper cells leads to the conversion of these cells to "induced" Treg cells, which exhibit Treg cell phenotype and function (175). Further, we have clearly demonstrated that Foxp3 is induced in $CD8^+$ T cell targets following Treg cell / $CD8^+$ T cell co-culture and that Foxp3 binds the IL-2 promoter in $CD8^+$ T cells (117).

Based upon our previous work using the FIV model of lentiviral persistence, we hypothesized that Treg-induced Foxp3 contributes to the progressive loss $CD8^+$ T cell function in a manner reminiscent of the LCMV mechanism described above. Here we demonstrate that mRNA levels of IL-2, TNF-α, and IFN-γ decreased in virus-specific $CD8^+$ T cells following *ex vivo* Treg / $CD8^+$ T cell co-culture. Further, we report the induction of Foxp3 in virus-specific $CD8^+$ T cells following *ex vivo* Treg / $CD8^+$ T cell co-culture. Most importantly, we demonstrate that Foxp3 binds the IL-2, TNF-α, and IFN-γ promoter regions in virus-specific $CD8^+$ T cells from FIV+ cats following Treg cell co-culture. We believe this is the first report demonstrating Treg induced Foxp3 binding of all three of these promoter regions in virus-specific $CD8^+$ T cells during the course of lentiviral infection. These results help explain, in part, the progressive $CD8^+$ T cell dysfunction that is associated with persistent lentiviral infection.

Materials and Methods

Cats and FIV infection

Specific pathogen-free cats were obtained from Liberty Labs (Liberty Corners, NJ) and housed at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Cats were inoculated with $1x10^5$ $TCID_{50}$ cell free $NCSU_1$ FIV as described by Bucci et al. (176). FIV-infection was confirmed using a commercially available ELISA kit (IDEXX Laboratories Inc, Westbrook ME) and by provirus detection by PCR using primers specific for the FIV-p24 GAG sequence as described previously (104, 177). Age matched, non-infected control cats were housed separately from FIV-infected cats. Control cats were sham infected with the same type of culture medium (sterile) used to culture the virus. All protocols

were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Sample collection and preparation

Single-cell suspensions were prepared from popliteal or submandibular peripheral lymph nodes (PLNs) obtained through surgical biopsies or following euthanasia, by gently and repeatedly injecting sterile cell culture media into the tissue using an 18G needle until the cells were released from the tissue. Cell viability was determined by trypan blue dye exclusion.

$CD8^+$ T cell co-culture and CFSE cell proliferation assays

Both anti-feline CD4 and anti-feline CD8 monoclonal antibodies were developed by our feline lentivirus research group as described previously in Tompkins et al. (178). The feline anti-CD25 monoclonal antibody was developed by Ohno as described previously in Fogle et al. (115). Single cells from LNs were suspended at 1×10^8 cells/ml in HBSS with 2% FBS and stained with anti-feline CD8 PE antibody (clone 3.357) at 4^oC for 30mins. EasySep® PE Selection Cocktail was added at 100µL/mL of cell suspension at RT for 15mins, then EasySep® Magnetic Nanoparticles were added at 50µL/mL at RT for 10mins. $CD8^+$ PE^+ cells were separated by using the magnet provided in the Kit (Stem Cell, Vancouver, BC, Canada). The rest of the cell suspension was stained with mouse anti-feline CD4 APC antibody to isolate $CD4^+$ cells by using EasySep® APC Selection kit (Stem Cell). Isolated $CD4^+$ cells were then stained with mouse anti-feline CD25 FITC antibody to sort $CD4^+$ $CD25^+$ double positive Treg cells using the MoFlo XDP high speed cell sorter (Beckman Coulter). DAPI (BioLegend) was used as the cell viability dye to ensure we obtained live cells at the end of each of the sorts. $CD8^+$ T cells were

resuspended in pre-warmed PBS/0.1% BSA and stained with 10 μM CFSE dye from the Cell Trace^{TM} CFSE Cell Proliferation Kit (Life Technologies). $CD8^+$ T cells were returned to LN culture without $CD4^+CD25^+$ Treg cells, and stimulated *in vitro* with UV inactivated-FIV-$NCSU_1$. The cells were co-cultured for 72 hours. Following stimulation, the virus specific proliferating $CFSE^{int/low}$ cells and non-specific $CD8^+$ T cells $CFSE^{high}$ (internal control) were isolated by re-sorting using a high-speed cell sorter. For all of the co-culture studies presented here, $CD8^+$ lymphocytes were co-cultured at a 1:1 (Treg: $CD8^+$) ratio with autologous $CD4^+CD25^+$ Treg cells for 24 h. After co-culture, the cells were washed and then resorted into $CD8^+$ populations for analysis by qPCR or ChIP. The purity of magnetic bead sorted cells was $\geq$ 95% and Moflo XDP sorted cell populations was >99%.

RNA extraction, RT and real-time PCR quantification

Total RNA was extracted from cells using PureLink^{TM} RNA Micro Kit (Life Technologies).The concentration was quantified using a Nano Drop Spectrophotometer. qPCR was performed for mRNA using qScript cDNA Synthesis Kit (Quanta Biosciences). 15 uL reactions were incubated for 5 min at 22^{o}C, 40 min at 42^{o}C, and 5 min at 85^{o}C to inactivate the reverse transcriptase. Feline specific primers as shown in Table 2.1 were used to detect the Foxp3, IL-2, TNF-α and IFN-γ mRNA levels using LightCycler 480 System (Roche) qPCR. GAPDH mRNA expression was used as a normalizing control. For qPCR experiments, a ΔΔCt ratio was used to quantify relative mRNA expression. 8 uL of diluted cDNA, 10 uL PerfeCTa SYBR Green SuperMix Reaction Mix (Quanta Biosciences), 1uL forward primer and 1ul reverse primer were run in triplicates under the following cycling conditions: hot start enzyme (Qiagen) activation at 95^{o}C for 5 mins, denatured at 94°C for 45s, annealed at 60°C for 45s, and elongated at 72°C for 1

minute with 35 cycles, and final extension at 72°C for 10 minutes.

Table 2.1: Feline primers used for qPCR for cytokine amplification.

Primer Target	Forward	Reverse
Foxp3	5'-GCCTGCCACCTGGAATCAAC-3'	5'-GTGTGCTGGGGCTTGGGA-3'
IL2	5'-ACAGTGCACCTGCTTCAAGCTCT-3'	5'-CCTGGAGAGTTTGGGGTTCTCAGG-3'
TNFα	5'-ATGCCCTCCTGGCCAATGGCG-3'	5'-TAGACCTGCCCGGACTCGGC-3'
IFNγ	5'-TGGTGGGTCGCTTTTCGTAG-3'	5'-GAAGGAGACAATTTGGCTTTGAA-3'
GAPDH	5'-GGAGAAGGCTGGGGCTCAC-3'	5'-GGTGCAGGAGGCATTGCTGA-3'

Chromatin Immunoprecipitation (ChIP) assay

The ChIP was performed using Chroma Flash High-Sensitivity ChIP Kit (Epigentek). The protocol was followed according to manufacturer's specifications. In brief, anti-Foxp3 (Abcam), anti-RNA polymerase II (positive control) and non-immune IgG (negative control) antibodies were first bound to Assay Strip Wells. The sorted cells were cross-linked by adding CTL media containing formaldehyde to a final concentration of 1% with incubation at room temperature (20-25°C) for 10 minutes on a rocking platform (50-100 rpm). To each tube, pre-warmed 1.25M Glycine (1:10) was added to a final concentration of 125mM and incubated at room temperature for 5 minutes. After washing with ice-cold PBS, Working Lysis Buffer was added to re-suspend the cell pellet and incubated on ice for 10 minutes. After carefully removing the supernatant, Working ChIP Buffer was added to re-suspend the chromatin pellet and the chromatin sheared by sonication. ChIP samples were centrifuged at 12,000 rpm at 4°C for 10 minutes after shearing and the supernatant was transferred to a new vial. The ChIP samples were added to the wells bound with antibodies, positive control, or negative control. The reaction wells were incubated at 4°C overnight. ChIP samples were then washed according to the protocol and subjected to reverse cross-linking at 42°C for 30 min, 60°C for 45 min. DNA release was at 95°C

for 15 min in a thermocycler. Finally, the DNA samples were purified by spin column for quantitative PCR (qPCR) using the ChIP primers shown in Table 2.2. The relative expression of the target gene was calculated. The relative expression was calculated by using a ratio of amplification efficiency of the ChIP sample over that of Non-Immune IgG,

$FE\ \% = 2^{(\text{IgG Ct} - \text{Sample Ct})} \times 100\%$.

Table 2.2: Feline primers used for ChIP to measure Foxp3 binding.

Primer Target	Forward	Reverse
IL2 promoter-1	5'-ACTCAACTTGCATCCCCTTG-3'	5'-ACCCAGGAAAGGATTTGCAT-3'
IL2 promoter-2	5'-TGCTCCACATGTTCAACACA-3'	5'-CCCACACTTAGGTGGCAGTT-3'
TNFα promoter-1	5'-AGGGTTGCTTTCACTCCCAC-3'	5'-GGGAGCTTGAGAGAAGGCTG-3'
TNFα promoter-2	5'-GAGCTCATGGGTTTCTCCAC-3'	5'-AGCTTCTGCTGACTGGGTGT-3'
IFNγ promoter-1	5'-GCTTTCAAAGGATCCCACAA-3'	5'-TTTGTGGCATTTTGGTGTTG-3'
IFNγ promoter-2	5'-CTTCCTCACCACCTTGGTCT-3'	5'-AGGGGTGCTCCAACCTTTAC-3'

Data Analysis

Data (Figures 2.2-2.4) are presented in the text as the mean + SEM and were analyzed using an unpaired t-test with significance set at $p < 0.05$. All data were analyzed using GraphPad Prism software.

Results

Isolation of virus-specific $CD8^+$ T cells from FIV+ cats

Specific Pathogen-Free (SPF) cats were infected with FIV-$NCSU_1$ and an equal number of cats were sham infected with sterile cell culture medium as described in the methods section. Two peripheral (submandibular and popliteal) lymph nodes (LN) were collected at 1 week, 4 weeks and 8 weeks post infection during the acute stage of infection and between 6 to 12 months post-

infection. $CD4^+CD25^+$ Treg cells were depleted from LN suspensions and kept separately in culture. Isolated $CD8^+$ T cells were CFSE labeled, returned to culture, and stimulated with virus *in vitro* as described in the methods. Following stimulation, virus-specific $CD8^+$ T cells ($CFSE^{int/low}$) were isolated from non-specific $CD8^+$ T cells ($CFSE^{hi}$) for subsequent co-culture experiments with autologous $CD4^+CD25^+$ Treg cells. Figure 2.1A shows non-specific $CD8^+$ T cells in FIV- control cats after FIV stimulation. Figure 2.1B shows the proliferation of $CD8^+$ T cells from FIV+ cats through approximately four generations, in response to viral stimulation.

IL-2, IFN-γ, and TNF-α mRNA levels from virus-specific $CD8^+$ T cells were decreased following autologous $CD4^+CD25^+$ Treg cell co-culture

Following Treg cell co-culture, $CD8^+$ T cells were re-isolated and analyzed by qPCR for IL-2, TNF-α and IFN-γ mRNA expression at 1, 4, and 8 weeks post infection. $CFSE^{hi}$ (non-virus specific) $CD8^+$ T cells were compared to virus-specific $CD8^+$ ($CFSE^{int/low}$) T cells. There was no change observed in the mRNA levels of IL-2, IFN-γ, and TNF-α in non-specific $CD8^+$ T cells ($CFSE^{hi}$) upon co-culture with autologous Treg cells. As reported in Figure 2. 2, TNF-α mRNA expression was decreased in virus-specific $CD8^+$ T cells from FIV+ cats following autologous Treg cell co-culture at 4 weeks post infection. IL-2 and IFN-γ mRNA expression were decreased in virus-specific $CD8^+$ T cells following autologous Treg cell co-culture at both 4 and 8 weeks post-infection.

Foxp3 mRNA levels were increased in virus-specific $CD8^+$ T cells following autologous $CD4^+CD25^+$ Treg cell co-culture.

Virus-specific $CD8^+$ cells ($CFSE^{int/low}$) from FIV+ cats and non-virus specific ($CFSE^{hi}$) were co-cultured with or without autologous $CD4^+CD25^+$ Treg suppressor cells for 24 hours. After co-culture, cells were re-sorted by high speed cell sorting and analyzed by qPCR for Foxp3 mRNA expression. Following co-culture with autologous Treg cells, increased Foxp3 mRNA levels were observed in virus-specific $CD8^+$ T cells at 1, 4, and 8 weeks post infection in FIV+ cats (Fig. 2.3A-C). Foxp3 induction was also noted in non-virus specific ($CFSE^{hi}$) CD8+ T cells at 1 and 8 weeks post infection, but to a much lesser degree than virus specific $CD8^+$ T cells. These results demonstrate that lentivirus-activated Treg cells in FIV+ cats are able to induce Foxp3 mRNA expression in virus-specific $CD8^+$ T cell targets.

Foxp3 binds the IL-2, IFN-γ, and TNF-α promoter regions in virus-specific $CD8^+$ T cells

We performed ChIP on virus-specific $CD8^+$ T cells using a feline-specific anti-Foxp3 Ab, followed by qPCR for the IL-2, TNF-α and IFN-γ promoters. FIV- cats did not show any significant change in Foxp3 binding to the cytokine promoter regions before and after co-culture (Figure 2.4A-C). Our data clearly show Foxp3 binding to the IL-2, TNF-α and IFN-γ promoters at 8 weeks and 6 months (Figure 2.4A-C) post-infection in virus-specific $CD8^+$ T cells following Treg cell co-culture.

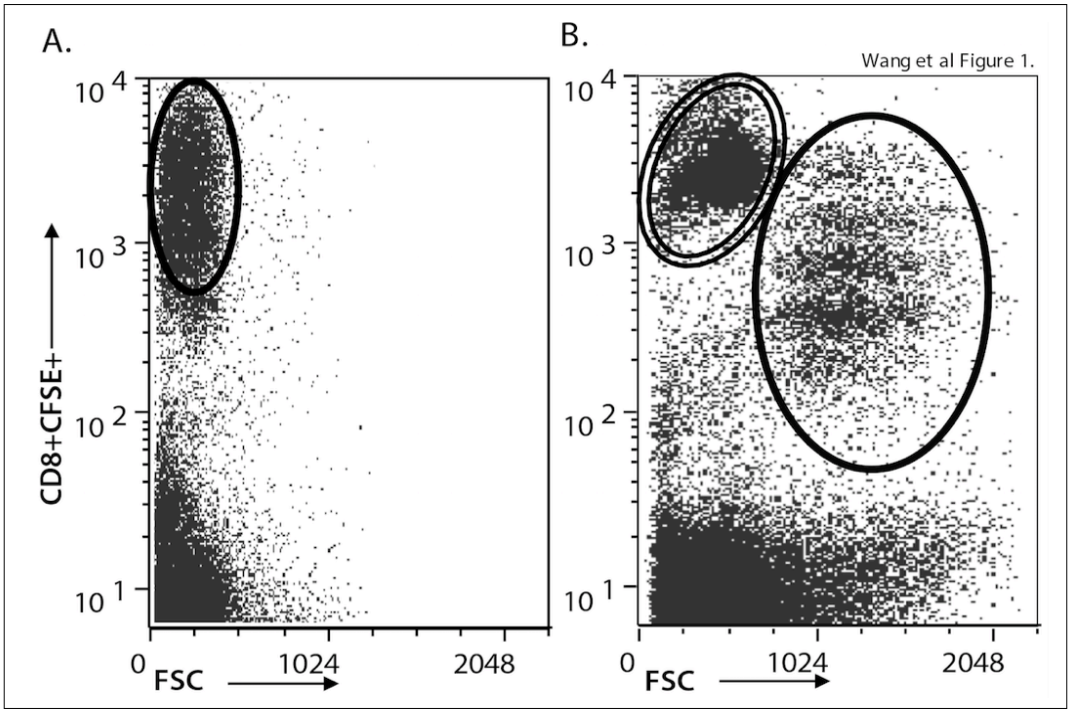

Figure 2.1. Isolation of virus specific CD8$^+$ T cells from FIV+ cats.
CD8$^+$ T cells were isolated from peripheral lymph nodes (LNs) of FIV- control cats (A) and FIV+ cats (B). The cells were CFSE stained and returned to CD4$^+$CD25$^+$ depleted lymph node cultures. The cultures were stimulated with UV inactivated FIV for 72 hours and resorted. CD8$^+$ T cells from FIV- control cats exhibited some CFSE dilution in culture (circled, A). Dead cells were gated by DAPI exclusion (not shown). Virus specific CD8$^+$ T cells from FIV+ cats were identified by CFSE dilution and increased forward scatter (single circle, B) as compared to virus non-specific CD8$^+$ T cells that did not divide in response to FIV (double circle, B).

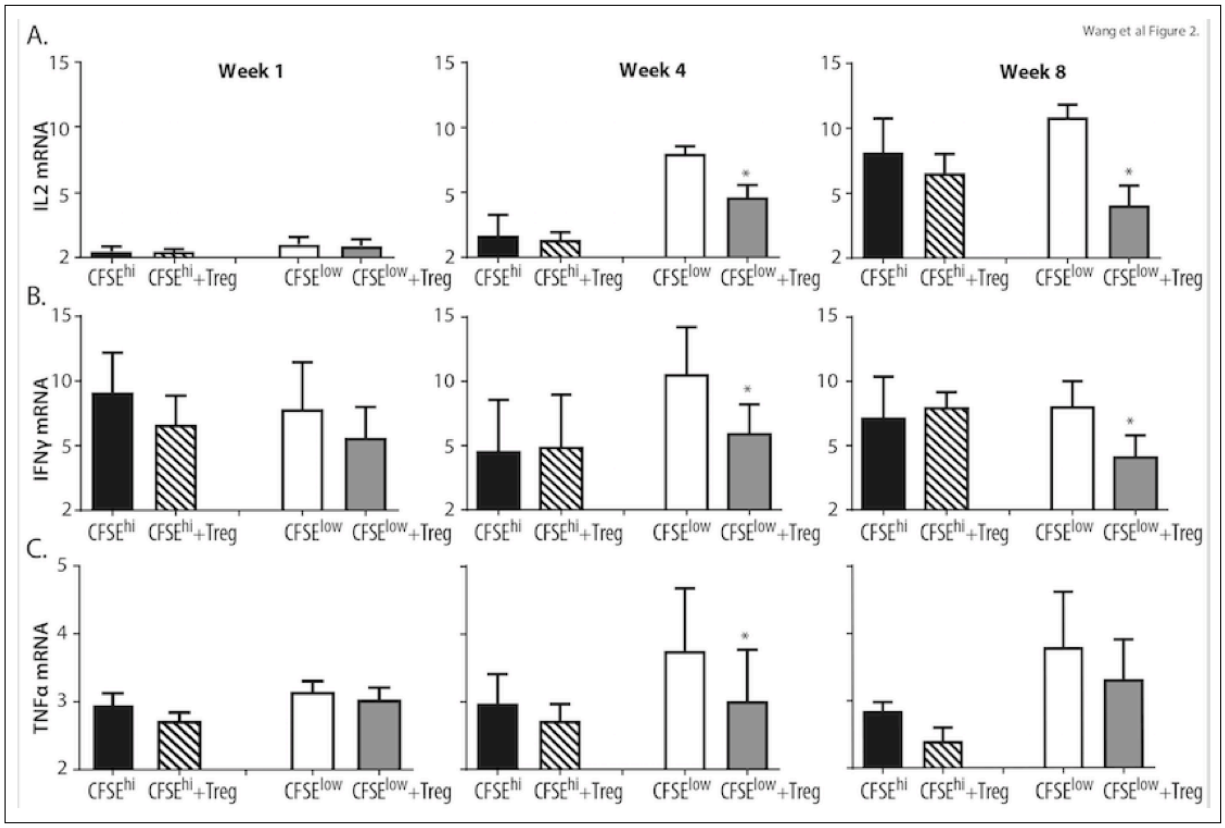

Figure 2.2. IL-2, IFN-γ, and TNF-α mRNA from virus-specific $CD8^+$ T cells following autologous $CD4^+CD25^+$ Treg cell co-culture.

Virus non-specific and virus specific $CD8^+$ T cells from FIV+ cats were sorted as described in Figure 2.1. Virus non-specific $CD8^+$ T cells (black bars, $CFSE^{hi}$) from FIV+ cats were cultured with autologous Treg cells (striped bars, $CFSE^{hi}$+Treg) and virus specific $CD8^+$ T cells from FIV+ cats (white bars, $CFSE^{low}$) were cultured with autologous Treg cells (grey bars, $CFSE^{low}$+Treg). Cells were isolated and cultured at 1 week (left column), 4 weeks (middle column), and 8 weeks (right column) post infection. (A). Following Treg cell co-culture, IL-2 mRNA is decreased in virus specific $CD8^+$ T cells at 4 and 8 weeks post infection. (B.) Following Treg cell co-culture, IFN-γ mRNA is decreased in virus-specific $CD8^+$ T cells at 4 and 8 weeks post infection. (C.) TNF-α is decreased in virus specific $CD8^+$ T cells at 4 weeks post infection following Treg cell co-culture. (n = 3 to 7 cats for each time point). Bars represent the mean + SEM. Data was analyzed using an unpaired t-test with significance set at *$p < 0.05$.

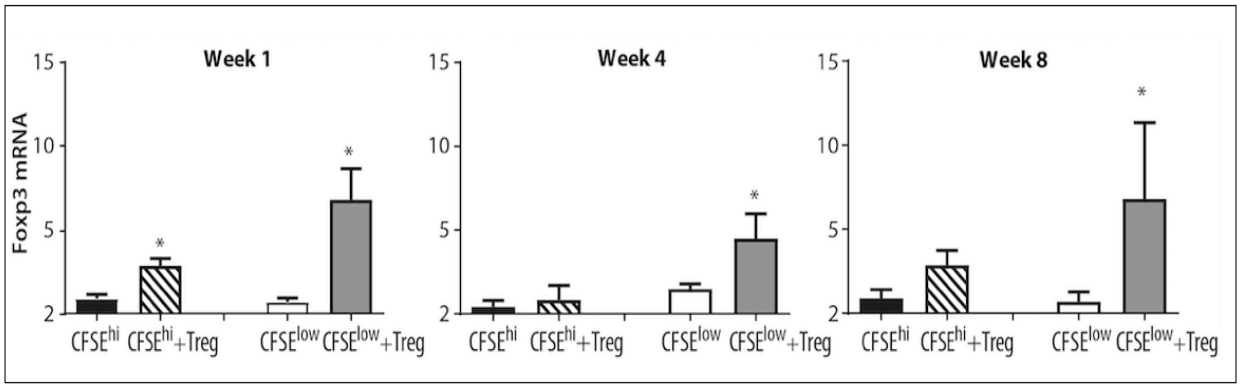

Figure 2.3. Foxp3 mRNA expression is increased in virus-specific CD8$^+$ T cells following co-culture with lentivirus activated Treg cells.
Virus non-specific and virus specific CD8$^+$ T cells from FIV+ cats were sorted as described in Figure 2.1. Virus non-specific CD8$^+$ T cells (black bars, CFSE^{hi}) from FIV+ cats were cultured with autologous Treg cells (striped bars, CFSE^{hi}+Treg) and virus specific CD8$^+$ T cells from FIV+ cats (white bars, CFSE^{low}) were cultured with autologous Treg cells (grey bars, CFSE^{low}+Treg). Cells were isolated and cultured at 1 week (left), 4 weeks (middle), and 8 weeks (right) post infection. Following Treg co-culture, Foxp3 is increased in virus specific CD8$^+$ T cells at all time points and in nonspecific CD8$^+$ T cells at week 1 post infection (n=4-6 cats for each time point). Bars represent the mean + SEM. Data was analyzed using an unpaired t-test with significance set at *$p < 0.05$.

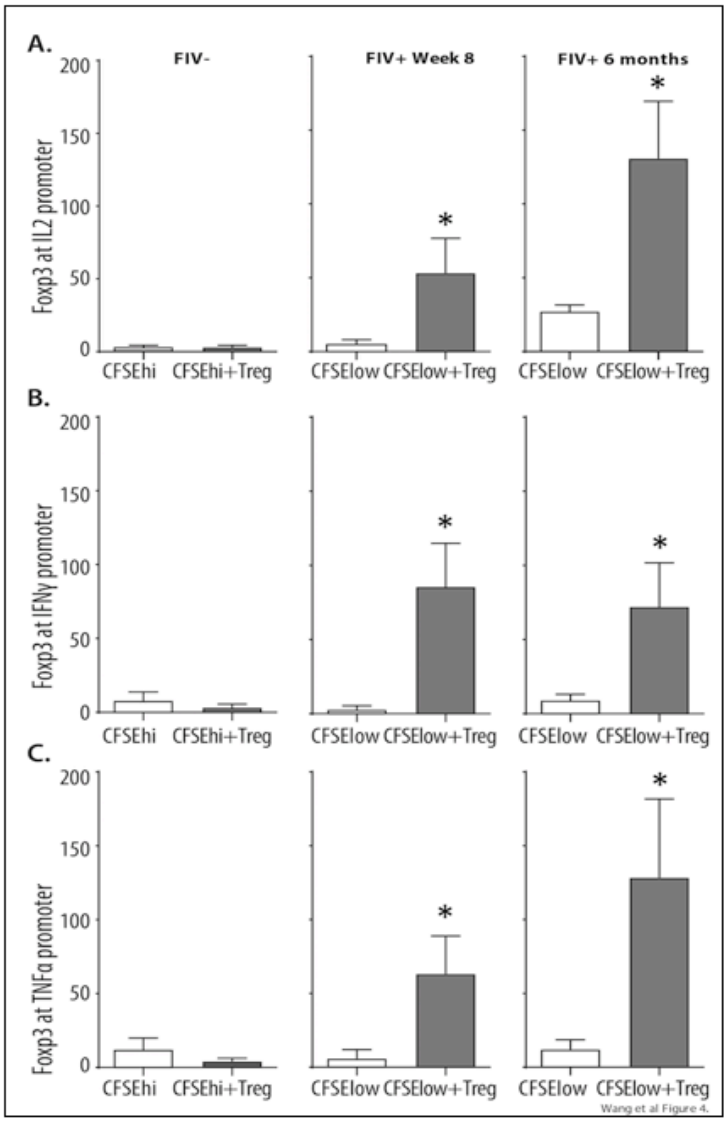

Figure 2.4. Foxp3 binds the IL-2, IFN-γ and TNF-α promoters in virus-specific $CD8^+$ T cells.

$CD8^+$ T cells from FIV- cats were co-cultured with autologous $CD4^+CD25^+$ Treg cells (left column). Virus-specific $CD8^+$ T cells from FIV+ cats 8 weeks (center column) and 6 months (right column) were either untreated (white bars, $CFSE^{low}$) or co-cultured with autologous $CD4^+CD25^+$ Treg cells ($CFSE^{low}$+Treg). After 24 hrs., Foxp3 ChIP followed by qPCR for IL-2, IFN-γ and TNF-α promoters was performed. Results show increased Foxp3 binding to the IL-2, IFN-γ, and TNF-α promoters and (A-C) after co-culture with autologous Treg cells at both 8 weeks and 6 months post infection for FIV+ cats (n= 5 to 7 cats). FIV negative cats exhibited almost no Foxp3 binding to the various promoters following autologous Treg cell co-culture. Bars represent the mean + SEM. Data was analyzed using an unpaired t-test with significance set at *$p < 0.05$.

Discussion

The purpose of the study here was to explore the molecular mechanisms associated with Treg-mediated suppression of virus-specific $CD8^+$ T cells. Specifically, we looked at IL-2 as an indicator of proliferative capacity in combination with TNF-α and IFN-γ as indicators of antiviral function. In the murine LCMV system, chronic infection leads to a predictable inactivation of virus-specific $CD8^+$ T cell responses. Fuller et al. clearly showed the sequential decrease and then loss of IL-2, TNF-α, and IFN-γ in virus-specific $CD8^+$ T cells (173). More importantly, $CD8^+$ T cell antiviral function was restored following a reduction in LCMV viral load (173). Polyfunctional $CD8^+$ T cells have been recognized as important to controlling virus in HIV ECs and in other HIV patient cohorts (179, 180). A recent study by Deng et al. clearly showed that chronically infected HIV patients retain broad spectrum CTL responses capable of eliminating viral reservoirs (181). However, these CTLs were dysfunctional, requiring extensive priming to eliminate autologous $CD4^+$ reservoirs *in vitro* and in humanized mice *in vivo.* In the pivotal study by Shan et al. utilizing HIV latency reversal drugs, $CD8^+$ T cells were unable to kill infected $CD4^+$ T cells following latency reversal alone; however, following Gag-stimulation prior to latency reversal, autologous $CD8^+$ T cells efficiently killed virus infected $CD4^+$ targets (182). Taken together, these results suggest that dysfunctional virus-specific $CD8^+$ T cells are maintained during the course of AIDS lentiviral infection and may be rescued under the right conditions. Because feline $CD8^+$ T cell tetramers are currently not available, we identified virus-specific $CD8^+$ T cells via proliferation in response to FIV, *ex vivo* (Figure 2.1). Our investigations here indicate a reduction in IL-2, TNF-α and IFN-γ mRNA in virus-specific $CD8^+$ T cells, following Treg cell co-culture, as early as 4 weeks post infection (Figure 2.2). The

reduction in IL-2 and IFN-γ mRNA persisted in virus specific $CD8^+$ T cells at 8 weeks post-infection, while TNF-α exhibited a trend decreased mRNA but did not reach significance.

Infection with a recombinant SIV/HIV virus (R5-SHIV) in macaques leads to accumulation of Treg cells in LNs and Treg cell depletion restores $CD8^+$ T cell responses to Gag epitopes (183). Miles et al. recently described accumulation of Treg cells and TGFβ-dependent suppression of follicular Th cells in LN follicles and in a previous investigation, the same group clearly demonstrated lower numbers of SIV-specific CTLs within LN follicles was associated with increased SIV RNA (184, 185). Using the FIV lentiviral model, we have clearly demonstrated that activated Treg cells display membrane-bound TGF-β (mTGF-β), activated Th and $CD8^+$ T cells upregulate TGF-βRII, and that Treg-mediated suppression is TGF-β-dependent (115, 116). Treg-mediated signaling through the T cell TGF-βRII leads to downstream Smad phosphorylation and increased Foxp3 expression (115). We have previously documented TGF-β-dependent Foxp3 induction in $CD4^+$ and $CD8^+$ T cells following Treg cell co-culture (109, 175). We have demonstrated that Treg-induced Foxp3 expression in $CD4^+$ T helper target cells leads to the induction of Treg cell suppressor function in the $CD4^+$ T helper targets (175). We have also described Treg-induced Foxp3 binding to the $CD8^+$ T cell IL-2 promoter in virus nonspecific $CD8^+$ T cells (117). Although others have demonstrated that $CD8^+Foxp3^+$ cells are indeed suppressors, our group was unable to document that $CD8^+$ lymphocytes exhibited suppressor function following Treg co-culture, despite the induction of $CD8^+$ Foxp3 expression (109). Here, we clearly demonstrate Treg-induced Foxp3 mRNA in virus-specific $CD8^+$ T cells at 1, 4, and 8 post-infection (Figure 2.3). It is interesting to note that Foxp3 mRNA in virus-specific $CD8^+$ T cells is induced quite early during the course of lentiviral infection. When taken together with

Figure 2.2, the results suggest an inverse correlation between Foxp3 mRNA and IL-2, TNF-α and IFN-γ mRNA in virus-specific $CD8^+$ T cells, following Treg cell co-culture. These results also suggest a short lag period between Foxp3 mRNA upregulation (1week post infection, Figure 2.2) and inhibition of cytokine mRNA (4 weeks post infection, Figure 2.3) as Foxp3 protein binds these promoter regions in the nucleus.

The transcription factor Foxp3 serves as a "master molecule" for Treg function. Foxp3 alters gene expression by binding to specific promoters and regulating their transcription (117). For example, Foxp3 binds the IL-2 promoter and decreases transcription of IL-2 while increasing the expression of the high affinity IL-2 receptor alpha (CD25) (71). In $CD4^+$ T cells, Foxp3 binding to the IL-2, TNF-α, and IFN-γ promoter regions has been described (186, 187). Transient expression of Foxp3 in activated $CD8^+$ lymphocytes is most likely a normal regulatory feedback mechanism (188, 189). However less is known about the role persistent Foxp3 signaling might play in modulating $CD8^+$ T cell function (109, 188). Based upon the collective evidence described above (Figures 2.2 and 2.3), we asked if Treg-induced Foxp3 could bind to the IL-2, TNF-α, and IFN-γ promoter regions in virus-specific $CD8^+$ T cells from FIV+ cats. We were able to document Foxp3 binding to the IL-2, TNF-α and IFN-γ promoters at 8 weeks and 6 months post-infection in virus-specific $CD8^+$ T cells following Treg cell co-culture (Figure 2.4).

There were several limitations to this study. The assessment of mRNA (Figures 2.2 and 2.3) and the ChIP assays required a relatively high number of virus-specific $CD8^+$ T cells. Therefore, we did not examine surface $CD8^+$ T cell phenotype nor polyfunctionality by flow cytometry for these studies. Based upon the mRNA and ChIP data presented here and our previous studies

examining Treg-mediated inhibition of cytokine secretion in FIV+ cats, it is likely that there was a reduction in virus specific $CD8^+$ T cell polyfunctionality following autologous Treg cell co-culture (104, 109, 115, 116, 175). We were limited by the total number of lymphocytes available after harvesting peripheral lymph nodes. Therefore, we chose 1, 4, and 8 weeks post infection for mRNA assessment and 8 weeks post and 6 months post infection to perform the Foxp3 ChIP assays. As shown in Figure 2.2, TNF-α mRNA was not decreased at 8 weeks post infection. However, Figure 2.4 demonstrated a high but variable degree of Foxp3 binding to the TNF-α promoter region 8 weeks post infection. Taken together, these results suggest a more complex relationship between Foxp3 and TNF-α expression.

Our previous findings demonstrate that lentivirus-activated Treg cells are activated during the course of FIV infection and upregulate mTGF-β, and that $CD4^+$ and $CD8^+$ T cells upregulate TGF-βRII during the course of infection (115, 116, 175). Further, we have demonstrated that Foxp3 induction is mediated via TGF-β signaling in both $CD4^+$ and $CD8^+$ T cells and that Foxp3 binds the IL-2 promoter in virus-non-specific $CD8^+$ T cells (115, 117, 175). The results here clearly demonstrate that lentivirus-activated Treg cells interact with virus-specific $CD8^+$ T cells to induce Foxp3 expression. Further, these results demonstrate that Foxp3 inhibits IL-2, TNF-α and IFN-γ transcription by binding to these gene promoter regions in lentivirus-specific $CD8^+$ T cells. We believe this is the first description of this process during the course of lentiviral infection. More importantly, these findings demonstrate a potential mechanism for the progressive loss of function in virus-specific $CD8^+$ T cells. Heightened Treg cell suppressor function has been clearly documented for lentiviral infection, including FIV, SIV, and HIV (109, 183, 190). However, Treg cell activation is not unique to lentiviral infection. Treg cells are

activated during the course of other types of chronic infections, such as hepatitis C virus and Leishmania infection and neoplastic diseases such as melanoma (171, 191, 192). Therefore, it is likely that the mechanisms described here may also contribute to T cell immune dysfunction in other diseases with heightened Treg cell function. The studies by Deng et al. and Shan et al. mentioned above, suggest that $CD8^+$ T cell dysfunction is reversible and that augmenting antiviral activity in these cells is possible (181, 182). Our previous studies and the studies presented here demonstrate that lentivirus-activated Treg cells mediate $CD8^+$ T cell dysfunction via Foxp3 induction. Based upon these findings, our group is exploring mechanisms for blocking Foxp3 promoter binding in an effort to augment $CD8^+$ T cell antiviral function. These results may yield new therapeutic approaches for enhancing virus-specific $CD8^+$ T cell function in vaccination and latency reversal strategies.

CHAPTER 3

Histone modulation blocks Treg-induced Foxp3 binding to the IL-2 promoter of virus-specific $CD8^+$ T cells from feline immunodeficiency virus infected cats.

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Introduction

Human Immunodeficiency Virus (HIV) /Feline Immunodeficiency Virus (FIV) infection is marked by robust expansion of $CD8^+$ T cells during the acute phase which is followed by a steady state lower level of virus-specific $CD8^+$ T cells during the chronic phase of infection. $CD8^+$ T cells in the chronic phase of infection are characterized by impaired proliferative capacity, reduced cytolytic function and dysfunctional effector function (5, 8, 9). IL-2 signals are important for the differentiation and homeostasis of various lymphocyte subsets including $CD8^+$ T cells. Naive $CD8^+$ T cells undergo rapid expansion and differentiation based on the strength and duration of IL-2 signals into either short-lived effector T cells or long lived memory T cells (193-195). Antigen- specific $CD8^+$ T cells respond to autocrine and paracrine IL-2 signals, via the high affinity trimer IL-2-R, to expand and differentiate to mount a potent immune response (196). During chronic infection, virus-specific $CD8^+$ T cells show a decrease and then loss of IL-2 expression along with progressive loss of antiviral cytokines such as IFN-γ and TNF-α (173, 197). The accumulation of dysfunctional $CD8^+$ T cells contributes to viral persistence and is a major obstacle to HIV cure strategies. However, recent studies suggest these dysfunctional $CD8^+$ T cells may be rescued and contribute to the elimination of viral reservoirs. For example, it was reported that lymphocytes latently infected with HIV could not be cleared by $CD8^+$ T cells after virus reactivation (with latency reversing drugs) unless the $CD8^+$ T cells were also primed

with gag-1 peptide (182). Similarly, another study reported that dysfunctional $CD8^+$ T cells from chronically infected HIV patients could eliminate the $CD4^+$ T cell reservoir only once they were primed either *in vitro* or using a humanized mouse model (181).

$CD4^+$ $CD25^+$ T regulatory cells (Treg) are progressively activated during FIV and HIV infection and suppress the proliferation and effector functions of both $CD4^+$ and $CD8^+$ T cells (90, 92, 198, 199). $CD4^+$ $CD25^+$ Treg cells stably express high levels of the transcription factor Forkhead Box P3 (Foxp3) that is critical for the development and suppressive function of Treg cells (200). Using the feline immunodeficiency virus model (FIV) for AIDS lentiviral persistence, our group investigates the interaction between lentivirus-activated $CD4^+CD25^+$ Treg cells and $CD4^+$ and $CD8^+$ target cells. Specifically, we are focused upon the molecular events that occur in the target cells that contribute to dysfunction, following interaction with Treg cells (109, 117, 118). Treg cells from FIV+ cats exhibit heightened suppression of T cell function when compared to Treg cells from uninfected control cats (104, 115). This Treg-mediated suppression of $CD4^+$ and $CD8^+$ T cells is TGF-β dependent. Membrane-bound TGF-β expressed on FIV-activated Treg cells ligates TGF-βRII displayed on the surface of target effector cells (115, 116). This ligation leads to Smad phosphorylation, contributing to Foxp3 induction in effector cells (115, 116). Previously, we have demonstrated that the induction of Foxp3 in $CD4^+$ T helper cells leads to the conversion of these cells into "induced" Treg cells that exhibit Treg cell phenotype and function (175).

Foxp3 is known to bind to the IL-2 and IFN-γ promoters and suppress transcription of these important cytokines (71, 186). Transient expression of Foxp3 in activated $CD8^+$ T cells is most

likely a mechanism to control the $CD8^+$ T cell response during FIV/ HIV infection (109, 188). However, during FIV infection, TGF-β –TGF-βRII binding in activated $CD8^+$ T cells can induce stable Foxp3 expression (115, 116). We have demonstrated that Foxp3 binds the IL-2 promoter in virus-nonspecific $CD8^+$ T cells following Treg cell / $CD8^+$ T cell co-culture (117). More recently, we have demonstrated Foxp3 binding to the IL-2, IFN-γ and TNF-α promoter regions in virus-specific $CD8^+$ T cells from FIV+ cats (and not FIV- control cats) following Treg cell co-culture (118).

Epigenetic modifications are heritable alterations in the genome that are not due to changes in DNA sequence. These molecular changes, e.g. DNA methylation, post- translational histone methylation and histone acetylation, along with chromatin remodeling, alter DNA accessibility and chromatin structure, thereby changing gene expression (201-203). Genes poised for active transcription have in general, hypo-methylated DNA and possess hyper-acetylated histones in their promoters. Virus-specific $CD8^+$ T cells actively transcribe genes essential to differentiation and antiviral function and thus exhibit an "open" chromatin conformation at essential promoter regions. At the same time, this "open" chromatin formation allows repressive transcription factors such as Foxp3 to bind to IL-2, TNF-α, and IFN-γ promoter regions and cause suppression of these genes (117, 118). Epigenetic modifications including de-methylated DNA and acetylation of histones H3 and H4 have been reported in murine $CD4^+$ $CD25^+$ Treg cells, murine $CD8^+$ T cells, feline $CD8^+$ T cells isolated from PLNs and human $CD4^+$ T cells isolated from PBMCs for several chronic viral infections, including LCMV, FIV and HIV (117, 150, 186, 204). These epigenetic modifications are, however, reversible. We demonstrate that blocking de-methylation of mitogen-activated $CD8^+$ T cells prior to autologous Treg co-culture blocked

Foxp3 binding to the IL-2 promoter in $CD8^+$ T cells (117). Based upon these findings, we asked if promoting histone de-acetylation, could be exploited as another means to prevent Treg cell-induced Foxp3 binding to the IL-2 promoter in virus-specific $CD8^+$ T cells.

In the current study, we used a Histone Acetyltransferase (HAT) inhibitor, Anacardic acid (AA) to block histone acetylation *in-vitro* and *ex-vivo.* We show that AA can block Foxp3 binding to the IL-2 promoter and result in a concurrent increase in IL-2 mRNA levels *in vitro*. We also demonstrated that treatment of virus-specific $CD8^+$ T cells of FIV-infected cats with AA for 24 hrs followed by co-culture with autologous Treg cells resulted in a reduction in Foxp3 binding at the IL-2 promoter. Taken together, these data suggest that histone acetylation contributes to Treg- induced Foxp3-mediated suppression of $CD8^+$ T cell function during lentiviral infection. These results further imply that epigenetic modulations of dysfunctional $CD8^+$ T cells could be employed to boost $CD8^+$ T cell function and enhance the clearance of virally-infected cells in HIV cure strategies.

Materials and Methods

Cats and FIV infection

Specific pathogen-free (SPF) cats were obtained from Liberty Labs (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA) and housed at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Cats (n= 5) were inoculated with 10^5 $TCID_{50}$ of FIV-$NCSU_1$ intravenously as described in (109). FIV infection was confirmed by ELISA (*S*NAP FIV/FeLV; Idexx Laboratories). Samples were collected from

cats at 8 weeks and 6-12 months (chronic phase) post infection. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. Single-cell suspensions were prepared from popliteal or submandibular peripheral lymph nodes (PLNs) obtained through surgical biopsies by gently and repeatedly injecting sterile CTL media (1640-RPMI with 2 mM L-glutamine, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1.5 g/L sodium bicarbonate, 10 mM HEPES and 1.0 mM sodium pyruvate) into the tissue using an 18G needle until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion using the Luna II cell counter (Logos Biosystems), and viability was always found to be >90%.

$CD8^+$ T cell co-culture and CFSE cell proliferation assays

Both anti-feline CD4 and anti-feline CD8 monoclonal antibodies were developed by our feline lentivirus research group as described previously (178). The feline anti-CD25 monoclonal antibody was developed by K. Ohno from University of Tokyo as described previously (109). Single cells from LNs were suspended at 1×10^8 cells/ml in HBSS (Thermo Fisher) with 2% FBS and stained with anti-feline CD8 PE antibody (clone 3.357) at 4ºC for 30mins. EasySep® PE Selection Cocktail was added at 100µL/mL of cell suspension at RT for 15mins, then EasySep® Magnetic Nanoparticles were added at 50µL/mL at RT for 10mins. $CD8^+$PE+ cells were separated by using the magnet provided in the Kit (Stem Cell, Vancouver, BC, Canada). The rest of the cell suspension was stained with mouse anti-feline CD4 APC antibody to isolate $CD4^+$ cells by using EasySep® APC Selection kit (Stem Cell). Isolated $CD4^+$ cells were then stained with mouse anti-feline CD25 FITC antibody to sort $CD4^+$ $CD25^+$ double positive Treg cells using the MoFlo XDP high-speed cell sorter (Beckman Coulter). DAPI (BioLegend) was used as

the cell viability dye to ensure we obtained live cells at the end of each of the sorts. $CD8^+$ T cells were resuspended in pre -warmed PBS/0.1% BSA and stained with 10 μM CFSE dye from the Cell Trace™ CFSE Cell Proliferation Kit (Life Technologies). $CD8^+$ T cells were returned to LN culture without $CD4^+CD25^+$ Treg cells, stimulated *in vitro* with UV-inactivated FIV-NCSU1, and were then co-cultured for 72 hours. Following stimulation, the virus specific proliferating $CFSE^{int/lo}$ cells and non-specific $CD8^+$ T cells $CFSE^{high}$ were isolated by re-sorting using a high-speed cell sorter. For all of the co-culture studies presented here, $CD8^+$ lymphocytes were co-cultured at a 1:1 (Treg : $CD8^+$) ratio with autologous $CD4^+CD25^+$ Treg cells for 24 hr. After co-culture, the cells were washed and then resorted into $CD8^+$ populations for analysis by qPCR or Chromatin immunoprecipitation (ChIP). The purity of magnetic bead sorted cells was > 95% and Moflo XDP sorted cell populations was >99%.

Mya-1 cell culture and cell viability

Mya-1 feline $CD4^+$ T cells were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 10% FBS and 1% penicillin and streptomycin and supplemented with 0.05 mM 2-mercaptoethanol and 100 units/ml recombinant human IL-2 (R&D). Cultures were maintained by the addition of fresh medium to cells every 2-3 days maintained at 37°C in a humidified atmosphere containing 7% CO_2. Cells were stained with Trypan Blue and counted with Luna II cell counter (Logos Biosystems) for cell viability assessment. The viability was always found to be > 90%.

Chromatin Immunoprecipitation (ChIP) and Acetyl-Histone 3 (AcH3) ChIP assay

The ChIP was performed using Chroma Flash High-Sensitivity ChIP Kit (Epigentek, Farmingdale, NY) according to manufacturer's specifications. In brief, 2μg of anti-Foxp3 (Abcam, ab2481), anti-Acetyl-histone 3 (Millipore), anti-RNA polymerase II (positive control) and non-immune IgG (negative control) antibodies were first bound to Assay Strip Wells. The sorted cells were cross-linked by adding CTL media containing formaldehyde to a final concentration of 1% with incubation at room temperature (20-25°C) for 10 min on a rocking platform (50-100 rpm). To each tube, pre-warmed 1.25M Glycine (1:10) was added to a final concentration of 125mM and incubated at room temperature for 5 min. After washing with ice-cold PBS, Working Lysis Buffer was added to re-suspend the cell pellet and incubated on ice for 10 min. After carefully removing the supernatant, Working ChIP Buffer was added to re-suspend the chromatin pellet. Sonic Dismembrator 50 (Fisher Scientific) was used to shear chromatin. The program was set to 25% power output and sonication was done with 4 pulses of 15 seconds each, with 30 seconds rest on ice between each pulse. ChIP samples were centrifuged at 12,000 rpm at 4°C for 10 min after shearing and the supernatant was transferred to a new vial. The ChIP samples were added to the wells bound with antibodies, positive control, or negative control. The reaction wells were incubated at 4°C overnight. ChIP samples were then washed according to the protocol and subjected to reverse cross-linking at 42°C for 30 min, 60°C for 45 min. DNA release was at 95°C for 15 min in a thermocycler. Finally, the DNA samples were purified by spin column for quantitative PCR (qPCR) using the ChIP primers for IL-2 Promoter: Forward Primer: 5' –TGCTCCACATGTTCAACACA- 3', Reverse Primer: 5' – CCCACACTTAGGTGGCAGTT- 3'. The relative enrichment of the target gene was calculated. Fold enrichment (FE) can be calculated by using a ratio of amplification efficiency of the ChIP

sample over that of Non-Immune IgG, FE % = $2^{(\text{IgG Ct} - \text{Sample Ct})} \times 100\%$.

RNA extraction, RT and real-time PCR quantification

Total RNA was extracted from cells using PureLink ™ RNA Micro Kit (Life Technologies). The concentration was quantified using a Nano Drop Spectrophotometer. RT PCR was performed for mRNA using qScript cDNA Synthesis Kit (Quanta Biosciences). 15 µL reactions were incubated for 5 min at 22°C, 40 min at 42°C, and 5 min at 85°C to inactivate the reverse transcriptase. Feline specific primers as shown in Table 1 were used to detect the Foxp3 and IL-2 mRNA levels using LightCycler® 480 System (Roche) qPCR. GAPDH mRNA expression was used as a normalizing control. For qPCR experiments, a ΔΔCt ratio was used to quantify relative mRNA expression. 8 µL of diluted cDNA, 10 µL PerfeCTa SYBR Green Super Mix Reaction Mix (Quanta Biosciences), 1µL forward primer and 1µL reverse primer were run in triplicates under the following cycling conditions: hot start enzyme activation at 95°C for 5 mins, denatured at 94°C for 45s, annealed at 60°C for 45s, and elongated at 72°C for 1 min with 35 cycles, and final extension at 72°C for 10 min.

Table 3.1: Feline primers used for Foxp3 and IL-2 qPCR.

Primer Target	Forward	Reverse
Foxp3	5'-GCCTGCCACCTGGAATCAAC-3'	5'-GTGTGCTGGGGCTTGGGA-3'
IL-2	5'-ACAGTGCACCTGCTTCAAGCTCT-3'	5'-CCTGGAGAGTTTGGGGTTCTCAGG-3'
GAPDH	5'-GGAGAAGGCTGGGGCTCAC-3'	5'GGTGCAGGAGGCATTGCTGA-3'

Anacardic Acid (AA) treatment

$1x10^6$ Mya-1 cells cultured in 12-well plates were treated with 0μM, 10μM, 20μM and 50μM of Anacardic Acid (Sigma Aldrich) for 24 hr**.** Viability was measured by flow cytometry. DAPI (BioLegend) was used to stain the cells. The data was acquired on BD LSRII and analyzed using FCS expression version 6 software when determining the appropriate concentration of AA (Figure 3.1A). For the rest of the experiments with AA treatment, Trypan Blue was used to determine viability and cells were counted with Luna II cell counter (Logos Biosystems)**.** The viability was found to be > 90% for each experiment.

Data Analysis

Statistical analysis was performed using GraphPad Prism software version 7.0. Samples of two different groups were compared by Mann-Whitney test, paired samples were analyzed by Wilcoxon matched -pairs signed rank test. Viability data was analyzed using paired t-test. P values of < 0.05 were considered statistically significant.

Results

Epigenetic modulation by AA promotes histone de-acetylation and blocks Foxp3 binding to the IL-2 promoter leading to higher IL-2 mRNA levels *in-vitro*.

Based upon our previous studies modulating DNA methylation, we asked if reducing histone acetylation also blocks Foxp3 binding to the IL-2 promoter. Anacardic acid is reported to reduce histone deacetylation by altering the activity of histone deacetylase. To determine the potential toxicity of AA, we first treated Mya-1 cells, a feline $CD4^+$ T cell line, with increasing concentrations of AA (Figure 3.1A). At the same time, we measured histone 3 acetylation at the

IL-2 promoter (Figure 3.1B). The results demonstrated that a 20μM concentration of AA had limited toxicity to feline lymphocytes while promoting histone deacetylation at the IL-2 promoter, and this concentration was selected to perform the subsequent experiments.

To determine if AA blocks the binding of the repressive transcription factor Foxp3 to the IL-2 promoter, feline Mya-1 cells were either untreated or treated with 20 μM AA for 24 hrs. ChIP followed by qPCR demonstrated that 20 μM AA treatment significantly inhibited endogenous Foxp3 binding to the IL-2 promoter (Figure 3.2A). To validate the biological significance of this finding, we investigated the effect of AA on the transcription of IL-2. qPCR data confirmed that IL-2 mRNA levels increased with 20 μM AA treatment (Figure 3.2B). Foxp3 mRNA levels were also enhanced by AA treatment, and therefore reduced Foxp3 binding to the IL-2 promoter was not due to reduced Foxp3 transcription in AA-treated Mya-1 cells (Figure 3.2B). Collectively these results demonstrated that AA blocks Foxp3 binding to the IL-2 promoter concomitant with a reciprocal increase in IL-2 transcription.

AA blocks Foxp3 binding to IL-2 promoter in virus-specific $CD8^+$ T cells co-cultured with autologous Treg cells in FIV+ cats.

The potential importance of this epigenetic modification *in vivo* during lentiviral infection was assessed by treating virus-specific $CD8^+$ T cells isolated from the PLNs of FIV-infected cats with or without 20 μM AA for 24 hrs, followed by co-culture with autologous Treg cells. Subsequently, the $CD8^+$ T cells were re-isolated for Foxp3 ChIP followed by qPCR for the IL-2 promoter. Consistent with our *in vitro* findings using Mya-1 cells, Foxp3 binding to the IL-2 promoter was decreased when virus-specific $CD8^+$ T cells were pre-treated with AA (Figure 3.3). Although the difference in Foxp3 binding to the IL-2 promoter between treated and

untreated $CD8^+$ T cells did not reach statistical significance, reduced Foxp3 binding was observed in each paired sample set, indicative of biological significance.

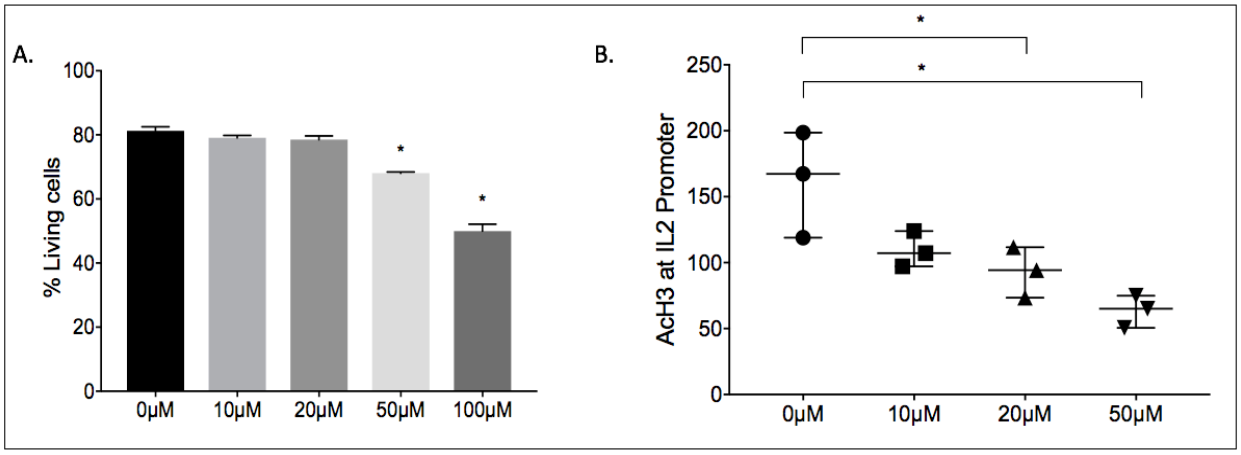


Figure 3.1. Epigenetic modulation by Anacardic acid (AA) promotes histone de-acetylation. Feline Mya-1 cells, a $CD4^+$ T cell line, were treated with anacardic acid (AA) at 0, 10, 20, 50 and 100 μM for 24 hours. (A.) Cell viability was measured by flow cytometry as described in the methods using DAPI as the live-dead stain. Data are represented as the mean+ SD, n=3 (B). AcH3 ChIP, followed by qPCR demonstrated a reduction in histone acetylation at the IL-2 promoter. Data are represented as the median with range, n=3. Statistical differences were determined by a paired t-test in 3.1A and Wilcoxon matched- pairs signed rank test in 3.1B, with * representing $p<0.05$.

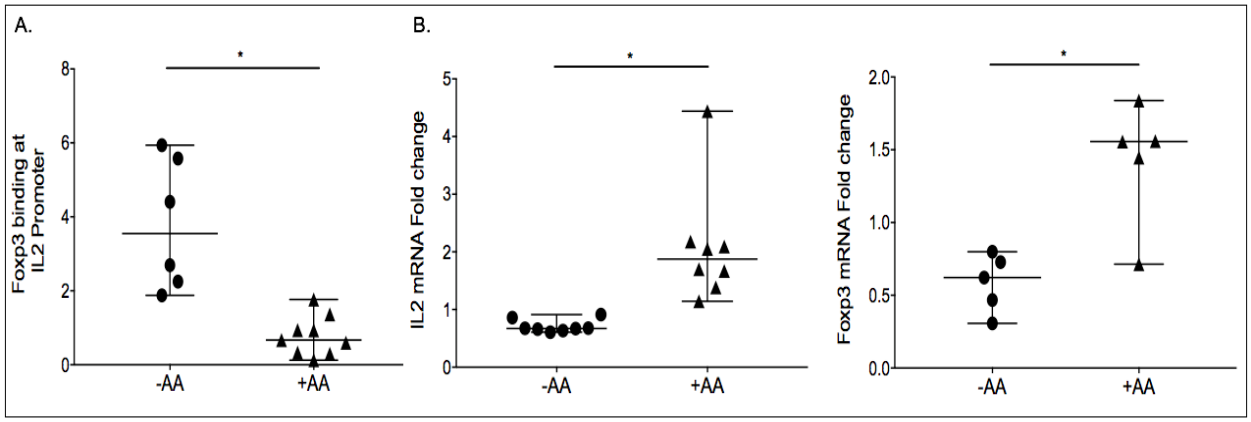


Figure 3.2: Anacardic acid blocks endogenous Foxp3 binding to the IL-2 promoter and increases IL-2 mRNA levels *in-vitro*.
(A.) Mya-1 cells were either untreated (circles, n=6) or treated with 20 µM AA (triangles, n=9); AA treatment inhibited Foxp3 binding at the IL-2 promoter. (B.) Mya-1 cells were either untreated (circles) or treated with 20 µM AA (triangles) and assessed by RT-qPCR. Both IL-2 mRNA (n=8) and Foxp3 mRNA (n=5) were increased following AA treatment. All data are presented as the median with range; with each symbol represents an individual animal. Statistical differences were determined by Mann-Whitney test, with * representing $p<0.05$.

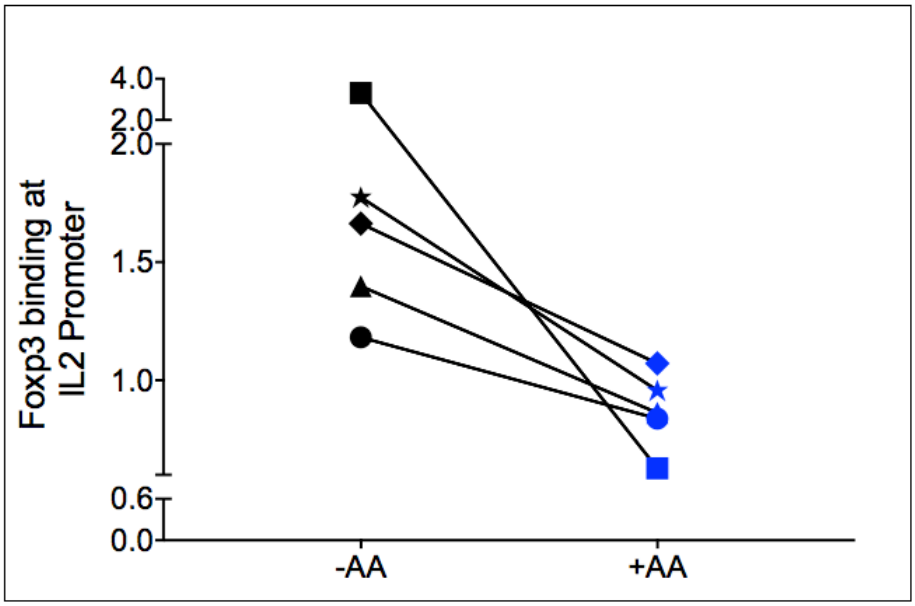


Figure 3.3: Anacardic acid blocks Foxp3 binding to the IL-2 promoter in virus-specific CD8$^+$ T cells.
Virus-specific CD8$^+$ lymphocytes isolated from PLNs of FIV+ cats (> 6mo infection) were either untreated (black) or treated (blue) with AA at 20μM for 24 hr followed by co-culture with autologous Treg cells. Foxp3 ChIP followed by qPCR demonstrated a reduction in Foxp3 binding to the IL-2 promoter. (n=5, each symbol represents an individual animal).

Discussion

Our group and others have reported that FIV-infected Treg cells induce Foxp3 expression in activated CD8$^+$ T cells via a TGF-β -dependent mechanism. We also demonstrated that Foxp3 binds to the IL-2, IFN-γ and TNF-α promoters in virus-specific CD8$^+$ T cells, and that blocking DNA de-methylation reduces Foxp3 binding to the IL-2 promoter (115-118). These results suggest that hyper-methylation of the IL-2 promoter prevents Foxp3 binding to the promoter region. In the current study, we asked whether histone modulation also prevents Foxp3-mediated binding to the IL-2 promoter. The Histone H3 tail is susceptible to different covalent modifications such as acetylation, phosphorylation, methylation of lysine and serine residues, thereby regulating gene activation or suppression. Histone acetylation has been related to gene

activation and has been shown to induce a transcriptionally active chromatin (205, 206). Therefore, we hypothesized that reducing histone acetylation might also prevent Foxp3 binding. To address our hypothesis, we tested whether AA, a well-documented natural HAT inhibitor inhibits Foxp3 binding to the IL-2 promoter (207). Consistent with the function of AA as a HAT inhibitor, we demonstrate that AA treatment reduced histone 3 acetylation in the feline cell line Mya-1 (Figure 3.1B). Further, AA treatment of Mya-1 cells reduced Foxp3 binding to the IL-2 promoter (Figure 3.2A). IL-2 mRNA levels were higher in AA-treated versus untreated Mya-1 cells further supporting our model of Foxp3-mediated suppression (Figure 3.2B). Previous studies have shown that the distal enhancer region of the IL-2 gene undergoes increased H3 acetylation upon activation, and that the IL-2 promoter at the proximal region exhibits decreased histone acetylation due to the loss of H3 and H4 proteins in response to T cell activation (208, 209). We and others have demonstrated that $CD8^+$ T cells exhibit an activated phenotype during FIV infection (109, 176). Here, we indicate the role of histone acetylation in lentiviral infection by demonstrating the reduction in Foxp3 binding to the IL-2 promoter upon AA treatment of FIV-specific $CD8^+$ T cells isolated from each of the five animals (Figure 3.3). Even though the difference between the two groups was not significant, a consistent reduction in Foxp3 binding at the IL-2 promoter after AA treatment was observed for every animal suggesting the biological relevance of our data. A low sample size could explain the lack of statistical significance. Overall, these data suggest that epigenetic modulation of dysfunctional $CD8^+$ T cells during lentivirus infection can block Foxp3 binding. Future studies are needed to evaluate whether AA could be applied *in vivo* to restore, or at a minimum, enhance the function of antiviral $CD8^+$ T cells at different stages of FIV infection. AA can also activate Aurora kinase A (ARK) and mediate phosphorylation of histone H3 *in vitro* thereby impacting post-transcriptional

modifications (210). Therefore, “off-target” effects of AA cannot be ruled out. Despite these limitations, to our knowledge, this is the first account indicating the involvement of histone acetylation in the suppression of IL-2 in virus- specific $CD8^+$ T cells using the FIV model. This opens up a new area of investigation to advance our understanding of virus-specific $CD8^+$ T cell dysfunction and avenues to boost $CD8^+$ T cell function for potential cure strategies.

Based upon our findings presented here, we propose the following model (Figure 3.4). Lentivirus-induced activation of virus-specific $CD8^+$ T cells results in chromatin remodeling as these T cells upregulate the expression of genes essential to antiviral function. During this process, histone acetyltransferases add acetyl groups to histone tails, relaxing the chromatin to its euchromatin form, and DNA becomes accessible for transcription factors to bind. Our results suggest that Treg- induced Foxp3 can bind to the IL-2 promoter and suppress its expression. These results suggest that the potential exists to de-acetylate histones and prevent Foxp3 binding to the IL-2 promoter *in vivo* during the course of lentiviral infection. The reduction in Foxp3 binding at the IL-2 promoter may translate into restoring or enhancing IL-2 function in the dysfunctional $CD8^+$ T cells during lentivirus infections.

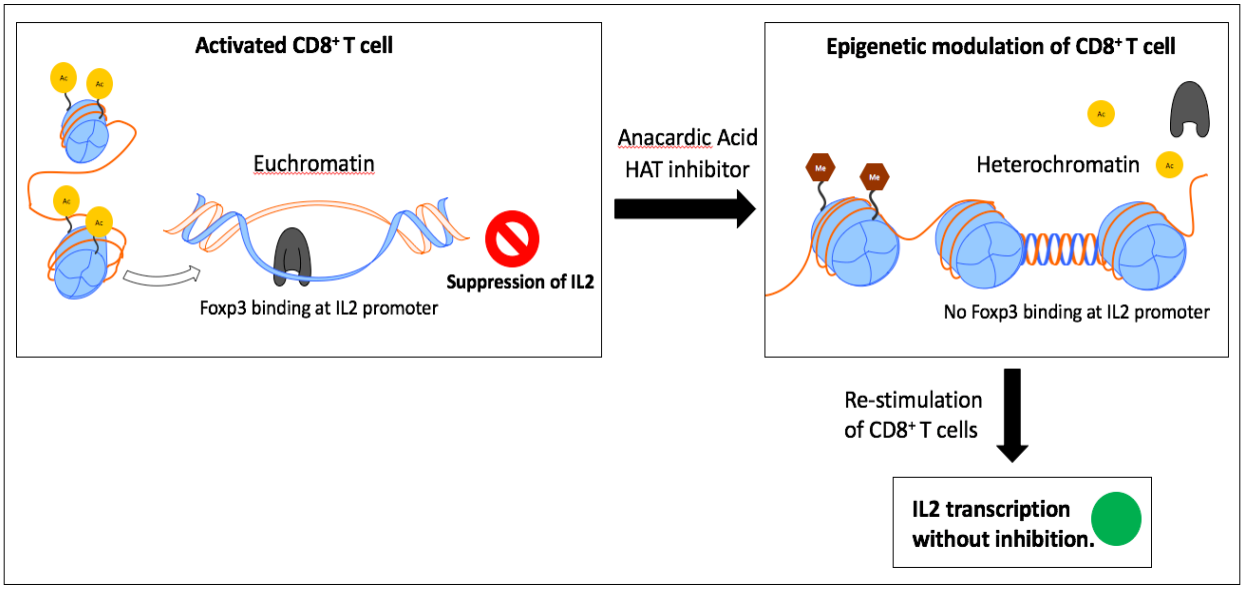


Figure 3.4. A model of blocking Foxp3 binding to the IL-2 promoter through histone modulation.
The chromatin configuration within a lentivirus activated $CD8^+$ lymphocyte showing histone acetylation (small yellow beads) leading to a "relaxed" conformation (euchromatin, left side). Here, the IL-2 promoter region is open and accessible, allowing the repressive transcription factor Foxp3 access to inhibit IL-2 transcription, following interaction with an activated Treg cell. Treatment with AA promotes histone de-acetylation (heterochromatin formation), preventing Foxp3 access to the IL-2 promoter region (right). If the cell is re-stimulated, IL-2 transcription and translation can proceed without inhibition.

CHAPTER 4

TGF-β induced Foxp3 binds to the IL-2, IFN-γ, and TNF-α promoters in rhesus macaque $CD8^+$ T cells.

Introduction

$CD8^+$ T cells play a critical role in the control of viremia during SIV infection of rhesus macaques (211, 212). A robust cytotoxic $CD8^+$ T cell response during the acute phase of SIV infection contributes to a sharp decline in viremia (213). In the chronic phase of SIV infection, similar to HIV and FIV infection, $CD8^+$ T cells progressively become functionally impaired (214-216). Impairment of IFNγ-producing SIV-specific $CD8^+$ T cells is detected as early as 4 months post infection (215), and the cytolytic function of SIV-specific $CD8^+$ T cells starts to decrease by the end of the acute phase of infection (214). As the infection proceeds, these dysfunctional $CD8^+$ T cells are unable to eliminate virally infected cells.

$CD8^+$ T cell responses are suppressed by $CD4^+$ $CD25^+$ T regulatory (Treg) cells during HIV, FIV and SIV infections (92, 109, 198, 217). The detrimental effect of the interaction of Treg cells with $CD8^+$ T cells is emphasized by a recent study demonstrating that *in vivo* depletion of Treg cells in SIV–infected controller macaques resulted not only in $CD4^+$ T cell activation and reactivation of latent SIV, but also boosted SIV-specific $CD8^+$ T cells that were able to rapidly clear the reactivated cells (25, 218).

Treg frequencies increase early during SIV infection, coinciding with the onset of the attenuation of SIV-specific $CD8^+$ T cell response (99). High frequencies of both thymus-derived (nTreg) and induced Treg (iTreg) cells are reported during the acute phase of HIV and FIV infections as well

(98, 104, 219, 220). Treg cells accumulate in blood, peripheral lymph nodes and mucosal tissues within 24 hours to 2 weeks post SIV infection (94, 95, 103, 221). Despite increased frequencies of Treg cells at viral replication sites during acute and chronic HIV/SIV infections, the overall number of $CD4^+$ Treg cells decrease because they also represent viral target cells (222, 223). Multiple mechanisms elucidating Treg-mediated suppression have been described, such as CTLA-4 binding, expression of CD39 and CD73 surface ectonucleotidases, expression of IL-10, IL-35 and transforming growth factor β (TGF-β) (reviewed in Vignali et al., 2008 (224)). These suppressor functions of Treg cells are heightened as they get activated during lentivirus infections (104, 220, 222, 223). Treg cells are, therefore, important in inducing and maintaining $CD8^+$ T cell dysfunction during HIV/SIV infection.

Using the FIV model, we have demonstrated that lentivirus-activated Treg cells induce stable Foxp3 in $CD8^+$ T cells and that Foxp3 induction is TGF-β dependent (115-117). Recently, we reported that Foxp3 directly binds to the promoters of IL-2, IFN-γ and TNF-α in virus-specific $CD8^+$ T cells, thereby inhibiting cytokine production (118). In addition, the IL-2 promoter exhibits epigenetic modulation, specifically de-methylation in the distal promoter region indicating a euchromatin conformation in activated $CD8^+$ T cells from FIV-infected cats (117). Upon modulating the chromatin to its heterochromatin form by blocking DNA de-methylation and histone acetylation, we demonstrated a reduction in Foxp3 binding at the IL-2 promoter ((117), Chapter 3). The epigenetics data suggest that chromatin remodeling at the IL-2 promoter, while essential for $CD8^+$ T cell activation, allows the binding of Foxp3 resulting in the suppression of IL-2. Therefore, epigenetically controlled Foxp3 induction and binding at antiviral cytokine promoters represent a mechanism of Treg-mediated $CD8^+$ T cell suppression.

To extend our results from the FIV model, we next investigated whether Foxp3 also mediates $CD8^+$ T cell dysfunction in the NHP SIV infection model. As a first step, we investigated the molecular events occurring in $CD8^+$ T cells with Foxp3 induced by *in vitro* stimulation of rhesus macaque PBMCs with TGF-β1 and α-CD3/α-CD28 (225). Based on our FIV studies, we hypothesized that Foxp3 binds the IL-2, IFN-γ, and TNF-α promoters in rhesus macaque $CD8^+$ T cells stimulated with TGF-β1.

Our results illustrate that stimulation with α-CD3/α-CD28+TGF-β1 results in the induction of intracellular Foxp3 in $CD8^+$ T cells. Consistent with our findings in the FIV model, we could further demonstrate increased binding of Foxp3 to IL-2, IFN-γ, and TNF-α promoters in stimulated compared to unstimulated $CD8^+$ T cells. In addition, Foxp3 induction and binding was associated with euchromatin conformation demonstrated by the suppression of the DNA methyltransferases DNMT1 and DNMT3b.

Materials and Methods

Sample collection and blood processing

Whole blood (50 ml/animal) was drawn from adult donor rhesus macaques in EDTA vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). PBMCs were isolated using the Lymphocyte Separation Medium (LSM) (MP Biomedicals, Santa Ana, CA) following the protocol described in Jensen et al., 2016 (226). PBMCs were resuspended in complete RPMI (cRPMI) consisting of 1640-RPMI (Cellgro, Corning, NY), 10% heat-inactivated fetal bovine serum (FBS) (Serum

Source International, Charlotte, NC), and 1% penicillin-streptomycin-L glutamine (Sigma, St. Louis, MO).

Anti-CD3/anti-CD28 and TGF-β1 stimulation of macaque PBMC

24-well cell culture plates or T75 tissue culture flasks (Corning, NY) were coated with 1μg/ml of α-CD3 antibody (clone FNI8, NHP Reagent Resource) in PBS overnight at 4ºC. Excess α-CD3 antibody was aspirated and the wells/flasks were rinsed twice with cRPMI for 10 mins. Then, 2μg /ml of soluble α-CD28 antibody (clone L293, BD Biosciences, Franklin Lakes, NJ), 5ng/ml of recombinant human TGF-β1 activated with 0.1N HCl per manufacturer's instructions (R&D Systems, Minneapolis, MN) and $1X10^6$ cells/ml of PBMCs were mixed and added to the wells/flasks. The culture was incubated for 5 days at 37ºC, 5%CO_2. 24- well cell culture plates were used for α-CD3/α-CD28 only, TGF-β1 only treated controls used for flow cytometry. T75 tissue culture flasks were used for stimulating sort samples. Cell density was maintained at $1X10^6$ cells/ml in both. α-CD3/α-CD28+TGF-β1 treated group was also included in the 24-well plate for comparison with the α-CD3/α-CD28+TGF-β1 stimulated sort sample. Foxp3 expression in both the samples was comparable (data not shown).

FACS-sorting of cell populations:

α-CD3/α-CD28+TGF-β1 stimulated and unstimulated (media control) PBMCs were stained with CD3-APC-Cy7(Clone SP34-2, BD), CD8-BV785 (clone RPA-T8, BD), CD4 PE-CF594 (clone L200, BD), and CD25-PE (clone 4E3, eBioscience, San Diego, CA) for 30 mins in the dark at room temperature (RT), followed by a wash with PBS at 1500rpm for 7 mins. PBMCs were resuspended in PBS at 10^7 cells/ml. DAPI (BioLegend, San Diego, CA) was used to

discriminate between live and dead cells. The stained cell suspension was filtered into a 5ml polystyrene tube with a cell strainer cap (StemCell, Vancouver, Canada). Compensation was set up using compensation bead controls (eBioscience) and unstained cells. Single, live, $CD3^+$ lymphocytes were sorted on BD FACS Aria II or FACS Aria III instruments into $CD8^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cell populations that were collected into 5ml polypropylene tubes containing 500µl of FBS. Fluorescence minus one (FMO) controls for CD25 were included as controls.

Flow cytometry analysis

$1x10^6$ cells of sorted α-CD3/α-CD28+TGF-β1 stimulated and unstimulated PBMCs and control cells stimulated with α-CD3/α-CD28 only or TGF-β1 only were stained for CD3-APC Cy7 (clone SP34-2, BD), CD8-BV785 (clone RPA-T8, BD), CD4-PE-CF594 (clone L200, BD), CD25-PE (clone 4E3, eBioscience), and fixable live/dead stain (Invitrogen, Carlsbad, CA) at RT for 30 mins in the dark. Intracellular Foxp3 staining was performed according to the protocol by Law et al., 2009 using the Human Foxp3 Buffer set (BD) and the Foxp3- eFluor 450 antibody (clone PCH101, eBioscience). Cells were washed and fixed with 1% paraformaldehyde (PFA) as described in Jensen et al., 2016. Samples were acquired within 24-48 hrs on the BD LSR Fortessa and analyzed using FlowJo version 10.4 applying Boolean gating strategies. FMO controls were included for all samples.

Chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) assay

ChIP was performed using the Chroma Flash High-Sensitivity ChIP Kit (Epigentek, Farmingdale, NY) according to the manufacturer's specifications. In brief, 2µg of anti-Foxp3

(Abcam, Cambridge, United Kingdom), anti-RNA polymerase II (positive control) and non-immune IgG (negative control) antibodies were first bound to assay strip wells. The sorted cells were cross-linked by adding cRPMI containing formaldehyde to a final concentration of 1% and incubated at RT for 10 min on a rocking platform (50-100 rpm). To each tube, pre-warmed 1.25M Glycine (1:10) was added to a final concentration of 125mM and incubated at RT for 5 min. After washing with ice-cold PBS, Working Lysis Buffer was added to re-suspend the cell pellet, followed by incubation on ice for 10 min. After carefully removing the supernatant, Working ChIP Buffer was added to re-suspend the chromatin pellet. The Q500 Sonicator (QSonica, Newtown, CT) was used to shear chromatin. The program was set to 25% power output and sonication was done with 5 pulses of 15 seconds each, with 40 seconds rest on ice between each pulse. ChIP samples were centrifuged at 12,000 rpm at 4°C for 10 min after shearing and the supernatant was transferred to a new vial. The ChIP samples were added to the antibody-coated wells and incubated at 4°C overnight. ChIP samples were then washed according to the protocol and subjected to reverse cross-linking at 42°C for 30 min and 60°C for 45 min. DNA release was performed at 95°C for 15 min in a thermocycler. Finally, the DNA samples were purified by spin column for qPCR using the ChIP primers listed in Table 4.1. The promoter sequence (upto 2000 bp upstream of transcription start site) for each cytokine was obtained from UCSC genome browser. Primers were designed using NCBI Primer Blast and obtained from Thermo Scientific (Waltham, MA). Primers were validated using sanger sequencing from Genewiz (Morrisville, NC). 20µl qPCR reactions set up per well using 4 µL(100ng) of purified ChIP-DNA, 10µl of PowerUP SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 1µL forward primer, 1µL reverse primer and 4 µL nuclease free water were amplified under the following cycling conditions: 50°C for 2 mins, 95°C for 2 mins,

(95ºC for 15s, 60°C for 1 min) for 45 cycles, followed by a 4ºC hold step.

The relative increase in Foxp3 binding was calculated and reported as Foxp3 binding at each promoter. %Fold increase in Foxp3 binding = $2^{(\text{IgG Ct} - \text{Sample Ct})} \times 100\%$. GAPDH amplification in RNA Polymerase II-bound DNA was used as a control for PCR conditions. Ct values of Non-Immune IgG were used for normalization.

Table 4.1: Rhesus macaque ChIP primers.

Primer Target	**Forward**	**Reverse**
IL-2 Promoter	5'-CTTGGGGCAGCAATCACCTA-3'	5'-AGAACAGTGTACAGGCAGGC-3'
IFN-γ Promoter	5'-GGCTACTACTCTCAGGGGCA -3'	5'-GCACAAAAAGCCCTCCACTC-3'
TNF-α Promoter	5'-GTCTGTGAATTCCCGGTGGT-3'	5'-GGACACCCAAGCATCAAGGA-3'
GAPDH	5'-GCACCACCAACTGCTTAGCAC-3'	5'-TCTTCTGGGTGGCAGTGATG-3'

RNA extraction, reverse transcription and real-time PCR quantification

Total RNA was extracted from unstimulated and stimulated sorted $CD8^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells using the RNeasy Micro Kit (Qiagen, Hilden, Germany). The concentration was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific). cDNA was prepared using the qScript cDNA Synthesis Kit (Quanta Biosciences, Beverly, MA) in 20 µL reverse transcription reactions with the following conditions: 5 min at 22ºC, 40 min at 42ºC, and 5 min at 85ºC. To quantitate mRNA levels of DNMT1 and DNMT3b, cDNA was PCR-amplified using rhesus macaque-specific primers listed in Table 4.2. Macaque DNMT1 and 3b gene sequences were obtained from NCBI, the primers were designed using NCBI Primer Blast and obtained from Thermo Scientific. Primers were validated by sanger sequencing from Genewiz.

Specifically, 4 µL of diluted cDNA, 10 µL Power SYBR Green Master Mix (Applied Biosystems), 1µL forward primer, 1µL reverse primer and 4 µL of nuclease free water were amplified under the following cycling conditions: hot start enzyme activation at 95°C for 10 mins, denatured at 95ºC for 15s and annealed at 58°C for 1 min with 40 cycles, followed by a 4ºC hold step. The relative mRNA expression levels were calculated using the ΔΔCt method, with GAPDH serving as control.

Table 4.2: Rhesus macaque primers for qPCR.

Primer Target	**Forward**	**Reverse**
DNMT1	5'- ACCTGGCTAAAGTCAAATCCCT-3'	5'- GTCTCTCCATCGGACTTGCT-3'
DNMT3b	5'- GCCGCTTCCTCGCAACAG -3'	5'- CCTCTCCGTTGAGATGCCTG-3'
GAPDH	5'- GCACCACCAACTGCTTAGCAC -3'	5'- TCTTCTGGGTGGCAGTGATG -3'

Data Analysis

Statistical analysis was performed using GraphPad Prism software, version 7.0. Paired samples were analyzed by Wilcoxon matched -pairs signed rank test. P values of < 0.05 were considered statistically significant.

Results:

***In vitro* stimulation of rhesus macaque PBMCs with α-CD3/α-CD28+TGF-β1 results in the upregulation of Foxp3 in $CD8^+$ T cells**

Stimulation with TGF-β1 promotes the conversion of naive $CD4^+CD25^-$ T cells to $CD4^+CD25^+Foxp3^+$ iTreg cells in $CD4^+$ T cells isolated from FIV –infected cats, humans and

C57BL/6 mice (175, 225, 227). Therefore, to confirm the induction of Foxp3 by our stimulation conditions using cells from macaques, we quantitated the frequencies of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells and their Foxp3 expression after α-CD3/α-CD28+TGF-β1 stimulation including α-CD3/α-CD28 only, or TGF-β1 only stimulated cells as controls. Compared to culture in media only, the frequencies of $CD4^+$ T cells expressing Foxp3 were increased in α-CD3/α-CD28+TGF-β1 and α-CD3/α-CD28 only treated cells, but not in TGF-β1 only treated cells (Figure 4.1A). We also observed an increase in the frequency of $CD4^+CD25^+$ $Foxp3^+$ T cells in the α-CD3/α-CD28 only and α-CD3/α-CD28+TGF-β1 treated cells compared to media only and TGF-β1 only stimulated cells (Figure 4.1B). Treatment with TGF-β1 only did not change the frequency of $CD4^+CD25^+Foxp3^+$ T cells compared to the media only group, but resulted in an increase of Foxp3 expressing $CD4^+CD25^-$ T cells (Figure 4.1C). None of the other stimulation conditions induced Foxp3 in $CD4^+CD25^-$ T cells. Next, we assessed how these various stimulation conditions impacted Foxp3 induction in $CD8^+$ T cells. Stimulation with α-CD3/α-CD28+TGF-β1 resulted in higher frequencies of $CD8^+$ $Foxp3^+$ T cells compared to media only (Figure 4.1D). We also observed an increase in $CD8^+CD25^+$ T cells expressing Foxp3 upon stimulation with α-CD3/α-CD28 only or α-CD3/α-CD28+TGF-β1 compared to media only. When compared to TGF-β1 only treated cells, an increased frequency of $CD8^+CD25^+$ $Foxp3^+$ T cells was observed in α-CD3/α-CD28+TGF-β1 stimulated cells (Figure 4.1E). Foxp3 expression did not differ in $CD8^+$ $CD25^-$ T cells across the treatment groups (Figure 4.1F). Although the Foxp3 induction in $CD8^+$ and $CD4^+CD25^+$ T cells was comparable in TCR stimulated (α-CD3/α-CD28) and α-CD3/α-CD28+TGF-β1 stimulated groups, the addition of TGF-β1 most likely enabled stable Foxp3 expression (225, 228).

Foxp3 binding to the IL-2, IFN-γ and TNF-α promoters is increased in α-CD3/α-CD28 + TGF-β1 stimulated CD8$^+$ T cells.

In the FIV infection model, we demonstrated that Foxp3 binds to the IL-2, IFN-γ and TNF-α promoter regions in virus-specific CD8$^+$ T cells. Therefore, we performed ChIP for Foxp3 binding to the IL-2, IFN-γ and TNF-α promoters in stimulated and unstimulated rhesus macaque CD8$^+$, CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. We detected an increase in Foxp3 binding to all 3 cytokine promoters in all the cell populations (Figures 4.2 A-I). Although the differences in Foxp3 binding to the cytokine promoters did not reach statistical significance between unstimulated and stimulated cells, the increase in Foxp3 binding was detected in every animal, suggesting biological relevance. To identify the most modulated cytokine within each cell type, we measured the relative increase in Foxp3 binding at each of the promoters in the three cell populations (Figures 4.2J-L). Interestingly, the most pronounced difference in Foxp3 binding in CD8$^+$ T cells was observed at the IL-2 promoter, followed by the TNF-α promoter and then the IFN-γ promoter (Figure 4.2J). The IL-2 promoter was the most susceptible to increased Foxp3 binding in CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^+$ T cells exhibited the highest increase in Foxp3 binding at the IFN-γ promoter across all cell populations whereas TNF-α promoter was enriched with Foxp3 the most in CD4$^+$CD25$^-$ T cells (Figures 4.2K-L). Collectively, these data suggest that the cell type and its role in an immune response likely drives the differential Foxp3 binding at the cytokine promoters.

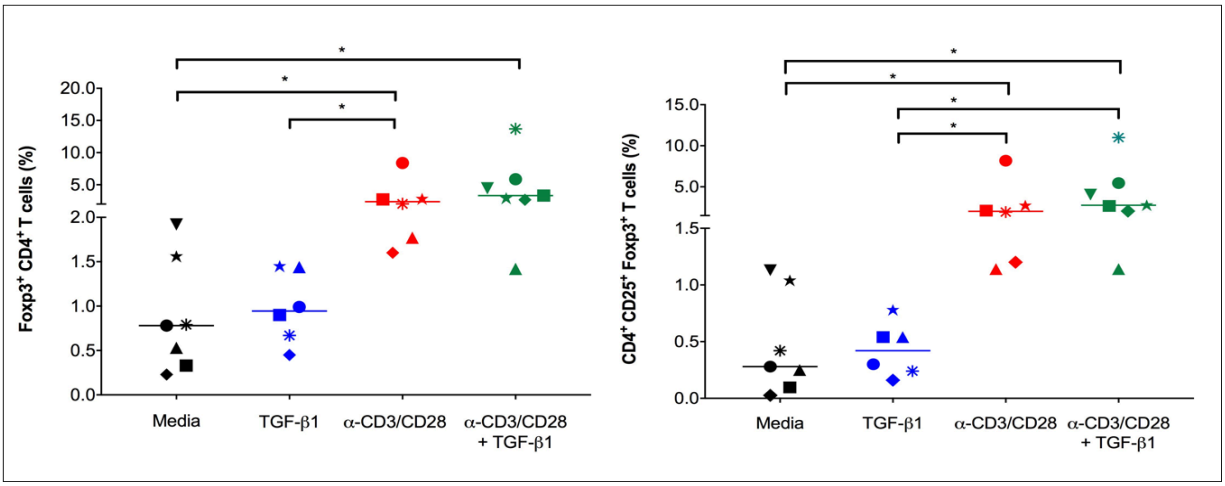
α-CD3/α-CD28+TGF-β1 stimulation suppresses DNMT1 and DNMT3b transcription in $CD8^+$ T cells.

In the FIV model, increased Foxp3-binding at the IL-2 promoter region was attributed to euchromatin conformation with reduced DNA methylation of lentivirus-activated $CD8^+$ T cells. Therefore, we tested the mRNA expression levels of 2 DNMTs: DNMT1, a constitutively expressed DNMT that maintains methylation after DNA replication, and DNMT3b, a DNMT induced upon cellular activation and responsible for *de novo* methylation of DNA (229-231). Stimulation with α-CD3/α-CD28+TGF-β1 resulted in reduced DNMT1 mRNA levels in $CD8^+$ T cells and in $CD4^+CD25^+$ T cells compared to media control cells (Figure 4.3A). In contrast, DNMT1 levels in $CD4^+CD25^-$ T cells remained elevated even after α-CD3/α-CD28+TGF-β1 stimulation (Figure 4.3A). A similar trend was observed for DNMT3b, with reduced mRNA levels in $CD8^+$ T cells and $CD4^+$ $CD25^+$ T cells, while mRNA levels were elevated in $CD4^+CD25^-$ T cells (Figure 4.3B). The elevated DNMT levels do not explain increased Foxp3 binding in $CD4^+CD25^-$ T cells and need further investigation.

Figure 4.1: Foxp3 expression is upregulated in α-CD3/α-CD28+TGF-β1 stimulated $CD8^+$ T cells *in vitro*.

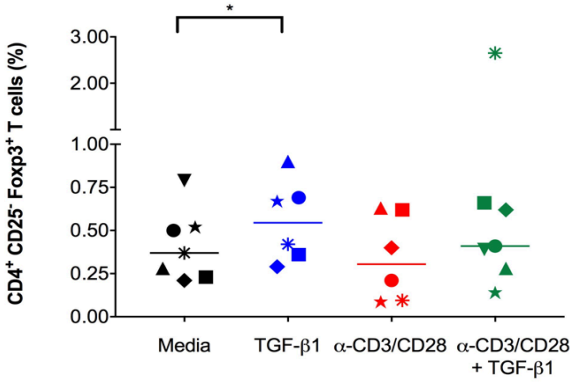
Donor rhesus macaque PBMCs were stimulated with media (negative control; black, n=7), 5ng/ml of TGF-β1 only (blue; n=6), 1 μg/ml α-CD3 and 2 μg/ml α-CD28 only (red; n=6), or stimulated with α-CD3/ α-CD28 + 5ng/ml TGF-β1 (green, n=7) for 5 days at 37ºC, 5%CO_2. Intracellular Foxp3 expression was assessed by gating on single, live, $CD3^+$ lymphocytes. Panels A-F illustrate the frequencies of $CD4^+$ $Foxp3^+$ T cells, $CD4^+$ $CD25^+$ $Foxp3^+$ T cells, $CD4^+$ $CD25^-$ $Foxp3^+$ T cells, $CD8^+$ $Foxp3^+$ T cells, $CD8^+$ $CD25^+$ $Foxp3^+$ T cells and $CD8^+$ $CD25^-$ $Foxp3^+$ T cells respectively for each treatment group. Individual animals are represented by the same specific symbol in each treatment condition. Data are represented as median with range. Wilcoxon matched- pairs signed rank test was used for statistical analysis with *$p<0.05$.

1A.

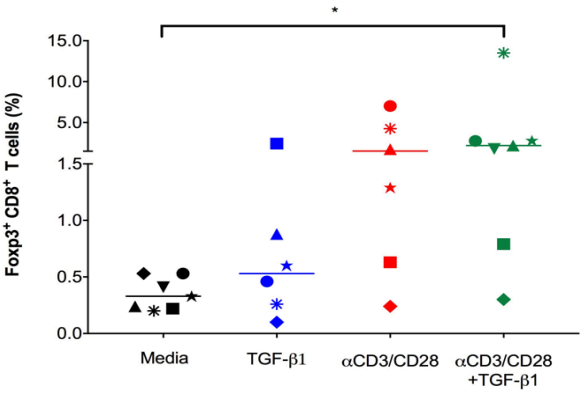


1B.

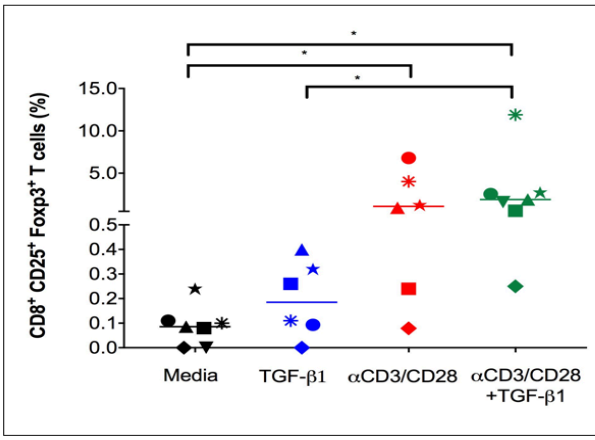
1C.



1D.



1E.



1F.

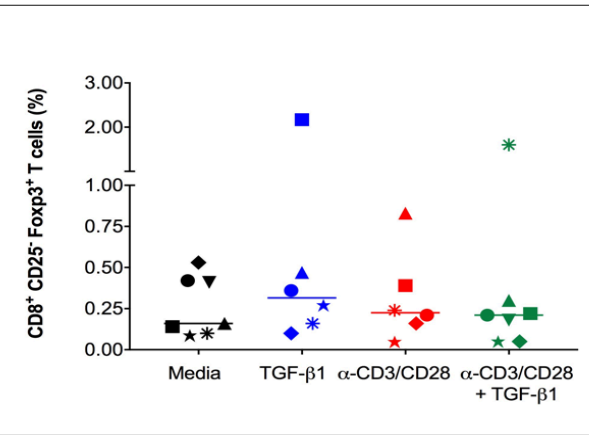


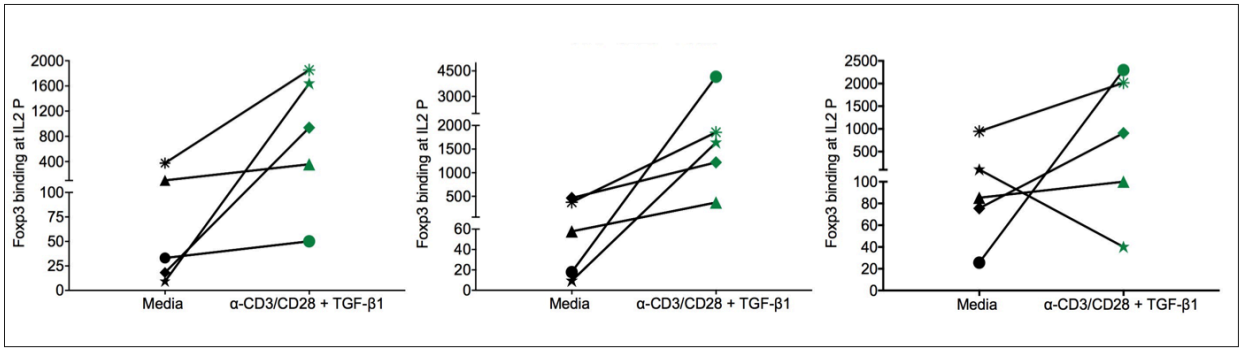
Figure 4.2: Foxp3 binding is increased to the IL-2, IFN-γ and TNF-α promoter in α-CD3/α-CD28 + TGF-β1 stimulated $CD8^+$ T cells *in vitro*.

ChIP for Foxp3 followed by qPCR for IL-2, IFN-γ and TNF-α promoter was performed on media treated (negative control; black, n=5) and α-CD3/α-CD28 + TGF-β1 stimulated (green; n=5) FACS sorted $CD8^+$, $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells. Panels 4.2 A-C, 4.2 D-F and 4.2 G-I illustrate Foxp3 binding to the IL-2, IFN-γ and TNF-α promoters respectively in each of the cell populations when treated with media only or stimulated. Panels 4.2 J-L report the increase in Foxp3 binding at each of the cytokine promoters in $CD8^+$, $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells respectively. Individual animals are represented by the same specific symbol in each treatment condition (Panels 4.2A-I). Each bar represents the mean + SD for 5 animals (Panels 4.2J-L). Wilcoxon matched- pairs signed rank test used for statistical analysis with ($p>0.05$ for all groups).

CD8⁺ T Cell	CD4⁺CD25⁺ T Cell	CD4⁺CD25⁻ T Cell

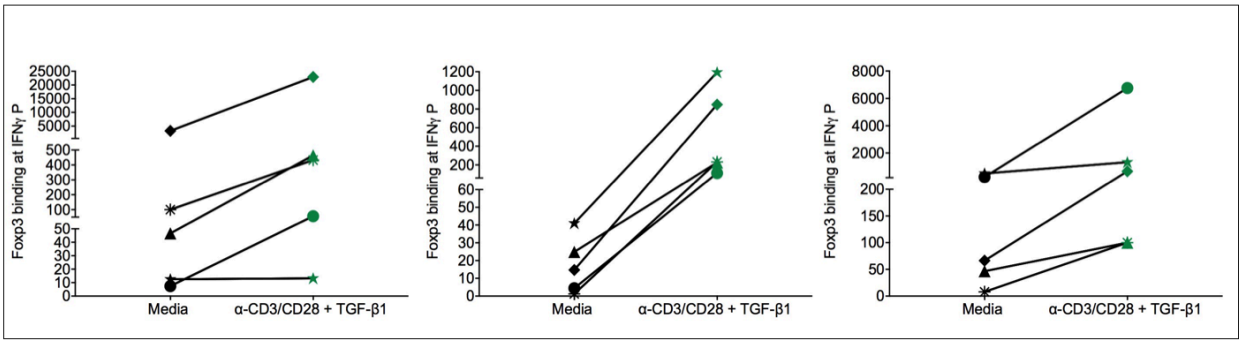
IL-2 Promoter

2A. 2B. 2C.



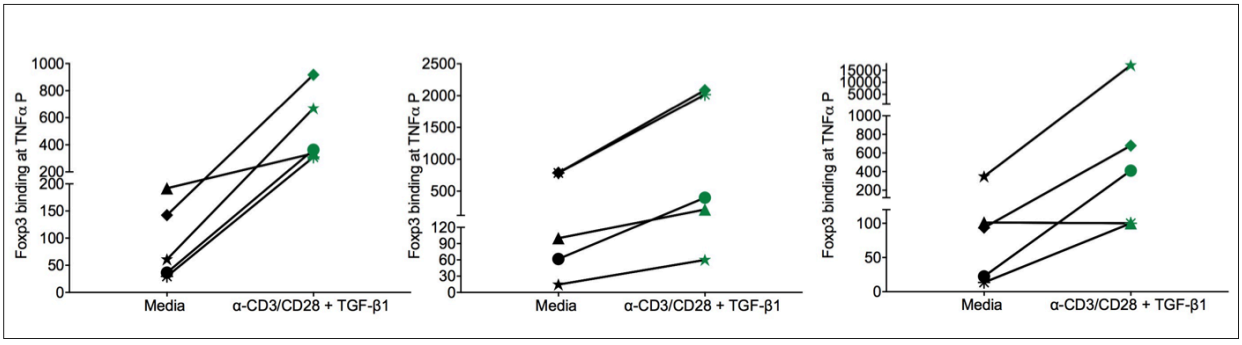
IFN-γ Promoter

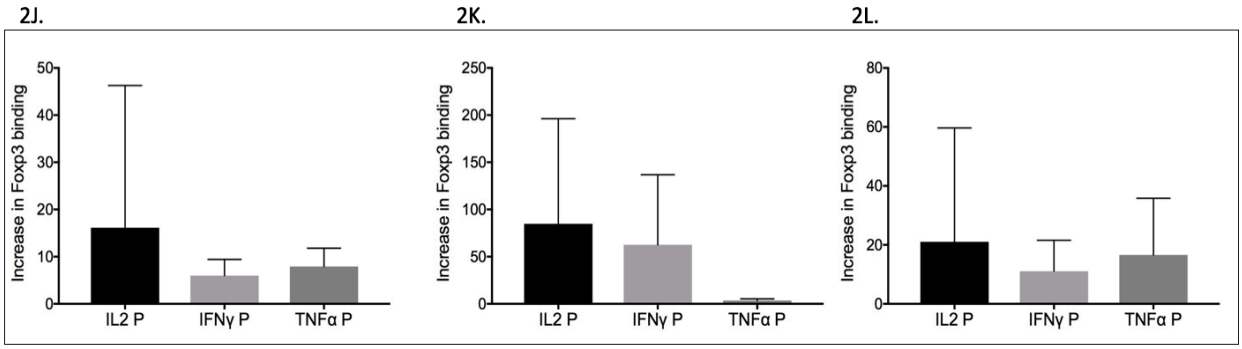
2D. 2E. 2F.



TNF-α Promoter

2G. 2H. 2I.





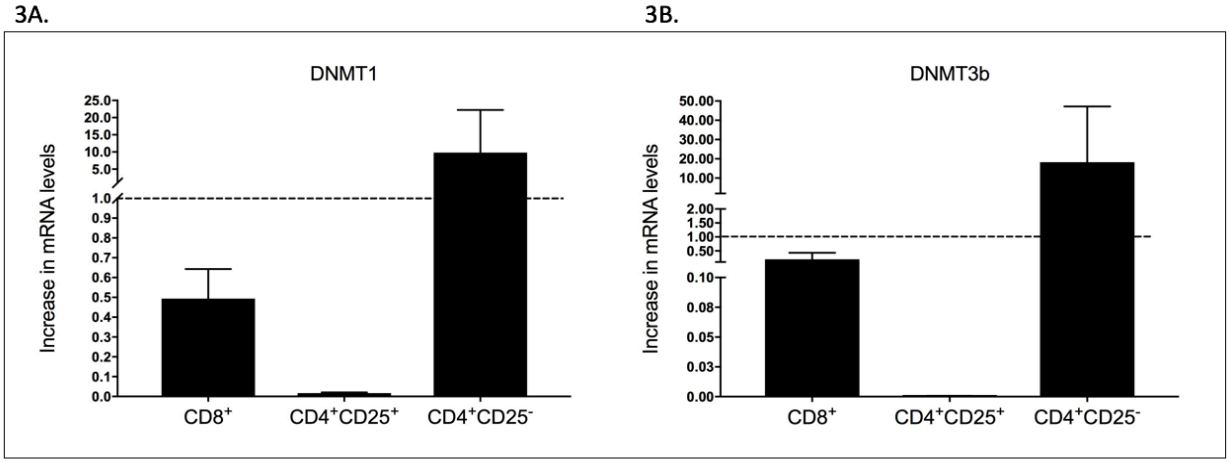


Figure 4.3: DNMT1 and DNMT3b mRNA expression is suppressed in α-CD3/α-CD28+TGF-β1 stimulated $CD8^+$ T cells *in vitro*.
Panels A and B report the increase in DNMT1 (Panel A) and DNMT3b (Panel B) in $CD8^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells stimulated with α-CD3/α-CD28+TGF-β1 compared to media controls. Each bar represents the mean + SD for 3 separate experiments. mRNA levels in media controls were assigned a value of "1" (dotted line); therefore values <1 indicate suppression and values>1 indicate induction.

Discussion

Using the FIV model, we have demonstrated that during lentivirus infection, Treg cells induce Foxp3 expression in $CD8^+$ T cells and that Foxp3 directly binds to the IL-2, IFN-γ and TNF-α promoters resulting in their suppression (115-118). The epigenetic remodeling of chromatin to a "relaxed" euchromatin form enables the binding of Foxp3 at antiviral cytokines in $CD8^+$ T cells from FIV-infected cats (117). This represents a novel Treg-induced Foxp3-mediated mechanism of $CD8^+$ T cell suppression in lentivirus infections. Our aim is to recapitulate our findings from the FIV model using the NHP model of SIV infection. As a first step in defining the role of Foxp3 in macaque $CD8^+$ T cell dysfunction, we investigated the molecular events occurring in $CD8^+$ T cells upon Foxp3 induction *in vitro*. We hypothesized that Foxp3 would bind to the IL-2, IFN-γ, and TNF-α promoters in rhesus macaque $CD8^+$ T cells stimulated with TGF-β1.

During the course of SIV infection, Treg cells exhibit progressive depletion (96, 97, 102). Therefore, for our study, we were unable to perform *ex vivo* Treg/$CD8^+$ T cell co-culture experiments to induce Foxp3 in $CD8^+$ T cells similar to the FIV study. Instead, we induced Foxp3 in $CD8^+$ T cells using α-CD3/α-CD28+TGF-β1 stimulation to address our hypothesis. In our *in vitro* cultures, we treated PBMCs isolated from donor rhesus macaques with α-CD3/α-CD28+TGF-β1 for 5 days to facilitate the induction of Foxp3. TCR (α-CD3/α-CD28) + TGF-β1 stimulation of T cells results in the induction of Foxp3 in $CD4^+CD25^-$ T cells converting them into iTreg cells (175, 225, 227). In Figure 4.1, we demonstrate intracellular Foxp3 expression in $CD8^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells stimulated with α-CD3/α-CD28+TGF-β1 or media only, TGF-β1 only and α-CD3/α-CD28 only as controls. The increased relative frequency of Foxp3 expressing $CD4^+$ and $CD4^+$ $CD25^+$ T cells in the α-CD3/α-CD28 only and α-CD3/α-

CD28+TGF-β1 treated groups compared to media only control confirmed the ability of these conditions to induce Foxp3 *in vitro* (Figure 4.1A and 4.1B). We demonstrated the induction of Foxp3 expression in $CD8^+$ and $CD8^+$ $CD25^+$ T cells upon stimulation with α-CD3/α-CD28+TGF-β1 (Figure 4.1D and 4.1E). The increased frequency of Foxp3 expressing $CD4^+$ $CD25^-$ T cells with TGF-β1 only treatment suggests that TGF-β1 treatment might modulate $CD4^+CD25^-$ T cells differently than $CD4^+CD25^+$ T cells. It is important to note that in our *in vitro* cultures, Foxp3 induction was most likely reinforced by the TGF-β secreted by iTreg cells in culture interacting with activated $CD8^+$ T cells. As described above, stimulation with α-CD3/α-CD28 only could induce comparable Foxp3 producing cells to α-CD3/α-CD28 + TGF-β1 group, however the addition of TGF-β1 ensured stable Foxp3 expression by enabling Smad3 binding to the TGF-β sensor (232). Overall, the Foxp3 expression results indicate that stimulation with α-CD3/α-CD28+TGF-β1 could induce Foxp3 expression in $CD8^+$ T cells.

In the FIV model, we demonstrated the direct binding of Foxp3 to IL-2, IFN-γ and TNF-α promoters in virus-specific $CD8^+$ T cells induced to express Foxp3 by Treg co-culture (118). Therefore, we performed ChIP followed by qPCR to measure Foxp3 binding at the IL-2, IFN-γ and TNF-α promoters in α-CD3/α-CD28+TGF-β1 stimulated macaque $CD8^+$ T cells. We used Foxp3 binding at $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells as controls. Consistent with our feline data, we demonstrated increased Foxp3 binding α-CD3/α-CD28 + TGF-β1 stimulation compared to media only control at the various cytokine promoters in all the cell populations (Figure 4.2A-I). It was interesting to observe that there was enhanced Foxp3 binding to the cytokine promoters in $CD4^+CD25^-$ T cells even though there was no Foxp3 induction observed after stimulation of these cells. Although the difference in Foxp3 binding before and after

stimulation was not statistically significant, the consistent increase in Foxp3 binding at the cytokine promoters in $CD8^+$ and $CD4^+CD25^+$ T cells in each animal suggested biological relevance. These data represent the first step in understanding the molecular events, specifically Foxp3 binding at the antiviral cytokine promoters in activated macaque $CD8^+$ T cells.

Next, from the ChIP data, we determined that Foxp3 binding data also indicated that the cell type might drive the differential enrichment of Foxp3 at each of these cytokine promoters. It is known that activated $CD4^+$ T and $CD8^+$ T cells are the main producers of IL-2 during viral infections. The development and strength of antiviral response of virus-specific $CD8^+$ T cells is controlled, in part by IL-2 in the milieu (reviewed in Cox et al., 2013 (233)). IL-2 is also indispensable for the activation and suppressive role of $CD4^+$ $CD25^+$ Treg cells during HIV infection (234, 235). Given the important role of IL-2 in the expansion and strength of anti-viral response, it was intriguing to observe the highest increase in Foxp3 binding at the IL-2 promoter in $CD8^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells (Figure 4.2J-L). The IFN-γ promoter exhibited the maximum increase in Foxp3 binding in the $CD4^+CD25^+$ T cells (Figure 4.2K). IFN-γ is critical in the induction and expansion of Treg cells (236). The TNF-α promoter was most modulated by Foxp3 in $CD4^+CD25^-$ T cells (Figure 4.2L). These data collectively suggested that Foxp3 binding at the cytokine promoters required for their function might be a negative feedback mechanism to control immune activation, further lending support to Treg-mediated homeostasis of the immune response. However, to establish the biological relevance of Foxp3 binding at these cytokine promoters, the transcription levels of these cytokines post stimulation should be quantified.

We demonstrated that de-methylated "relaxed" DNA enabled the binding of Foxp3 in $CD8^+$ T cells isolated from FIV-infected cats. Therefore, we tested the changes in chromatin accessibility of the macaque $CD8^+$ T cells after treatment with α-CD3/α-CD28 + TGF-β1. Activated cells exhibit lower DNA methylation compared to transcriptionally inactive cells. DNMT 1 and DNMT 3b are the enzymes responsible for maintaining and adding methyl groups (230, 231). Suppression of DNMT1 has been reported in HIV-infected patients with detectable viremia compared to HIV-infected patients with undetectable viremia and uninfected patients (155, 237). Lower protein levels of DNMT1 and 3b in HIV –infected patients compared to uninfected have also been reported (238). These studies indicate the overall de-methylation of DNA in activated cells during lentivirus infections. Therefore, the expression of DNMT 1 and DNMT 3b was examined in the stimulated and unstimulated sorted $CD8^+$ T cells along with $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells. Suppression of DNMT1 and DNMT3b in $CD8^+$ and $CD4^+CD25^+$ T cells suggested the increased chromatin accessibility post α-CD3/α-CD28 + TGF-β1 stimulation (Figure 4.3A-B). Consistent with the FIV model, these data suggest that the euchromatin conformation in activated $CD8^+$ T cells likely enables the binding of Foxp3. This study indicates the role of epigenetic modulation upon activation in Foxp3 binding to cytokine promoters in $CD8^+$ T cells. To further characterize the epigenetic remodeling of activated $CD8^+$ T cells, we are currently investigating SIV-induced epigenetic signatures in activated $CD8^+$ T cells.

Overall, the results of this study clearly demonstrate the α-CD3/α-CD28 + TGF-β1 –mediated induction of Foxp3 and the direct binding of Foxp3 to IL-2, IFN-γ and TNF-α promoters in epigenetically modulated activated $CD8^+$ T cells in the NHP model. This study represents the

first step in investigating Treg-induced Foxp3-binding as a mechanism of $CD8^+$ T cell dysfunction in the NHP model.

CHAPTER 5

Summary, Conclusions and Future Directions

Summary and conclusions

$CD8^+$ T cells are instrumental in controlling HIV, SIV and FIV lentivirus infections (4, 211, 239). Efficient viral control during the acute phase of infection by $CD8^+$ T cells is attributed to their cytolytic response and polyfunctionality, i.e. their ability to produce antiviral cytokines such as IFN-γ, TNF-α and IL-2 (7). However, despite the robust $CD8^+$ T cell response, lentiviruses escape immune recognition and set up viral reservoirs that persist for years. $CD8^+$ T cells progressively lose their proliferative capacity and function due to persistent antigenic stimulation resulting in the accumulation of dysfunctional $CD8^+$ T cells that are unable to eliminate infected cells (8). Current HIV cure strategies are geared towards reactivating the latently infected cells. However, upon reactivation, these infected cells cannot be cleared by the large pool of dysfunctional $CD8^+$ T cells. Therefore, a multi-pronged approach, including both the reactivation of latently infected cells and restoring $CD8^+$ T cell function, needs to be an essential part of the HIV cure strategy. In this dissertation, we focus on advancing our understanding of $CD8^+$ T cell dysfunction using the FIV and the SIV models of HIV infection. Our aim is to identify targets that may be manipulated to rescue $CD8^+$ T cell function during chronic stages of lentiviral infections.

Different mechanisms have been studied to explain $CD8^+$ T cell dysfunction. Dysfunctional $CD8^+$ T cells upregulate inhibitory surface markers such as PD-1, CTLA-4, Tim-3, TIGIT, Lag-3 and 2B4.The upregulation of these markers negatively impacts $CD8^+$ T cell proliferation and

function (18, 240). T regulatory cells, important for maintaining a balance during an immune response, represent another mechanism of dampening $CD8^+$ T cell function. The studies presented here are focused upon the $CD4^+CD25^+$ $Foxp3^+$ T regulatory cell-mediated mechanisms that induce $CD8^+$ T cell dysfunction.

Our group has extensively studied the interplay between Treg cells and effector $CD4^+$ and $CD8^+$ T cells. Using the FIV model, we have previously reported that lentivirus activated Treg cells induce Foxp3 expression in activated $CD8^+$ T cells (115). This induction occurs due to the ligation of membrane bound TGF-β present on activated Treg cells to the TGF-βRII expressed on $CD8^+$ T cells (115, 116). Induced Foxp3 binds to the IL-2 promoter in $CD8^+$ T cells after co-culture with autologous Treg cells resulting in its suppression (117). Based on these studies in the FIV model, we hypothesized that Treg-induced Foxp3 contributes to the progressive loss of $CD8^+$ T cell function (Chapter 2). In Figure 2.1 of Chapter 2, we describe a novel method to identify virus-specific $CD8^+$ T cells in the FIV model. *Ex vivo* $CD8^+$ T cells were stained with CFSE, a cell proliferation dye, combined with the rest of the cell suspension and cultured with FIV to facilitate $CD8^+$ T cell response to the virus. The actively proliferating $CD8^+$ T cells with attenuated CFSE signal were identified as virus-specific $CD8^+$ T cells. The results shown in Figure 2.2 demonstrated the progressive suppression of IL-2, IFN-γ and TNF-α in virus-specific $CD8^+$ T cells post Treg co-culture. Loss of function was detected as early as 4 weeks post infection consistent with the reports in the LCMV model (173). Increased transcription of Foxp3 was demonstrated in Figure 2.3 in virus-specific $CD8^+$ T cells 1 week, 4 weeks and 8 weeks post infection after Treg co-culture. Next, Foxp3 binding at these cytokine promoters was demonstrated in virus-specific $CD8^+$ T cells 8 weeks and 6 months post-infection after co-culture

with Treg cells (Figure 2.4.). $CD8^+$ T cells ($CFSE^{Hi}$) from FIV-uninfected cats did not exhibit Foxp3 binding at the cytokine promoters after Treg co-culture. These data in Chapter 2 describe the role of Treg-induced Foxp3-mediated suppression of virus-specific $CD8^+$ T cells in FIV-infected cats. This suggests a novel mechanism explaining the suppressive action of Treg cells. In Chapter 4, we recapitulated the above findings in the rhesus macaque NHP model to further investigate the mechanism of Foxp3-mediated Treg suppression of $CD8^+$ T cells. Due to low numbers of natural Treg cells in SIV-infected macaques, we were unable set up $CD8^+$/ autologous Treg cell co-culture experiments to assess the induction and binding of Foxp3 in $CD8^+$ T cells. Therefore, we induced Foxp3 expression in $CD8^+$ T cells *in vitro* with anti-CD3/anti-CD28+TGF-β1 stimulation to investigate Foxp3 binding at cytokine promoters. We hypothesized that Foxp3 binds to the IL-2, IFN-γ and TNF-α promoters in rhesus macaque $CD8^+$ T cells stimulated with TGF-β1. Figure 4.1 demonstrated the induction of intracellular Foxp3 protein expression in total $CD8^+$ T cells post stimulation. Figure 4.2 and 4.3 clearly demonstrated that the stimulated $CD8^+$ T cells exhibit a transcriptionally activated state allowing direct Foxp3 binding at the IL-2, IFN-γ, and TNF-α promoters post stimulation. Collectively, the data in Chapter 2 and Chapter 4 suggest the important role of Treg-induced Foxp3 in the suppression of $CD8^+$ T cells during lentiviral infections. The *in vitro* results in the NHP model indicate the translational relevance of this mechanism.

Next, we investigated the molecular events occurring at these cytokine promoters enabling the binding of Foxp3 and hence the suppression of IL-2 in $CD8^+$ T cells responding to lentiviral infections. Epigenetic modifications such as DNA de-methylation and histone acetylation at the IL-2 promoter during HIV and other chronic viral infections such as HBV and HCV have been

reported (136, 138). Previously, a study by our group reported that chronic infection during FIV results in chromatin remodeling at the IL-2 promoter, specifically de-methylation of the distal IL-2 promoter region. The same study also demonstrated that, blocking DNA de-methylation using epigenetic modulators could reduce Foxp3 binding at the IL-2 promoter (117). Therefore, this study reported that DNA de-methylation in activated $CD8^+$ T cells, while required for mounting an antiviral response, make the cells susceptible to Foxp3 binding resulting in their dysfunction. This led us to investigate the influence of another mechanism of epigenetic control, i.e. histone acetylation on the Foxp3-mediated suppression of $CD8^+$ T cells using the FIV model. In Chapter 3 we hypothesized that decreasing histone acetylation in virus-specific $CD8^+$ T cells would decrease Treg-induced Foxp3 binding to the IL-2 promoter. In Figure 3.1 we demonstrated that Anacardic Acid (AA) functions as a HAT inhibitor by promoting histone de-acetylation *in vitro.* By promoting histone de-acetylation, Foxp3 binding to the IL-2 promoter is blocked resulting in a concurrent increase in IL-2 transcription *in vitro* (Figure 3.2). In Figure 3.3, we tested the importance of histone acetylation in virus-specific $CD8^+$ T cells from FIV-infected cats. We demonstrated a reduction in Foxp3 binding to the IL-2 promoter upon AA treatment of virus-specific $CD8^+$ T cells. Therefore, from the data presented in Chapter 3, we concluded that histone modulation blocks Treg-induced Foxp3 binding to the IL-2 promoter of virus-specific $CD8^+$ T cells from FIV-infected cats. The results from Chapter 3 and the study performed by Miller et al. suggest that blocking DNA de-methylation and histone acetylation using epigenetic modulators reduce Foxp3 binding at the IL-2 promoter (117).

Taken together, the data in Chapters 2,3, and 4 enhance our understanding of Treg-mediated suppression of $CD8^+$ T cells in lentivirus infections using the FIV and the SIV models. These

studies determine the role of Foxp3 in Treg mediated suppression of $CD8^+$ T cells responding to lentivirus infections. TGF-β-induced Foxp3 binding at the IL-2, IFN-γ and TNF-α promoters of activated macaque $CD8^+$ T cells *in vitro* suggests the relevance of this mechanism of $CD8^+$ T cell dysfunction in HIV infection. Epigenetic rearrangements upon activation at these essential antiviral promoters enable the binding of Foxp3, hence explain Foxp3-mediated suppression of $CD8^+$ T cells. These studies help resolve one of the paradoxes of lentiviral infection: why virus-specific $CD8^+$ T cells exhibiting an activated phenotype often also exhibit progressive antiviral immune dysfunction. Our data also suggest that the reduction in Foxp3-mediated suppression of the antiviral cytokine promoters by epigenetic modulation, might result in enhanced cytokine expression in dysfunctional $CD8^+$ T cells, or restore function upon subsequent stimulation. Figure 5.1 summarizes the overall proposed model.

Several strategies to enhance $CD8^+$ T cell function during chronic HIV infection have been investigated. Immune checkpoint inhibitors to block inhibitory surface markers used in combination with stimulatory cytokines such as IL-15 and IL-2 have shown promising results in boosting $CD8^+$ T cell responses (156, 159). Immunotherapy using chimeric antigen receptor (CAR) T cells engineered to eliminate HIV infected T cells is being explored (163). Epigenetic studies presented here and reported by others using HDAC inhibitors clearly demonstrate their potential to rescue $CD8^+$ T cell function (154, 164). Integrating methods to restore $CD8^+$ T cell function with reactivating latent HIV-infected cells is critical to develop a cure for HIV. We believe that epigenetic modulation of dysfunctional $CD8^+$ T cells is likely part of the strategy to generate a pool of functionally competent, HIV-specific $CD8^+$ T cells. Adoptive transfer of these competent $CD8^+$ T cells has the potential to effectively eliminate reactivated HIV-infected cells,

while ART can prevent new cells from getting infected. This strategy represents an integrated approach to cure HIV.

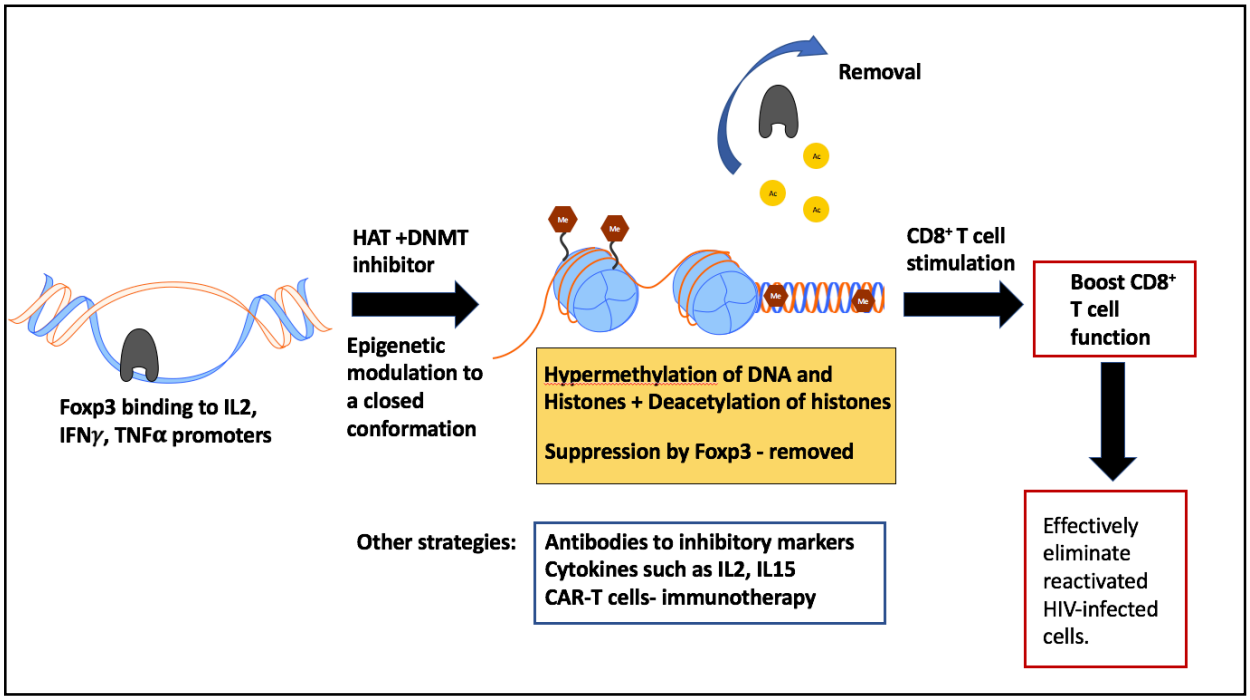


Figure 5.1: Proposed mechanism of epigenetic modulation to boost antiviral function of $CD8^+$ T cells.
In the FIV model, Treg-induced Foxp3 suppresses $CD8^+$ T cell function by directly binding to the IL-2, IFN-γ and TNF-α promoter regions. We have demonstrated that by blocking DNA de-methylation and histone acetylation using DNMT and HAT inhibitors respectively, Foxp3 binding to the IL-2 promoter is reduced. These data suggest that epigenetic modulations of dysfunctional $CD8^+$ T cells can alleviate Foxp3-mediated suppression. We propose that these rescued $CD8^+$ T cells, when stimulated, can restore their antiviral function and effectively eliminate reactivated virally infected cells.

Limitations

There were several limitations to the studies presented in Chapters 2,3 and 4. The mechanism of Treg-mediated induction of Foxp3 in $CD8^+$ T cells is mostly likely applicable to any chronic viral infection accompanied by the expansion and activation of Treg cells. To identify FIV-

specific events resulting from the induction of Foxp3 in $CD8^+$ T cells, we demonstrate Foxp3 binding to antiviral cytokine promoters in virus specific $CD8^+$ T cells from FIV-infected cats and not in the uninfected cats. However, we did not assess Foxp3 binding during other chronic viral infections. Polyfunctionality after Foxp3-mediated suppression and the differentiation status of virus-specific $CD8^+$ T cells was not measured due to limited tissue and cell recovery. However, attempts were made to phenotypically characterize the virus-specific $CD8^+$ T cells from FIV-infected cats (Appendix, Figure A.8). We next reported Foxp3- binding at the antiviral cytokine promoters in the NHP model shown in Chapter 4. However, we did not assess the consequence of Foxp3 binding at the promoters by measuring the cytokine levels. Based on our findings in the FIV model, we expect a suppression of these cytokines after Foxp3 binding to their promoters. To elucidate the mechanism of Foxp3 binding at the antiviral cytokine promoters, we explored the epigenetic modulation of $CD8^+$ T cells responding to lentiviral infections. The results are shown in Chapter 3. Although reduced Foxp3 binding at the IL-2 promoter after blocking histone acetylation was reported in virus (FIV)-specific $CD8^+$ T cells, these data did not reach statistical significance. Moreover, the "off target" effects of AA were not tested in our epigenetic studies. We were unable to report the modulation of *in vivo* $CD8^+$ T cell by lentivirus infection itself due to limited subjects and low cell recovery. Results of testing the AcH3 levels at the IL-2 promoter in virus-specific $CD8^+$ T cells from a few FIV infected cats demonstrated a trend towards transcriptional activation (Appendix, Figure A.1). Despite these limitations, the results presented here explain, in part, the mechanism underlying $CD8^+$ T cell dysfunction during lentiviral infections.

Future Directions

Treg-induced Foxp3-binding to the antiviral cytokine promoters enriched with acetylated histones in activated $CD8^+$ T cells represents a novel mechanism of $CD8^+$ T cell dysfunction during lentiviral infections. The studies presented in this dissertation necessitate further investigation to establish the role of Foxp3 and epigenetic modulation in lentivirus-associated $CD8^+$ T cell dysfunction.

To conclusively determine the role of Foxp3 in Treg-mediated $CD8^+$ T cell suppression, *in vitro* and *ex vivo* Foxp3 depletion studies in $CD8^+$/ Treg co-cultures need to be done in the FIV model. Foxp3 binding to cytokine promoters needs to be evaluated in other chronic viral infections or parasitic infections that promote Treg activation. These studies would determine the specificity of Foxp3 in mediating $CD8^+$ T cell dysfunction in the FIV model. The *in vitro* studies performed in the NHP model suggest the translational relevance of Foxp3-mediated $CD8^+$ T cell dysfunction. However, to validate our findings in the NHP model, *ex vivo* studies demonstrating the induction and binding of Foxp3 to antiviral cytokine promoters need to be tested in $CD8^+$ T cells isolated from SIV-infected macaques. Performing Foxp3 depletion studies *ex vivo*, followed by functional analysis of these $CD8^+$ T cells will definitively identify the role of Foxp3 in FIV and SIV infections. These follow up studies would determine the potential of Foxp3-mediated $CD8^+$ T cell suppression in HIV-infection.

The studies presented here also identify the importance of epigenetic modulation, specifically histone acetylation of $CD8^+$ T cells enabling Foxp3 binding during lentivirus infections. We use a HAT inhibitor, AA to block histone acetylation and demonstrate reduction in Foxp3 binding *in*

vitro. Ex vivo studies with AA demonstrating reduced Foxp3 binding at the antiviral cytokine promoters and a reciprocal increase in the cytokines need to be performed to determine the biological relevance of histone acetylation in modulating lentiviral response. To expand our understanding of the modulation of $CD8^+$ T cell response during lentivirus infection, differences in epigenetic signatures between uninfected and FIV- infected $CD8^+$ T cells need to be described. Differential enrichment of specific activation marks such as H3K4me3, H3K9ac need to be measured at the promoters of antiviral cytokine genes such as IFN-γ, TNF-α and granzyme B to indicate transcriptional activation during lentiviral infection. We are addressing the differences in epigenetic modifications in the SIV-infection model by identifying the differentially methylated regions within the antiviral cytokine promoters in $CD8^+$ T cells isolated from uninfected and infected macaques. Tissues from different anatomical locations are being tested to identify SIV-induced tissue specific epigenetic signatures. Collectively, these follow up studies will further characterize the epigenetic landscape of $CD8^+$ T cells induced by lentiviral infections. These studies will also help in the identification of potential therapeutic targets that can be modulated to restore $CD8^+$ T cell function.

Apart from the above mentioned follow up studies, several questions need to be addressed to fully understand the role of epigenetic modulations in lentivirus pathogenesis. Some of the open questions are- How early do these modifications appear during the course of infection? How informative will studies in animal models be? Are the epigenetic modifications in FIV, SIV and HIV infected cells conserved? Are there any differences in epigenetic signatures across $CD8^+$ T cells with distinct differentiation states? Are there differences in epigenetic signatures across different age groups, in HIV-infected people of different gender, or people in different

geographic locations? How do virus-specific and/or host-specific factors influence epigenetic changes? Many other confounding factors, such as co-infection, are likely to alter the epigenetic landscape, too. Our understanding of epigenetic changes in $CD8^+$ T cells is very limited. Specifically, the knowledge of epigenetic signatures that are unique to $CD8^+$ T cells remains relatively unknown. Therefore, we need to identify differences in the epigenetic landscape of $CD8^+$ T cells in acute and chronic lentiviral infection. Only then can we identify specific epigenetic targets that can be modulated to improve the cytokine production and cytolytic function of dysfunctional $CD8^+$ T cells during HIV infection.

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APPENDIX

The appendix comprises of all the *in vitro* and *ex vivo* studies performed in the FIV model that were not a part of the manuscripts.

Introduction

Epigenetic modulation of the antiviral cytokine promoters enables the binding of Foxp3 resulting in Foxp3-mediated dysfunction in virus specific $CD8^+$ T cells (117, 118). Using the FIV model, we have reported that Treg- induced Foxp3 binds to the promoter regions of IL-2, IFN-γ and TNF-α in activated virus-specific $CD8^+$ T cells (117, 118). Foxp3 binding at the promoter regions is possible because of a "relaxed" euchromatin conformation, with the DNA being de-methylated (117). In Chapter 3, we demonstrated a reduction in the acetylated histone 3 (AcH3) levels at the IL-2 promoter *in vitro* upon treatment with a HAT inhibitor, AA. We also demonstrated that histone acetylation most likely modulates Foxp3 binding at the IL-2 promoter *in vivo* during lentiviral infections (Chapter 3). To further understand the epigenetic modifications induced by lentivirus infections *in vivo*, we investigated the activation status of virus-specific $CD8^+$ T cells by measuring the levels of AcH3 at the IL-2 promoter in FIV-infected cats (Figure A.1). We have previously reported that blocking DNA de-methylation reduces Foxp3 binding at the IL-2 promoter in mitogen-activated $CD8^+$ T cells isolated from FIV-infected cats (117). We also reported that blocking histone acetylation prevents Foxp3 from binding at the IL-2 promoter in virus-specific $CD8^+$ T cells *in vitro* (Chapter 3, Figure 3.4). Collectively, these data suggest that epigenetic modulation at the IL-2 promoter can alleviate Foxp3-mediated suppression in mitogen-activated and virus specific $CD8^+$ T cells *in vitro*. Therefore, to establish the biological relevance of our *in vitro* findings, we determined if the reduction in Foxp3 binding at the IL-2 promoter translates into increased transcript levels of IL-2

in $CD8^+$ T cells isolated from FIV-infected cats (Figure A.2). Overall, our *ex vivo* data suggested that histone acetylation may, in part, explain Treg-induced Foxp3-mediated suppression of $CD8^+$ T cells during FIV infection, however, more *ex vivo* studies need to be performed to confirm the mechanism.

Polyfunctionality of $CD8^+$ T cells is important to mount a robust anti-viral response to lentiviral infections. Activated effector $CD8^+$ T cells produce large amounts of IFNγ and TNFα during viral infections in addition to IL-2 (8, 241-243). Dysfunctional $CD8^+$ T cells progressively lose their ability to produce these cytokines during FIV/SIV/HIV infections (8, 9). Here, we expand our understanding of the Treg- mediated suppression of these cytokines. As the first step, we demonstrated that Foxp3 binds to the promoter regions of IFN-γ and TNF-α in activated virus-specific $CD8^+$ T cells (118). Next, we investigated if the suppression of IFN-γ and TNF-α can be attributed to epigenetic modulations at their promoters similar to the IL-2 promoter. Previous studies reporting de-methylation and increased AcH3 levels post stimulation at the IFN-γ promoter; and increased histone acetylation at the TNF-α locus resulting in increased transcription provided the rationale for investigating epigenetic control of these cytokines during lentiviral infections (204, 244-246). Therefore, based on our findings at the IL-2 promoter, we hypothesized that blocking acetylation *in vitro* and *ex vivo* would prevent Foxp3 binding at the IFN-γ and TNF-α promoter regions. In Figures A.3- A.6 we provide preliminary data suggesting that blocking histone acetylation at the IFN-γ and TNF-α promoter regions *in vitro* and *ex vivo* may prevent Foxp3-mediated suppression of these cytokines.

Our epigenetic data indicated that the $CD8^+$ T cells responding to FIV infection exhibit an open chromatin conformation indicative of transcriptional activation. We rationalized that these events occur due to the activation of effector $CD8^+$ T cells. Therefore, we investigated whether $CD8^+$ T cells from FIV-infected cats exhibit an activated phenotype compared to uninfected control cats. We assessed the surface expression of CD25, TGF-βRII, CD27, CD45RA and CD62L. All these surface markers are known to be upregulated in effector $CD8^+$ T cells, whereas CD62L, which mediates the adhesion of lymphocytes to areas of high antigen-concentration in lymph nodes, is downregulated upon activation (116, 247-250). We evaluated whether there were any differences between the phenotype in $CD8^+$ T cells derived from peripheral blood and lymph node (Figure A.6-A.7). Importantly, we reported the phenotypic profile of virus-specific $CD8^+$ T cells responding to FIV infection (Figure A.8). None of the differences in surface marker expression were statistically significant. These phenotype studies, however, necessitate further investigation in phenotypically characterizing the lentivirus-responding $CD8^+$ T cells.

The studies presented in this dissertation confirmed the function of AA as a HAT inhibitor in the FIV model *in vitro* and *ex vivo* (Chapter 3). Here, we addressed other mechanisms of action of AA that might contribute to alleviating Foxp3-mediated suppression of antiviral cytokines using the FIV model. One of the other mechanisms of AA is the activation of Aurora kinase A (ARK)-mediated phosphorylation of histone H3 *in vitro* (210). These studies suggest that AA functions via multiple mechanisms towards promoting "closed" DNA conformation. Heterochromatin or "closed" DNA is typically characterized by hyper-methylated DNA and the presence of acetylated histones. This led us to investigate the effects of AA on DNA methylation. We hypothesized that AA inhibits DNA de-methylation, thereby preventing Treg-induced Foxp3

binding to the IL-2, IFN-γ and TNF-α promoters in $CD8^+$ T cells. However, with our data in Figures A.9-A.11 we reported that AA does not the change the global or promoter specific methylation of $CD8^+$ T cells *in vitro* and *ex vivo* in $CD8^+$ T cells. On the contrary, the promoter specific methylation data indicated that AA might decrease DNA methylation promoting active transcription.

Results

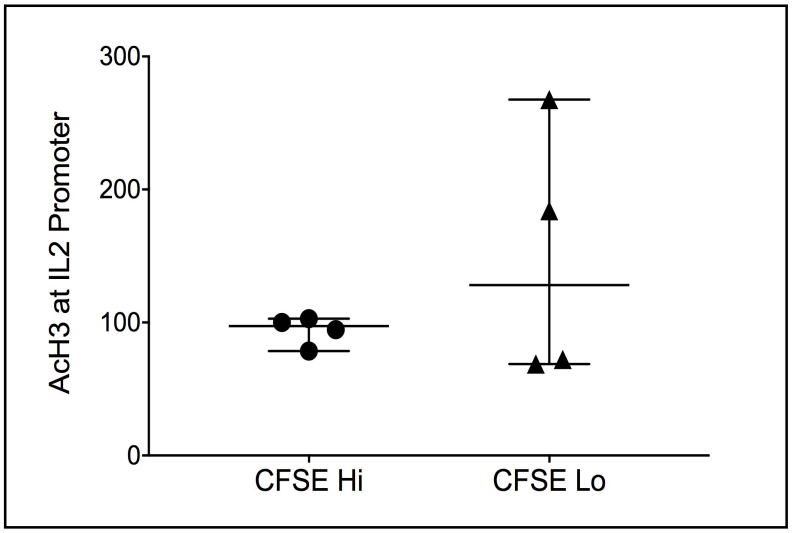


Figure A.1: Histone 3 acetylation at the IL-2 promoter in virus-specific $CD8^+$ T cells from FIV+ cats.
$CFSE^{hi}$ and $CFSE^{int/lo}$ $CD8^+$ T cells were isolated as described in the methods section in Chapter 3 from uninfected control cats and 8wk p.i FIV-infected cats respectively. Acetylated histone 3 (AcH3) ChIP followed by qPCR for the IL-2 promoter was performed for each treatment group. The data demonstrated a trend towards higher AcH3 at the IL-2 promoter in virus-specific ($CFSE^{int/lo}$) compared to virus-nonspecific ($CFSE^{hi}$) $CD8^+$ T cells at the IL-2 promoter indicative of transcriptional activation at the IL-2 promoter due to lentivirus infection. The difference, however, was not statistically significant, most likely due to the small sample size. (n=4 animals per group, $p>0.05$, Mann Whitney test used for statistical analysis. Data represented as median with range). IL-2 promoter primers listed in Chapter 3, Table 3.1.

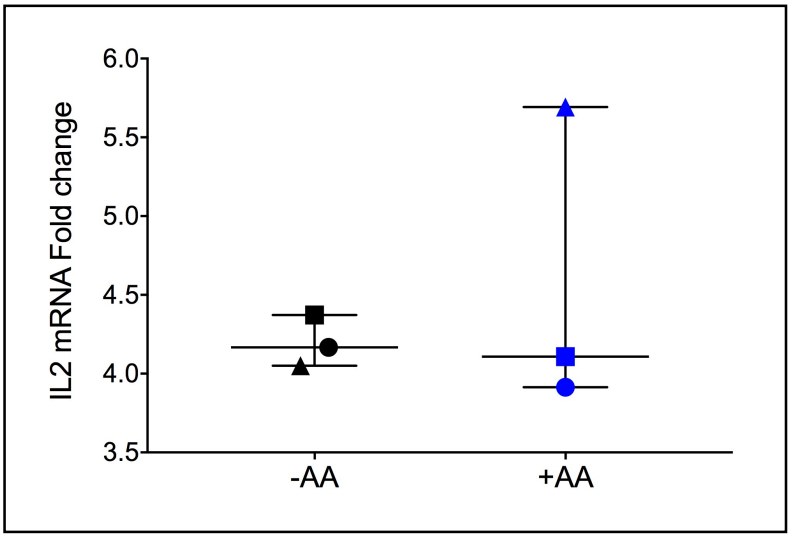


Figure A.2: AA does not affect the IL-2 mRNA levels in bulk CD8$^+$ T cells from FIV+ cats. Sorted bulk CD8$^+$ T cells (method described below) from chronically FIV-infected cats were either untreated (black) or treated with 20 µM AA (blue) for 24 hrs and assessed by RT-qPCR using primers listed in Chapter 3 (Table 3.2). IL-2 mRNA levels were not affected by AA treatment. The caveats of the study were that we only had 3 animals per group and that we used bulk CD8$^+$ T cells instead of FIV-specific CD8$^+$ T cells. (n=3 animals per group, $p>0.05$, Wilcoxon matched- pairs signed rank test used for statistical analysis. Each symbol represents individual animal. Data represented as median with range. Fold change data is reported relative to IL-2 mRNA levels in feline PBMCs).

Methods:

Bulk CD8$^+$ T cell isolation using EasySep® PE Selection Cocktail- Single cells from LNs of FIV+ and FIV – cats were suspended at a concentration of 1×10^8 cells/ml in HBSS (Thermo Fisher, Waltham, MA) with 2% FBS and stained with anti-feline CD8 PE antibody (clone 3.357) at 4ºC for 30mins. EasySep® PE Selection Cocktail was added at 100µL/mL of cell suspension at RT for 15mins, then EasySep® Magnetic Nanoparticles were added at 50µL/mL at RT for 10mins. CD8+PE+ cells were separated by using the magnet provided in the Kit (Stem Cell, Vancouver, BC, Canada).

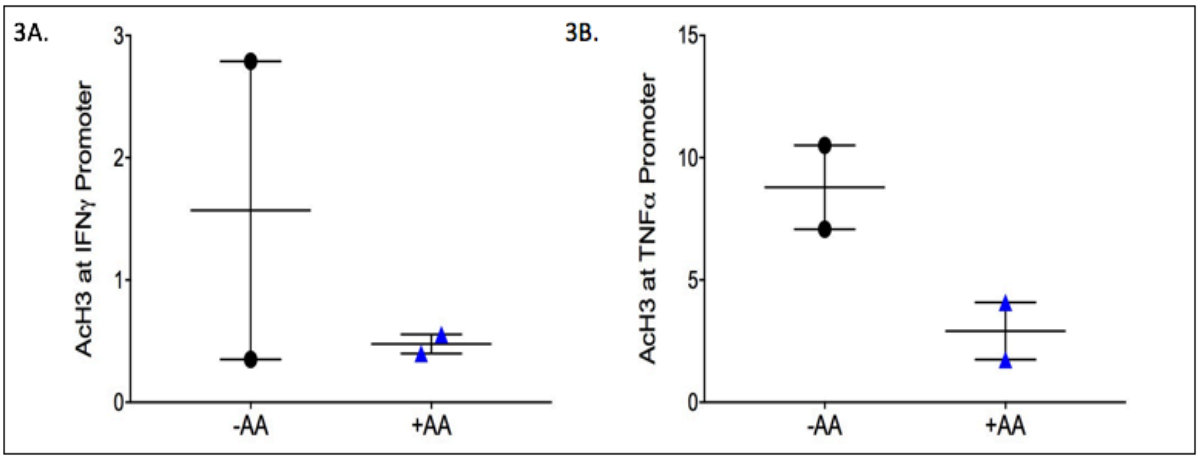


Figure A.3: The effect of AA on the de-acetylation of histones at the IFN-γ and TNF-α promoters *in vitro*.
Mya-1 cells were either untreated (black circles) or treated with 20 μM AA (blue triangles) for 24 hrs. ChIP for AcH3 followed by a qPCR demonstrated a reduction in AcH3 levels at the IFN-γ promoter for one replicate (A). A reduction in AcH3 levels was noted at the TNF-α promoter (B). However, as we had only two experimental samples, more experiments need to be performed to confirm the validity of this finding. (n=2, Data shown as median with range).
Primers sets: (i) IFN-γ promoter- Forward Primer: 5'-GCTTTCAAAGGATCCCACAA-3'; Reverse Primer: 5'-TTTGTGGCATTTTGGTGTTG-3', (ii) TNF-α promoter - Forward Primer: 5'- AGGGTTGCTTTCACTCCCAC -3'; Reverse Primer: 5'- GGGAGCTTGAGAGAAGGCTG -3'

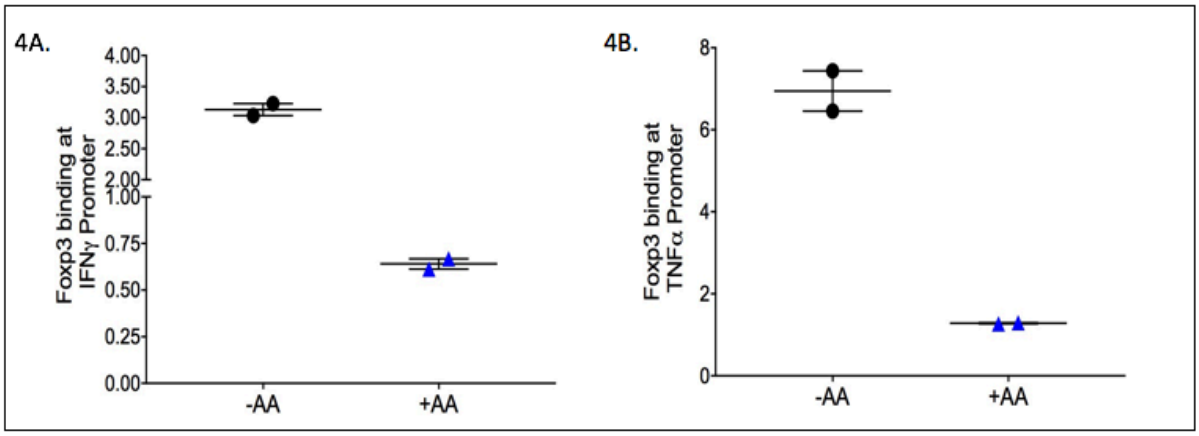


Figure A.4: The effect of AA on Foxp3 binding to the IFN-γ and TNF-α promoters *in vitro*. Mya-1 cells were either untreated (black circles) or treated with 20 μM AA (blue triangles) for 24hrs which inhibited Foxp3 binding at the IFN- γ (A) and TNF-α promoters (B). (n=2, Data shown as median with range).

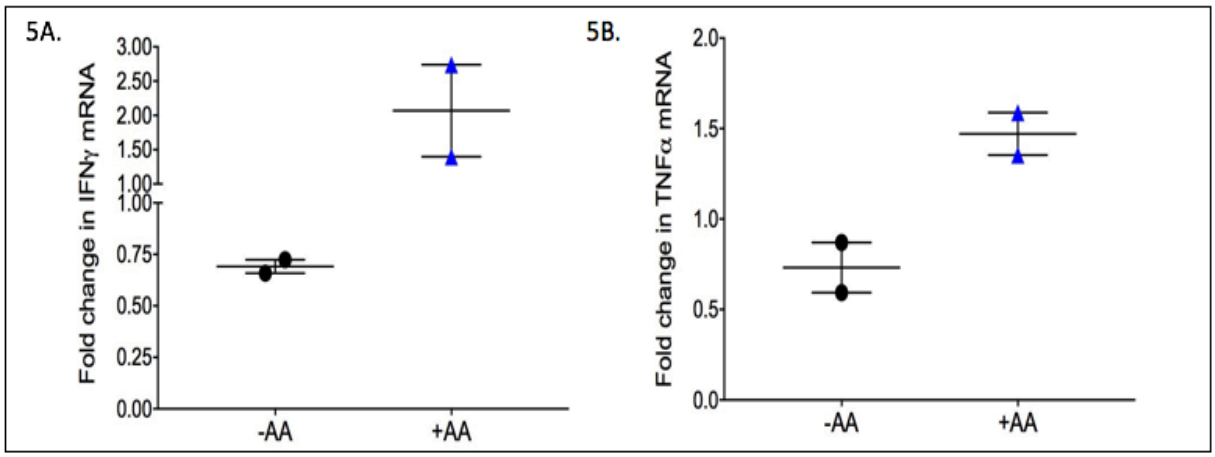


Figure A.5: The effect of AA on IFN-γ and TNF-α mRNA levels *in-vitro*.
Mya-1 cells were either untreated (black circles) or treated with 20 μM AA (blue triangles) for 24hrs and assessed by RT-qPCR. IFN-γ (A) and TNF-α (B) mRNA levels are increased following AA treatment. (n=2, Data shown as median with range. Fold change data is reported relative to mRNA levels in feline PBMCs).
Primers sets: (i) IFN-γ - Forward Primer: 5'-TGGTGGGTCGCTTTTCGTAG-3'; Reverse Primer: 5'- GAAGGAGACAATTTGGCTTTGAA-3', (ii) TNF-α - Forward Primer: 5'-ATGCCCTCCTGGCCAATGGCG -3'; Reverse Primer: 5'-TAGACCTGCCCGGACTCGGC -3'. GAPDH was used for normalization (primers listed in Chapter 3).

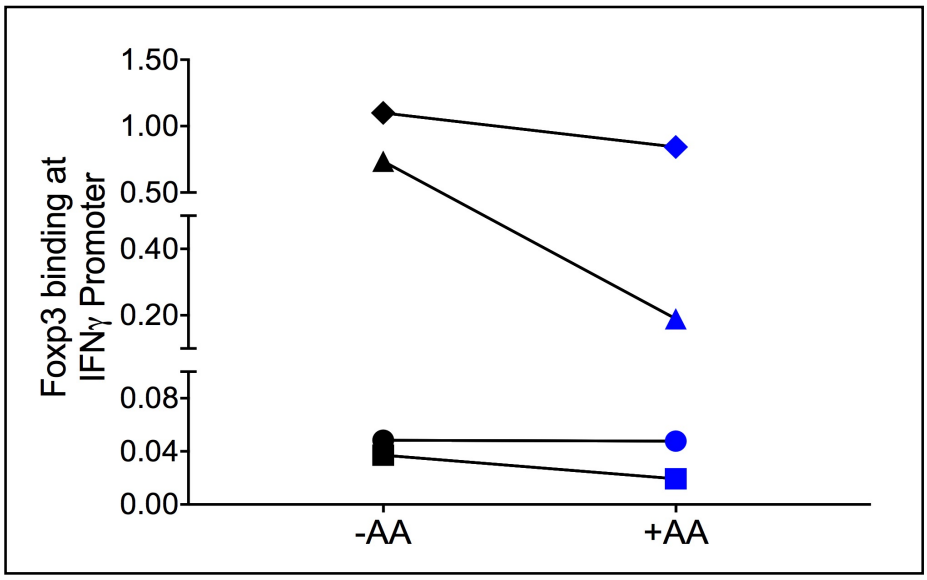


Figure A.6: The effect of AA on Foxp3 binding to IFN-γ promoter in virus-specific CD8$^+$ T cells co-cultured with autologous Treg cells in FIV+ cats.
Virus-specific CD8$^+$ lymphocytes from FIV+ cats (> 6mo infection) were either untreated (black) or treated(blue) with AA at 20μM for 24 hr followed by co-culture with autologous Treg cells. Foxp3 ChIP followed by qPCR demonstrated that in 3 of 4 cats, Foxp3 binding to the IFN-γ promoter was decreased when virus-specific CD8$^+$ T cells were pre-treated with AA. (n=4, $p>0.05$, Wilcoxon matched-pairs signed rank test done for statistics. Individual animals are represented by the same specific symbol in each treatment condition). The data for Foxp3 binding to the TNF-α promoter was inconclusive (data not shown).

Figure A.7: Immunophenotyping of total $CD8^+$ T cells in PBMCs and PLNs from FIV-infected cats.

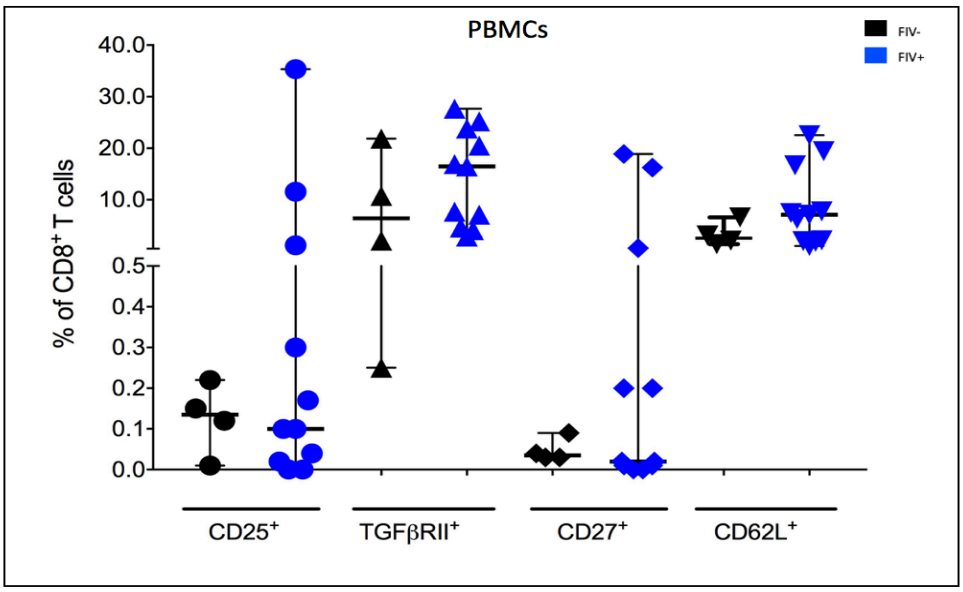
PBMCs were isolated from whole blood drawn from FIV- infected (blue, n=11) and uninfected (black, n=4) cats. Live $CD8^+$ T cells were gated to assess the surface expression of CD25, TGF-βRII, CD27 and CD62L markers. Flow data in 7A demonstrated the no difference in any of the markers between FIV-infected and uninfected cats. ($p>0.05$, Mann Whitney test done for statistics. Data shown as median with range). Figure 7B demonstrated the surface expression of CD25, TGF-βRII, CD27 and CD45RA in total $CD8^+$ T cells isolated from PLNs of FIV – infected (blue, n=10) and uninfected (black, n=2) cats. More control cats need to be tested to state the difference between marker expression. No statistical analysis could be performed as there were only 2 uninfected cats for comparison.

Methods:

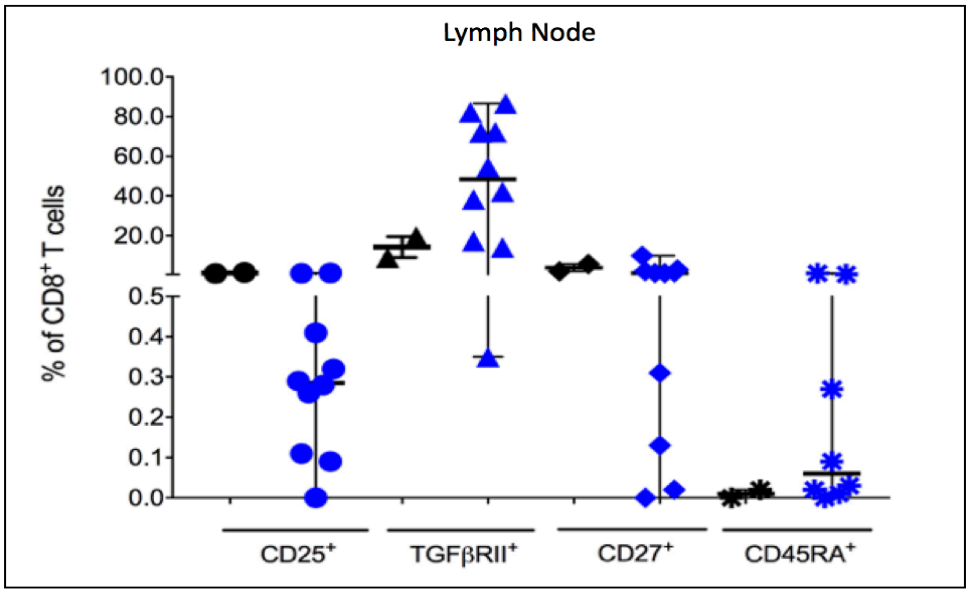
PBMC isolation: Whole blood (30 ml/cat) was collected by jugular venipuncture into EDTA vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). PBMCs were by Ficoll-Histopaque1077 density gradient centrifugation (Sigma-Aldrich, St. Louis, MO) following the manufacturer's guidelines. Blood was processed within 3-4 hrs of blood draw. PBMCs were washed with PBS and used for downstream flow experiments.

Flow cytometry analysis: Atleast $5X10^5$ bulk $CD8^+$ T cells from LNs or PBMCs were resuspended in HBSS+ 2% FBS and stained for surface expression for the following markers using specific antibodies: anti-feline CD8 PE (Clone: mAb 3.357, described in Tompkins *et al.,* (178)), anti-feline CD4 FITC (described in Tompkins *et al.,*(178)), anti-feline CD25 APC (clone: 9F23, described in Fogle *et al.,*(109)), Human-TGF-β-RII APC polyclonal antibody (RnD Sciences, Minneapolis ,MN), Brilliant Violet 570 anti-human CD27 (Clone: O323, BioLegend, San Diego, CA), APC anti-human CD45RA (Clone: HI100, BD). PBMCs were stained with the above-mentioned antibodies and PE Mouse anti-human CD62L (Clone: DREG-56, BD). The staining was done with antibody concentrations provided by the manufacturer/ as described previously in the literature at 4^oC for 30 min. Cells were washed in pre-warmed HBSS +2% FBS and acquired on the BD LSR II flow cytometer (BD Biosciences, Mountain View, CA). DAPI (BioLegend) was used as the live-dead stain. Live lymphocytes were gated on forward versus side scatter, and 20,000 gated events were acquired for each sample. Data was analyzed using FCS Express version 5 software. Gating was done using unstained and single color controls. Compensation beads (eBioscience, San Diego, CA) were used to compensate spectral overlap.

7A.


PBMCs
FIV-
FIV+
% of CD8+ T cells
40.0
30.0
20.0
10.0
0.5
0.4
0.3
0.2
0.1
0.0
CD25+
TGFβRII+
CD27+
CD62L+

7B.


Lymph Node
% of CD8+ T cells
100.0
80.0
60.0
40.0
20.0
0.5
0.4
0.3
0.2
0.1
0.0
CD25+
TGFβRII+
CD27+
CD45RA+

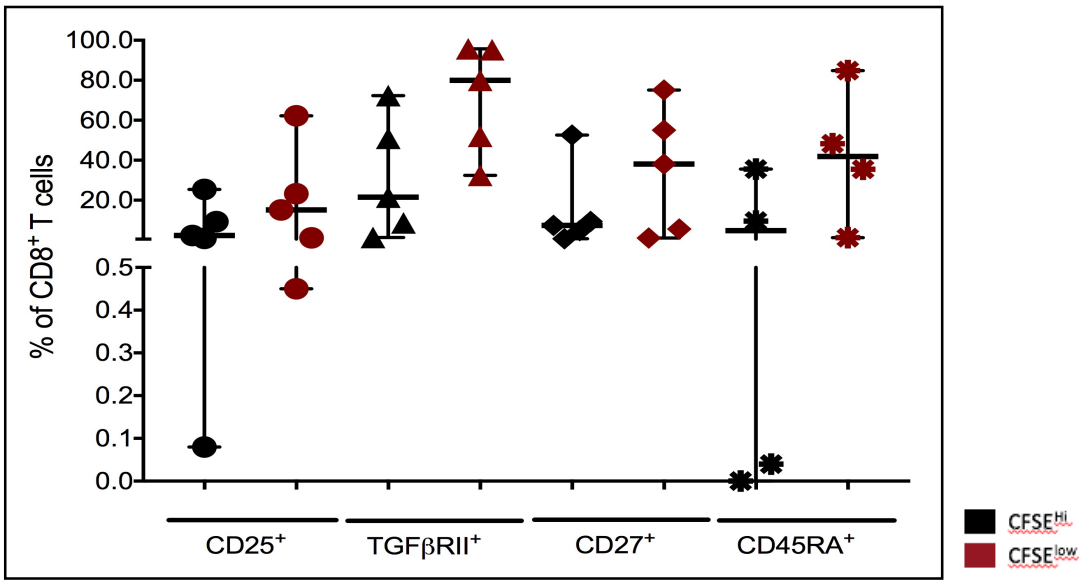


Figure A.8: Immunophenotyping of virus-specific $CD8^+$ T cells in PLN from FIV-infected cats.
Virus-specific $CFSE^{Low}$ and virus non-specific $CFSE^{Hi}$ $CD8^+$ T cells were isolated after CFSE-proliferation assay (described in methods section, Chapter 3). Figure A.8 demonstrated the surface expression of CD25, TGF-βRII, CD27 and CD45RA in the FIV-responding $CD8^+$ T cells (red, n=5) compared to the non-responders (black, n=5). Increased expression of CD25, TGF-βRII, CD27 and CD45RA in the $CFSE^{Low}$ cells suggested the "activation phenotype" of antigen-specific $CD8^+$ T cells. However, these data were not significant. ($p>0.05$, Wilcoxon matched-pairs signed rank test done for statistics. Data shown as median with range).

Methods:
Flow cytometry analysis: Atleast $5X10^5$ $CFSE^{int/low}$ virus-specific and $CFSE^{high}$ non-virus specific $CD8^+$ T cells were resuspended in HBSS+ 2% FBS and stained for surface expression for the following markers using specific antibodies: anti-feline CD8 PE (clone mAb 3.357), anti-feline CD4 FITC, anti-feline CD25 APC (clone: 9F23), Human-TGF-β-RII APC polyclonal antibody, Brilliant Violet 570 anti-human CD27 (clone O323), APC anti-human CD45RA (Clone: HI100, BD). Same staining protocol as figure A.7 was followed.

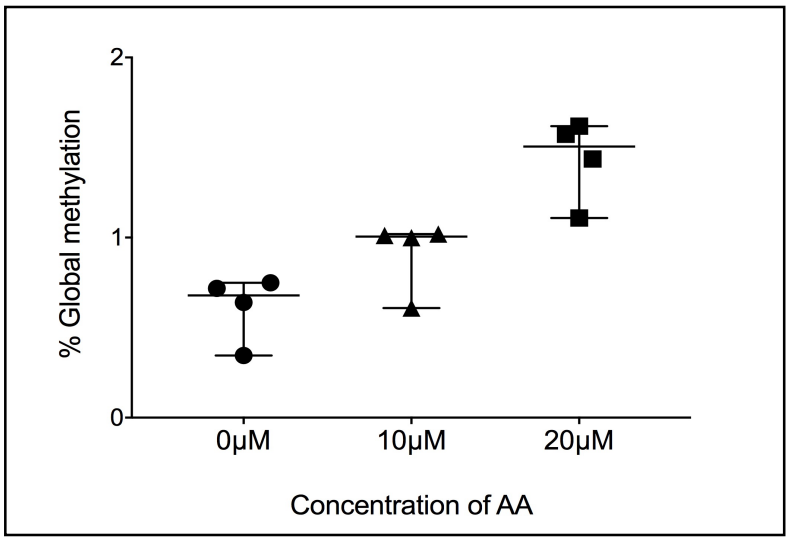


Figure A.9: AA treatment does not increase DNA methylation *in vitro*.
$1X10^6$ Mya-1 cells were treated with AA at 0, 10 and 20 μM for 24hr, genomic DNA of these cells was isolated and percentage global methylation was measured using Colorimetric Methyl Flash DNA Quantification kit (method described in Figure A.10). The methylation data showed a trend towards higher methylation in AA treated cells in a dose-dependent manner. The difference, however, was not significant. (n=4, $p>0.05$, Wilcoxon matched-pairs signed rank test done for statistics. Data shown as median with range).

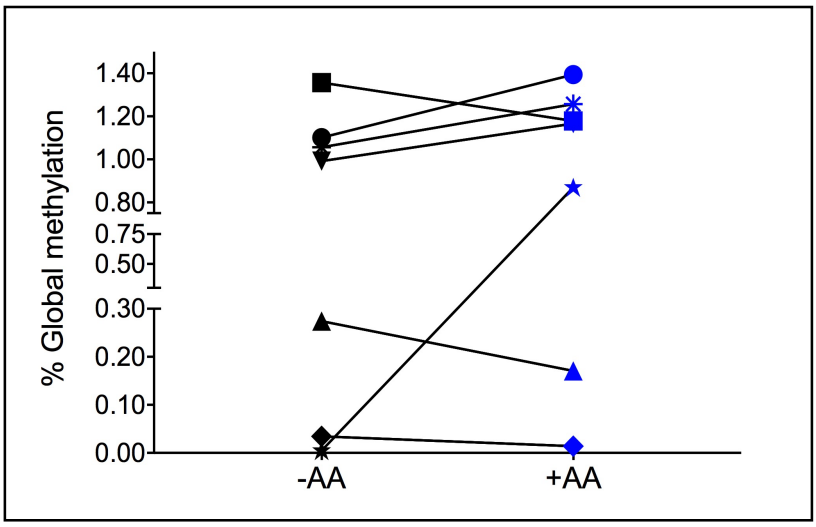


Figure A.10: AA treatment does not increase DNA methylation *ex vivo*.
$5X10^5$ $CD8^+$ T cells isolated from chronically infected (> 6 mo) were either untreated (black, n=7) or treated (blue, n=7) with AA at 20 µM for 24hr, genomic DNA of these cells was isolated and percentage global methylation was measured using Colorimetric MethylFlash DNA Quantification kit (method described below). The methylation data showed a trend towards higher methylation in 4 of 7 animals tested upon AA treatment. The difference, however, was not significant. ($p>0.05$, Wilcoxon matched-pairs signed rank test done for statistics. Individual animals are represented by the same specific symbol in each treatment condition).

Methods:
Global DNA methylation analysis- DNA was isolated from $1X10^6$ Mya-1 cells treated with 0µM, 10µM, 20µM and 50µM of Anacardic Acid (Sigma Aldrich, Cat# A7236-5mg) for 24 h. For the *ex vivo* studies, $1X10^6$ sorted bulk $CD8^+$ T cells isolated from FIV+ and FIV -cats were used for DNA isolation using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Isolated DNA was analyzed for global methylation using the MethylFlash Methylated DNA 5-mc quantification kit (Epigentek, Farmingdale, NY) according to the manufacturer's instructions.

Figure A.11: AA does not increase methylation at specific cytosine residues in the IL-2 promoter *in vitro*.

We identified the transcriptionally active regions in the IL-2, IFN-γ and TNF-α promoters (A). Within the IL-2 promoter, three cytosine residues (CG1 -1457, CG2 -1354, CG3 -1256) were identified for analysis in region 2 (-1681 to -1176) (B). Mya-1 cells either untreated (black) or treated (blue) with AA at 20 μM for 24hr, DNA was isolated and then bisulfite reduced wherein de-methylated cytosine residues were converted to uracil. The reduced DNA was amplified for the target regions of IL-2, IFN-γ and TNF-α using the bisulfite reduced primers listed in Table A.1. The amplicons were then cloned into TA vectors, transformed and sequenced. De-methylation of the CpG residues was identified by the replacement of the cytosine with a thymine residue in the sequencing readout. There were seven sequences that matched the reference IL-2 promoter (region 2). The methylation status for each of the cytosine residues was compared between the untreated and treated groups. All 7 sequences from untreated and AA treated groups exhibited methylation at CG1 and CG3 sites (100% methylated sequences). 66.7% methylated sequences without AA and 28.57% methylated sequences with AA treatment were reported (C). There were no methylation differences at any of the CpG residues in region 1 of the IL-2 promoter (data not shown). Sequencing of the IFN-γ and TNF-α promoters was not successful as there was no match between the sequencing readouts and the reference sequence. Although we did not observe an increase in methylation with AA treatment, this data demonstrated a trend towards distal de-methylation at the IL-2 promoter consistent with the previous studies (117). This data also suggests that AA might increase de-methylation at the DNA level.

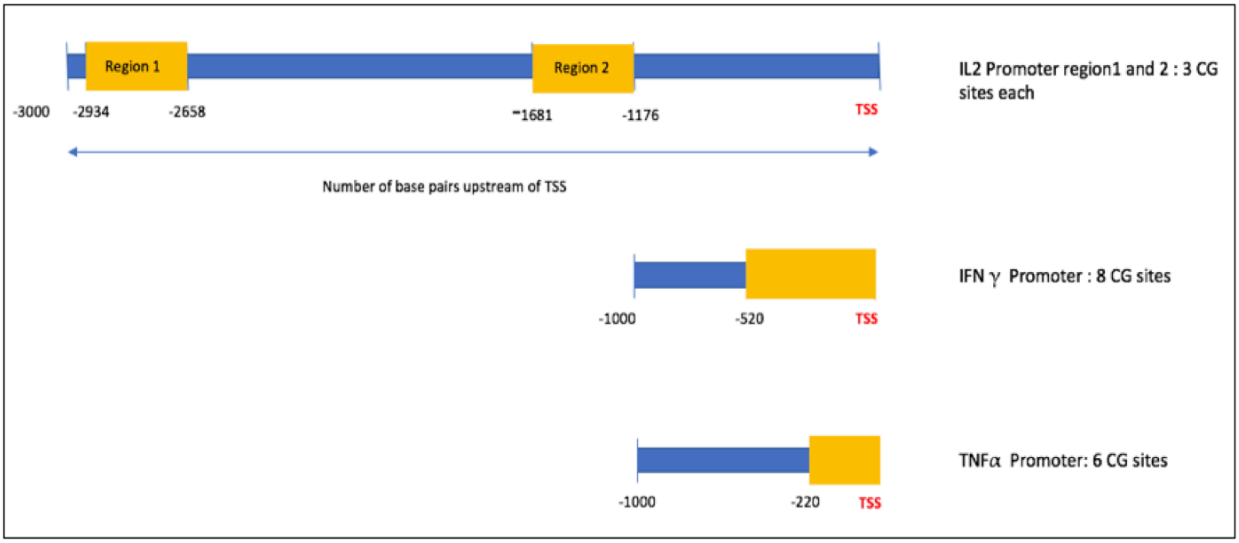
Methods:

Promoter specific DNA Methylation analysis and cloning: DNA was isolated from at least 5×10^5 Mya-1 cells (treated or untreated with AA) using the QIAamp DNA Mini kit (Qiagen) and then subjected to bisulfite modification using an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA). Bisulfite reduced DNA was used as a template for IL-2, IFN-γ and TNF-α promoter amplification using specific bisulfite primers (Table A.1). Primers were designed using Zymo Bisulfite Primer Seeker to amplify specific promoter segments up to 3 kb upstream of transcription start site (see Promoter Map, Figure 9A). The following cycling conditions were applied in the qPCR: 95^{o}C for 5 mins, 94°C for 45s, 58°C for 45s, and 72°C for 1 min with 40 cycles, and, for IL-2, a final extension step at 72^{o}C for 10 min. The annealing temperature was changed to 60°C for TNF-α and IFN-γ promoters, while all other cycling conditions were the same as for IL-2. PCR products were ligated into pCR® 4-TOPO® TA vectors using the TOPO TA cloning (Life Technologies), and the resulting recombinant molecule was transformed into One Shot® TOP10 Competent Cells (Life Technologies). 40 μl of transformed cells were plated on selective LB agar plates. Positive colonies were selected, cultured overnight in LB media and their plasmid was purified using the PureLink Quick Plasmid Miniprep Kit (Life Technologies). Individual clones upon plasmid purification were sent to Genewiz (Morrisville, NC) for sequencing. Sequences were analyzed using the Geneious software.

Table A.1: Feline bisulfite reduced primers for IL-2, IFN-γ and TNF-α promoters

Primer Target	**Forward (5'→3')**	**Reverse (5'→3')**
IL-2 Promoter region 1	AATTTTATTTTTAAAGGGGAAGTAGGGGGTGTG	AAAAAATTCRTTCCTAAACTCCTAACTTATCCC
IL-2 Promoter region 2	TGTTATATTTATAGTGTTTTAGATGTTGTAGTGG	ATAAAAACTATCCTCAAAACAAAAATATCAAAACTC
IFN-γ Promoter	TGTTTTATYGTTAAAAGATTTAGGGAGTTG	TCTAATAACCRACCAACAAACAATCAAAAAAATATAC
TNF-α Promoter	TTTTTTTAGYGAAGATATTAGGGATTAGTTAGGAG	AACRTTCTTCCCAAAAAACTCAAATCC

11A.



11B.

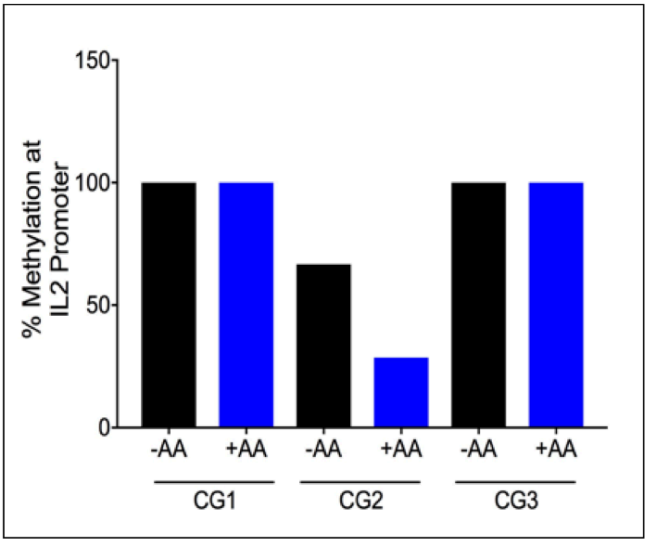
Region 2 of IL2 Promoter

-1681

CTACCATCACACTGGGGCAGTTAGTGCATTATACTGAGGA
CACAGTAACACATATCCTAAGTATCCTACCCATTCTACCAA
CTTTAAACATATCGATCTGATCTTATTTCAGAGCTACTATTG
TTACACTCATAGTGCCCTAGATGCTGTAGTGGAGAAACCC
AAACCTATTAGAGAGGTGAAAACATAACAAGTACAAACT
TAAGGATTGGTTTACATACAG**CG(1)**CATATAAAGTCTATT
ATAAAACTAGCATCAGTACTCTTGGATGCAAACCTTTTTC
TGAATATTTGTCTGTCATGAGTTGTTTTTAATACTTTATTTT
TTTT**CG(2)**TAAAAGGCACTCCAAAATTGTAATGGATACC
CCTTCTTAGTAACTTCTGTTGATTCAATTCTGACCTCTGTA
TAATAAGAATCATCTTGGAATAAA**CG(3)**AGTCCTGATATC
CCTGCTCTGAGGACAGCTCTTATTCCACAAGGAGCATGC
ACACACAAACACCCCTACCCTATGACCC

-1176

11C.



Summary

The studies in the appendix represent preliminary data collected to further elucidate the epigenetic events and phenotypic events enabling Treg-induced Foxp3-mediated $CD8^+$ T cell dysfunction during FIV infection.

Modulation of the IL-2 promoter influences IL-2 expression levels by changing the chromatin accessibility during activation (117). Here, we investigated the epigenetic events occurring at the IL-2 promoter in $CD8^+$ T cells *in vivo* in response to FIV infection. We determined the activation status of the IL-2 promoter in $CD8^+$ T cells responding to lentivirus infection by measuring the levels of AcH3 (Figure A.1). Based on the *in vitro* data in the FIV model, we rationalized that activation due to lentivirus would increase histone acetylation. Our results in Figure A.1 suggested a trend towards higher AcH3 levels in virus-specific $CD8^+$ T cells compared to non-specific $CD8^+$ T cells, indicating a transcriptionally active state. However, more *ex vivo* studies need to be performed to confirm these findings. In Chapter 3, we clearly demonstrated an increase in IL-2 transcription upon blocking histone acetylation with AA *in vitro.* Therefore, we next investigated the ability of blocking histone acetylation on the IL-2 transcript levels *in vivo.* Treatment with AA did not alter IL-2 transcription (Figure A.2). There are several limitations that might explain our findings: (i) we did not stimulate the cells after AA treatment to activate IL-2 production, (ii) the sample size was very small (n=3/group). Although, we still need extensive *ex vivo* studies to validate our proposed model (Chapter 5, Figure 5.1), this study, to our knowledge, is the first report exploring histone acetylation as a potential mechanism in Foxp3-mediated $CD8^+$ T cell dysfunction in FIV infection.

In the previous sections, we reported that DNA demethylation and histone acetylation play an important role in Foxp3 mediated - $CD8^+$ T cell dysfunction by enabling direct binding of Foxp3 to the IL-2 promoter in virus-specific $CD8^+$ T cells (117, 118) (unpublished data, 2018). We also demonstrated that Foxp3 binds to the promoter regions of IFN-γ and TNF-α in virus-specific $CD8^+$ T cells (118). Based on our findings of histone modulation at the IL-2 promoter, we examined the role of histone acetylation in the suppression of IFN-γ and TNF-α. In our preliminary data, we demonstrated that AA reduced Foxp3 binding to the IFN-γ and TNF-α promoters *in vitro* and resulted in a concurrent increase in their transcript levels (Figures A.4-A.5). Due to limited ChIP-DNA from the virus-specific $CD8^+$ T cells, we could only test the change in Foxp3 binding to the IFN-γ promoter upon AA treatment *ex vivo* (Figure A.6). Although there were no statistically significant differences between the groups, a reduction in Foxp3 binding was observed in three out of the four animals tested, indicating that this mechanism is most likely biologically relevant. Overall, the data in here provides the rationale for further investigating histone acetylation as a mechanism for Foxp3-mediated suppression of antiviral cytokines such as IFN-γ and TNF-α. Next, we asked whether these epigenetically activated $CD8^+$ T cells were predominantly effector cells in the FIV-infected cats. To address this question, we measured the surface markers expressed on $CD8^+$ T cells isolated from FIV-infected cats. A previous study described effector $CD8^+$ T cells from FIV- infected cats as $CD62L^-CD44^{hi}CD49d^{hi}CD18^{hi}CD57^-$ (251). Here, we further characterized total $CD8^+$ T cells isolated from peripheral blood and peripheral lymph nodes from FIV- infected cats. The flow data in Figure A.7 demonstrated no change in CD25, TGF-βRII, CD27 and CD62L expression in $CD8^+$ T cells in PBMCs between FIV-infected and uninfected cats. The phenotype profile of total $CD8^+$ T cells in PLNs from FIV –infected cats demonstrated high expression of TGF-βRII

and CD27, but these could not be compared to uninfected cats due to low subject count (n<3). Lack of adequate subjects explain our inconclusive findings. Next, we assessed the phenotype of virus-specific $CD8^+$ T cells. Due to the unavailability of FIV tetramers, no studies have been able to characterize FIV-specific $CD8^+$ T cells. The data presented in Figure A.8 represents the first attempt at defining FIV-specific cells phenotypically. $CFSE^{Low}$ $CD8^+$ T cells had a trend towards higher expression of CD25, TGF-βRII, CD27 and CD45RA compared to virus non-specific $CFSE^{Hi}$ $CD8^+$ T cells suggesting their activated state. This data was consistent with the phenotype of HIV- and SIV- specific $CD8^+$ T cells that have been well characterized (252). We were, however, unable to conclude the differentiation states of these cells as co-expression of surface markers was not assessed. To further characterize the virus-specific $CD8^+$ T cells, the expression of more activation and inhibitory markers need to be tested, such as CCR7, CD28, CTLA-4 and PD1. The biggest hurdle to expanding the phenotype panel in the feline model, is the lack of feline-cross reactive antibodies. For our phenotype studies, we tested multiple clones of different human, canine, equine and NHP cross reactive antibodies to identify a clone that could generate reproducible flow data in the feline model. Although limited, the flow data demonstrates the first attempt at characterizing virus-specific $CD8^+$ T cells in the FIV model.

The studies in Chapter 3 confirmed the action of AA as a HAT inhibitor. We next explored other mechanisms of action of AA, specifically, its effect on DNA methylation. Studies by our group and others demonstrated that AA functions via different post translational modifications to maintain chromatin in its inactive state. Therefore, we hypothesized that AA inhibits DNA de-methylation, thereby preventing Treg-induced Foxp3 binding at the IL-2, IFN-γ and TNF-α promoters. Data in Figures A.9-A.11 were unable to demonstrate an effect of AA on DNA

methylation *in vitro* and *ex vivo*. In fact, the data in Figure A.11 suggested that AA might increase DNA de-methylation which was contrary to our hypothesis.