

ABSTRACT

MIKKELSEN, SARAH ROCHELLE. The Role of Regulatory T Cells during Feline Immunodeficiency Virus Pathogenesis. (Under the direction of Dr. Gregg Dean.)

Human immunodeficiency virus-1 (HIV-1), simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) are members of the Retroviridae family in the Lentivirinae genus. Lentiviral pathogenesis involves a prolonged period of immune dysregulation that eventually results in acquired immunodeficiency syndrome (AIDS). Data exploring the roles of CD4⁺ T helper cells, cytotoxic T lymphocytes, B cells, natural killer cells, and dendritic cells during acute simian immunodeficiency virus (SIV) and feline immunodeficiency virus (FIV) infection suggest that dysregulation of the immune response begins early after lentiviral infection. Many studies suggest that CD4⁺CD25^{hi}FOXP3⁺ immunosuppressive regulatory T (Treg) cells are involved in regulation of the immune response during HIV-1 and SIV infection and could be involved in modulating immune dysfunction. Treg cells can suppress lentiviral-specific immune responses, and therefore may play a detrimental role during infection. However, several studies point to a protective role of Treg cells in lentiviral infection, as high PBMC Treg cell frequencies are correlated with high CD4⁺ T cell counts, low T cell activation, and/or low viremia. Treg cells have been described in the cat, and their immunosuppressive capabilities have been shown to be enhanced during FIV infection. Therefore, we sought to (1) validate a method of in vivo Treg cell depletion via anti-CD25 monoclonal antibody (mAb) in the cat, (2) determine the role of Treg cells during chronic FIV pathogenesis by depleting Treg cells in cats chronically infected with FIV, and (3) determine whether Treg cells play a role in acute FIV pathogenesis by depleting Treg cells prior to infection.

We found that anti-CD25 mAb administration in FIV naïve cats immunized with recombinant FIV p24 resulted in an 82% decrease in circulating CD4⁺CD25^{hi} Treg cells at the nadir of depletion. Treg cells and FOXP3 mRNA were reduced in the thymus, secondary lymphoid tissues, and the gut up to day 35 post-depletion. Anti-CD25 mAb treatment did not disturb anti-FIV p24 cellular and humoral immune responses. Anti-CD25 mAb treatment in cats chronically infected with FIV revealed significant FIV-specific immune responses as measured by interferon (IFN)- γ production. Cats that received the Treg cell-depleting mAb were able to produce a significantly more robust humoral response to new antigen as compared to cats receiving an isotype control mAb. Importantly, transient Treg cell depletion in cats chronically infected with FIV did not induce damaging proinflammatory cytokine production or viral replication. Treg cell depletion prior to FIV infection did not significantly alter acute FIV pathogenesis. The effects of anti-CD25 mAb treatment appeared to be truncated in cats acutely infected with FIV as compared to chronically infected cats or FIV naïve cats, as Treg cell levels were heightened in all treatment groups after FIV infection.

We propose that the influence of Treg cell suppression during FIV pathogenesis is most prominent after established infection, when Treg cells are activated and more functionally suppressive. Our findings suggest that short-term in vivo Treg cell depletion during chronic lentiviral infection could provide a window of opportunity for therapeutic vaccination in individuals with controlled viral replication.

The Role of Regulatory T Cells during Feline
Immunodeficiency Virus Pathogenesis

by
S. Rochelle Mikkelsen

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Immunology

Raleigh, North Carolina

2009

APPROVED BY:

Dr. Gregg A. Dean
Chair of Advisory Committee

Dr. Mary B. Tompkins

Dr. Scott M. Laster

Dr. Gregory D. Sempowski

DEDICATION

This dissertation is dedicated to my husband, Joel Mikkelsen. His examples of love and kindness give me more than reason enough to strive to be a better person.

BIOGRAPHY

Rochelle was born to Dana and Catherine Smithberg in Miami, Florida, where she was soon joined by a younger sister, Christy. A short time later, the family moved to Birmingham, Alabama where Rochelle would spend her formative years. While attending Jefferson County International Baccalaureate High School, she was given the opportunity to intern with the Cell Biology and Immunology group at Southern Research Institute (SRI) during her junior and senior years. There she developed her love of pipettes and immunology and completed a project that allowed her to compete in the 2001 Intel National Science Talent Search. After spending three summers with SRI and one year studying at the University of Miami, Rochelle entered the Microbiology program at the University of Alabama in Tuscaloosa, Alabama. There she performed research in the laboratories of Dr. David Oppenheimer and Dr. Gary Sloan and participated in the Howard Hughes Medical Research Intern Program. While she enjoyed her time working with Arabidopsis and gram-positive bacteria at the University of Alabama, she knew her heart truly belonged to antibodies and T cells; therefore, she applied for admittance to the Immunology Program at North Carolina State University (NCSU).

Rochelle joined the laboratory of Dr. Gregg Dean in 2005 and thus began the quest of understanding the mysterious regulatory T cell. She was awarded a National Institutes of Health traineeship with the Molecular Biotechnology Training Program at NCSU in 2006. She met her husband, Joel Mikkelsen, during her second year of graduate school, and they

were married a year later. She completed an internship with Global Vaccines, Inc., a not-for-profit company in Research Triangle Park, North Carolina, in 2008.

ACKNOWLEDGMENTS

I would like to thank my committee members, Dr. Gregg Dean, Dr. Mary Tompkins, Dr. Scott Laster, and Dr. Greg Sempowski, for providing me with direction and encouragement throughout the course of these studies. I gratefully acknowledge my major advisor, Dr. Gregg Dean for providing me with the opportunity to complete this research, for creating a laboratory environment conducive to scientific independence, for his insightful and uplifting comments and questions, and for his example of diligence and sound scientific analysis. I am incredibly grateful for the challenges and scientific understanding provided by Dr. Sue Tonkonogy and Dr. Scott Laster in the classroom and during journal club. I am indebted to Dr. Kristina Howard for her mentorship and training, without which my education would be incomplete.

The work described here would not have been possible without the expertise of Alora LaVoy, Stacie Reckling, Julie Long, Erin Egan, Lin Zhang, and Janet Dow – their friendship and opinions are invaluable. I thank Dr. Shila Nordone for her inquisitive comments and absolute honesty. I thank Dr. Mary Tompkins, Dr. Angie Mexas, and Dr. Jonathan Fogle for their support in working with the feline model. I am sincerely grateful to Rita de Cassia Simoes for her friendship and support, and to Laura Neuder, Jibing Yang, and Amy Heffelfinger for their companionship throughout our mutual experiences in graduate school.

I thank Dr. Rich May, Dr. Alex Aller, Mike Koratich, and Dr. John Farmer for starting me on the path to graduate school, and Dr. Gary Sloan and Dr. Paul LeBlanc for

encouraging me along that path. I am deeply grateful to my husband, family, and friends for their constant love and support - before, during, and after graduate school.

TABLE OF CONTENTS

LIST OF TABLES	ix
LIST OF FIGURES	x
1. INTRODUCTION	1
2. LITERATURE REVIEW	4
A. Introduction of the FIV Model.....	4
B. Current Theories of Lentiviral Immunopathogenesis	5
1. Cellular Receptors of Lentiviruses.....	5
2. Acute Phase Infection	7
3. Chronic Phase Infection.....	10
a. Overview	10
b. Immune activation marker expression correlates with disease progression.....	11
c. Microbial translocation as a cause of immune activation	11
d. Lentiviral-cellular interactions as a cause of immune activation and dysregulation	13
C. Regulatory T cells	14
1. Overview.....	14
2. The Transcription Factor FOXP3	15
a. Expression in naturally occurring, thymically derived Treg cells	15
b. Expression during Treg cell conversion	15
3. Treg Cell Activity during Infection	16
a. Adaptive Treg cells	16
b. Treg cell proliferation in vivo	17
c. Synchronous activation of Treg cells and T effector cells during infection	18
D. The Role of Treg Cells during Lentiviral Pathogenesis.....	19
1. Treg Cell Dynamics during Lentiviral Infection	19
a. Treg cells in lymphoid tissue	19
b. Treg cells in the periphery	21
c. Possible CD25 downregulation by Treg cells during lentiviral infection	21
2. Direct Lentiviral Infection of Treg Cells	22
3. Evidence for a Detrimental Role of Treg Cells during Lentiviral Pathogenesis.....	23
4. Evidence for a Protective Role of Treg Cells during Lentiviral Pathogenesis.....	25

E. Experimental Treg Cell Depletion	26
1. In Vitro CD25 ⁺ Cell Depletion	26
2. In Vivo CD25 ⁺ Cell Depletion.....	27
a. CD25 ⁺ cell depletion in cancer models or cancer patients.....	26
b. CD25 ⁺ cell depletion in models of pathogenic infection	28
c. Efficacy and limitations of in vivo CD25 ⁺ cell depletion using anti-CD25 monoclonal antibodies	29
3. Treg cell depletion or functional blockade during lentiviral infection	30
F. References.....	33
3. In Vivo Depletion of CD4 ⁺ CD25 ⁺ Regulatory T Cells in Cats	60
A. Abstract	61
B. Introduction	62
C. Materials and Methods	63
D. Results.....	70
E. Discussion.....	75
F. Figures	80
G. Tables.....	88
H. References.....	90
4. In Vivo Depletion of Regulatory T Cells Improves Antiviral and Novel Immune Responses in Cats Chronically Infected with Feline Immunodeficiency Virus	93
A. Abstract	94
B. Introduction	95
C. Materials and Methods	97
D. Results.....	103
E. Discussion.....	110
F. Figures	116
G. References.....	124
5. Treg Cell Depletion Prior to Acute Feline Immunodeficiency Virus Infection Does Not Alter Disease Pathogenesis	132
A. Abstract	133
B. Introduction	134
C. Materials and Methods	137
D. Results.....	143
E. Discussion.....	150
F. Figures	155
G. References.....	164
6. DISSERTATION SUMMARY	173
A. References.....	178

LIST OF TABLES

In Vivo Depletion of CD4⁺CD25⁺ Regulatory T Cells in Cats

Table 1. Feline-specific primer and probe sequences for real-time RT-PCR.....88

Table 2. Maintenance of anti-FIV p24-GST T cell proliferation after administration of 9F2389

LIST OF FIGURES

In Vivo Depletion of CD4⁺CD25⁺ Regulatory T Cells in Cats

Figure 1. In vivo depletion of circulating cells with mAb 9F23.....	80
Figure 2. CD25 ⁺ B cells and T cell subpopulations are depleted by 9F23	81
Figure 3. Anti-mouse antibody response precedes rebound in CD25 ⁺ cells.....	82
Figure 4. In vivo depletion of CD4 ⁺ CD25 ⁺ cells from lymphoid tissues.....	83
Figure 5. In vivo reduction of CD4 ⁺ CD25 ⁺ cells and FOXP3 by 9F23 in lymphoid tissues	84
Figure 6. Maintenance of anti-FIV p24-GST IgG titers after administration of 9F23	85
Figure 7. Suppression of CD25 ⁻ T cell proliferation by CD25 ⁺ peripheral lymph node T cells.....	86

In Vivo Depletion of Regulatory T Cells Improves Antiviral and Novel Immune Responses in Cats Chronically Infected with Feline Immunodeficiency Virus

Figure 1. Depletion of Treg cells by anti-CD25 mAb treatment.	116
Figure 2. Viral burden is unaffected by anti-CD25 mAb treatment	118
Figure 3. FIV-specific IFN- γ responses are transiently improved after anti-CD25 mAb treatment.....	120
Figure 4. In Anti-CD25 treatment does not induce systemic inflammatory cytokine production	121
Figure 5. Intranuclear FOXP3 is upregulated in CD4 ⁺ CD25 ⁺ cells of cats treated with nondepleting mouse mAb	122
Figure 6. Cats treated with anti-CD25 mAb produce a more robust humoral response against mouse immunoglobulin	123

**Treg Cell Depletion Prior to Acute Feline Immunodeficiency
Virus Infection Does Not Alter Disease Pathogenesis**

Figure 1. Treg cells are partially depleted by anti-CD25 mAb treatment.155

Figure 2. Anti-CD25 mAb treatment does not significantly alter
plasma viremia.....156

Figure 3. Proviral load is unaffected by anti-CD25 mAb treatment.....157

Figure 4. Peripheral lymphocyte dynamics during acute FIV-C36 infection
are not altered by anti-CD25 mAb administration.....158

Figure 5. Dendritic cell activation but not T cell proliferation is enhanced
by murine mAb treatment159

Figure 6. Cytokine-expressing CD8⁺ T cell levels are lower after CD25⁺ mAb
treatment at day 35 post-FIV infection160

Figure 7. FIV-specific IFN- γ responses during acute infection are not altered
by anti-CD25 mAb treatment.....161

Figure 8. Anti-FIV p24, but not anti-FIV gp95, IgG titers are significantly
lower after anti-CD25 mAb treatment during acute FIV infection.....162

Figure 9. A trend of higher anti-mouse IgG titers are found in cats treated
with anti-CD25 mAb during acute FIV infection.....163

INTRODUCTION

Feline immunodeficiency virus (FIV) is a member of the Retroviridae family in the Lentivirinae genus that infects domesticated and wild cats and is endemic in certain feline populations worldwide (1). Of the Lentivirinae, FIV is phylogenetically more closely related to equine infectious anemia virus and visna virus than to human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) (2). However, FIV pathogenesis is more similar to that of HIV-1 rather than any other lentivirus. FIV and HIV-1 pathogenesis is characterized by an acute phase anti-viral response involving CD8⁺ T lymphocytes and neutralizing antibodies that limits viremia but allows persistence of latent infection (3-5). This is followed by an asymptomatic phase that is perpetuated over the course of years and is associated with generalized immune dysregulation and lymphocyte activation (6-10). FIV infection in cats eventually results in acquired immunodeficiency syndrome (AIDS) and opportunistic infection in 10 years average post-infection, as does HIV-1 infection in humans (11, 12). The similarities between HIV-1 and FIV pathogenesis caused by natural infection of susceptible hosts supports the strength and soundness of the FIV/cat model as an appropriate analogue for understanding HIV-1 infection in humans.

Regulatory T (Treg) cells are potent regulators of many aspects of the immune response, including cytokine and chemokine release, T cell expansion and apoptosis, cytotoxic T lymphocyte responses, antibody production, lymphocyte trafficking, and dendritic cell function (13-19). Regulatory T cells are classically described as CD4⁺CD25^{hi}FOXP3⁺. Other cell surface proteins commonly associated with Treg cell

identification and function are the IL-7 receptor CD127, glucocorticoid-induced tumor necrosis factor receptor (GITR), cytotoxic T lymphocyte antigen 4 (CTLA-4), transforming growth factor β (TGF- β), and more recently, GARP (glycoprotein A repetitions predominant; LRRC32), which is associated with TGF- β surface expression (20-26). Treg cells originate in the thymus or are induced in the periphery from conventional naïve CD4⁺ cells and can expand in vivo when activated through their T cell receptor (TCR) (13, 27-31). Treg cells are fundamentally required for regulating autoreactive immune cells, as demonstrated by the fatal autoimmune disease that results in the absence of Treg cells when the lineage determining transcription factor gene, *Foxp3*, is corrupted (30, 32). In addition, many studies have elucidated Treg cell modulation of the immune response to pathogens (13, 17, 33-37). Collectively, these studies demonstrate that Treg cells are capable of limiting immunopathology and inflammation during infection. They also show that Treg cells are capable of suppressing immune responses to pathogens and influencing the development of adaptive memory immunity.

Immunosuppressive Treg cells have been described in the cat and are functionally activated during FIV infection (38). It is unknown whether Treg cells are harmful or helpful to feline hosts during the acute and chronic phases of lentiviral infection as they could potentially suppress both beneficial antiviral immune responses and harmful generalized immune activation simultaneously. This dissertation explores the effect of Treg cell depletion on the pathogenesis of FIV. The following specific aims are addressed:

1. Determine the in vivo kinetics of CD25⁺ Treg cell depletion and rebound in cats and identify any potential changes within effector cell populations after treatment with anti-CD25 monoclonal antibody.
2. Determine whether in vivo depletion of CD25⁺ Treg cells improves or diminishes the anti-FIV immune response in chronically FIV-infected cats.
3. Determine whether CD25⁺ Treg cell depletion prior to FIV infection boosts acute antiviral responses or affects viral pathogenesis.

LITERATURE REVIEW

A. Introduction of the FIV Model

Experimental FIV infection in domestic cats is a well established model for HIV-1 infection in humans. FIV was first described in 1987 by Niels Pedersen and colleagues, who recognized the usefulness of FIV as a model for HIV-1 (11). FIV, like HIV-1, can be transmitted horizontally through blood, saliva, and semen, and vertically through milk and placenta (12, 39-42). FIV pathogenesis is remarkably similar to HIV-1 pathogenesis, typified by a short acute phase, involving high levels of viremia, followed by a long asymptomatic phase, involving persistent latent viral infection, which eventually culminates in opportunistic infection and AIDS as the immune system becomes increasingly dysfunctional (7, 10, 43). FIV infection causes substantial CD4⁺ T cell depletion and inversion of the CD4:CD8 T cell ratio, which is one of the major hallmarks of HIV-1 infection (7). In addition to infecting CD4⁺ T cells, FIV infects CD8⁺ T cells, Treg cells, macrophages, dendritic cells, and thymocytes, as does HIV-1 (44-47). Clinical signs of FIV immunodeficiency are remarkably similar to human AIDS and include lymphadenopathy, diarrhea, wasting, neurologic syndromes, skin conditions, respiratory tract and urinary tract infections, and B cell lymphosarcomas (11, 48). FIV is genetically and molecularly similar to HIV-1, with genes encoding *gag*, *pol*, *vif*, *env*, and *rev* (48).

The advantages of using the FIV/cat model over other in vivo models of HIV-1 infection include affordability and readily available specific-pathogen-free animals.

Domestic cats are not endangered unlike some monkey species used in SIV research. FIV infects domestic cats naturally, without requiring manipulation of the virus or use of a host not normally susceptible to lentiviral infection. The FIV model has been employed to study lentiviral pathogenesis for two decades, and many FIV-specific and feline-specific assays and reagents have been developed.

FIV research continues to expand the knowledge base that contributes to understanding HIV-1 transmission, pathogenesis, and treatment. Importantly, research done in the FIV model has allowed elucidation of issues that must be addressed in order to develop a preventative lentiviral vaccine. This review will cover general theories of lentiviral pathogenesis advanced by HIV-1, FIV, and SIV research, and survey theories of Treg cell involvement in lentiviral pathogenesis.

B. Current Theories of Lentiviral Immunopathogenesis

1. Cellular Receptors of Lentiviruses

Most invasive pathogens are equipped with various weapons that allow them to delay obliteration by host immune defenses, but perhaps no pathogens are as directly destructive to the immune system as HIV-1, FIV, and SIV. These lentiviruses primarily target CD4⁺ T cells for infection, but CD8⁺ T cells, B cells, dendritic cells, and macrophages are also affected during lentiviral pathogenesis (45, 46). The cellular receptor for most HIV and SIV strains is CD4. The chemokine receptors CXCR4 or CCR5 serve as coreceptors for HIV/SIV infection.

The characteristic decline of CD4⁺ T cells that begins during HIV-1 acute infection, and continues through the asymptomatic phase, is mirrored in FIV infection, even though the cellular receptor for FIV is CD134. Because CD134 is predominantly expressed by CD4⁺ T cells in the cat, these cells are targeted first for FIV infection (44, 49). FIV infection is mediated by the coreceptor CXCR4 only.

Only cells that express a receptor and/or a coreceptor amenable to HIV-1 attachment can be infected directly by the virus, but cells not expressing viral receptors can also be infected through virological synapses involved in cell-to-cell transmission (50-52). Most HIV-1 strains use CCR5 as a coreceptor upon transmission to a new host. The majority of CD4⁺CCR5⁺ T cells are effector memory cells that reside in extra-lymphoid effector mucosal sites, especially the intestine. These cells are the first targets for CCR5 (R5)-tropic lentiviral infection (53-55). In association with this aspect of HIV-1 pathogenesis, $\alpha_4\beta_7$ integrin, a homing receptor that guides immune cells to gut-associated lymphoid tissue (GALT), has been suggested to be a putative HIV-1 coreceptor (56, 57). Arthos and colleagues showed that the HIV-1 envelope protein gp120 is able to bind $\alpha_4\beta_7$ in its active form on lymphocytes through a conserved Leu-Asp-Val motif (56). This may be part of the reason why HIV-1 is able to destroy gut effector memory T cells so quickly after infection. DC-SIGN and syndecan-3 have also been suggested as attachment coreceptors for HIV-1 on dendritic cells, and may play a key role in disseminating virus systemically as dendritic cells migrate from sites of mucosal viral exposure to draining lymph nodes (58-61). More in vivo studies are required to support the

theory that $\alpha_4\beta_7$, DC-SIGN, and syndecan-3 are involved in lentiviral infection and dissemination, but in vitro and ex vivo evidence indicates that this is the case.

It is important to note that during chronic phase HIV-1 infection, coreceptor tropism switches from predominantly CCR5 to predominantly CXCR4 in about half of HIV-1⁺ patients (62, 63). Coreceptor switching is associated with progressive disease, but is not necessary for progression to AIDS (64, 65). Multiple factors likely play a role in coreceptor switching, including changes in chemokine receptor expression as the immune system responds to viral presence, and increased CXCR4 (X4)-tropic virus survival as selective immunological pressures shift over time (66-69). Changes also occur in cell tropism of FIV during chronic infection. The expression of CD134 and/or CXCR4 correlates well with FIV infection in CD8⁺ T cells, B cells, dendritic cells, and macrophages as disease progresses and viral tropism expands beyond infection of CD4⁺CD134⁺ T cells (44-47). Lentiviral infection causes drastic changes in overall immune system composition as cell targets expand over time.

2. Acute Phase Infection

The loss of CD4⁺ T cells during acute R5-tropic lentiviral infection is typically most dramatic in the intestinal mucosa where CD4⁺CCR5⁺ memory T cells reside. During the asymptomatic phase of R5-tropic viral infection, CD4⁺ T cell counts gradually decline in peripheral blood and secondary lymphoid organs such as lymph node and spleen (53-55, 70). On the other hand, acute CXCR4 (X4)-tropic

lentiviral infection is characterized by a notable loss of CD4⁺ T cells in the periphery and secondary lymphoid organs, as these organs house higher proportions of naïve CXCR4⁺ T cells (70, 71). X4-tropic viruses eventually target and deplete CD4⁺ T cells situated in the GALT during chronic infection (72), as about 50% of memory T cells express CXCR4 (71, 73, 74).

It has been proposed that the initial depletion of effector memory T cells in the GALT by R5-tropic viruses during the acute phase is a definitive event in the corruption of immune responses during lentiviral infection. However, this event by itself is not sufficient or necessary for direct progression to AIDS, as demonstrated by the fact that rapid disease progression rarely occurs after mucosal CD4⁺ cell depletion during HIV-1 infection or during nonpathogenic SIV infection. In addition, X4-tropic virus infection can progress to AIDS without causing acute phase GALT CD4⁺ cell depletion (75, 76).

Immune activation during acute lentiviral infection includes antiviral responses that, at first glance, appear to be appropriately specific. The burst of viral replication that results in partial depletion of CD4⁺ T cells is countermanded by cytotoxic T lymphocyte (CTL) and humoral immune responses. Neutralizing antibodies can be detected as early as 14 days post infection in SIV-infected macaques (77) and are present in FIV-infected cats (78-80). In addition, Gag-specific CTL responses or tetramer⁺ CD8⁺ T cells can be demonstrated as early as 7 days post-SIV infection, 14 days post-FIV infection, and are present in HIV-1 infected acute phase patients (77, 81-83). Indeed, CD8⁺ T cell depletion via monoclonal antibody

prior to SIV infection allows relatively unchecked SIV replication, demonstrating the importance of CD8⁺ CTL activity in combating lentiviral replication (81).

Betts and colleagues demonstrated that up to 18% of CD8⁺ T cells in HIV patients are virus-specific (84). However, in the same study it was shown that high frequency of HIV-specific T cells does not correspond directly with reduced viral load, indicating that other factors in addition to CTL activity are involved in control of viral replication (84). Accordingly, SIV-specific CD8⁺ effector T cells are present at very low levels in gastrointestinal mucosa during acute infection and are most robust after peak viremia, alluding to the possibility that antiviral CTL responses are misdirected and delayed during early infection (85). Acute pathogenic SIV infection in rhesus macaques, but not nonpathogenic SIV infection of sooty mangabeys, is characterized by robust IFN- α production by plasmacytoid dendritic cells (pDCs), which implicates pDCs as potentiators of damaging immune activation during acute infection (86). Increased activation-induced cell death of CD4⁺ T cells is linked to increased IFN- α production by HIV-1 exposed human pDCs, suggesting that pDC activation is linked to immune dysregulation during HIV-1 infection (87, 88). Early natural killer cell responses during nonpathogenic SIV infection are linked to control of viral replication, but are rarely observed in pathogenic SIV infection (89). SIV infection of macaques, but not sooty mangabeys, is characterized by activation of non-SIV specific immune responses and increased bystander cell apoptosis (90). It appears that though immune responses eventually control early viral replication, acute

stage infection is characterized by a crippled immune response that continues into chronic infection.

3. Chronic Phase Infection

a. Overview

Chronic lentiviral infection is characterized by a sustained increase in cytokine production of IFN- α , IFN- γ , TNF- α , TGF- β , IL-1 β , IL-6, and IL-10 (8, 88, 91-96), macrophage activation (97), B cell activation (10, 98, 99), and NK cell dysfunction (89, 100). Increased frequencies of memory and activated T cells is concurrent with decreased naïve T cell frequency (6, 71, 101, 102). T cell proliferation due to generalized immune activation (103, 104), increased bystander cell apoptosis, and constant replacement of lymphocytes and NK cells (105, 106) lead to T cell exhaustion and general immune dysfunction during lentiviral infection. The generalized immune dysfunction caused by lentiviral infection is described by coinfection studies. FIV-infected cats exhibit significantly impaired immune responses to experimental *Listeria monocytogenes* and *Toxoplasma gondii* coinfection (8, 9).

b. Immune activation marker expression correlates with disease progression

CD38 is an enzyme that catalyzes multiple reactions involved in lymphocyte activation, proliferation, and cytokine production, and it is involved in lymphocyte adhesion to endothelial cells (107). Interestingly, data collected over the past 16

years conclusively shows that CD38 expression by CD8⁺ T cells correlates strongly with HIV-1 disease progression and can be used as a predictor of CD4⁺ T cell loss (108-112). However, the extent of viral infection of individual cells and levels of lentiviral antigen do not explain the extent of immune activation and CD4⁺ T cell depletion that is observed during chronic lentiviral infection. The frequency of infected T cells during chronic disease is relatively low, around 0.01 – 1% (113). The death rate of CD8⁺ T cells is about the same as CD4⁺ T cells during HIV-1 and SIV infection even though infected CD8⁺ T cells are far less frequent than infected CD4⁺ T cells (105, 106, 114-116). This indicates that direct cellular infection is not necessarily the main cause of cell death during lentiviral pathogenesis. In recent years, T cell activation as a major cause of cell death has been shown to be a hallmark of lentiviral pathogenesis (6, 117-119). But what factors unique to lentiviral infection could be responsible for sustaining immune activation during the asymptomatic phase, which is characterized by low viral load?

c. Microbial translocation as a cause of immune activation

One probable cause of immune activation during chronic lentiviral infection can be traced back to depletion of about half of mucosal CD4⁺CCR5⁺ T cells during acute infection (54, 55, 120, 121). Even after low viral set-point is reached, effector memory T cell compartments in the gut are never fully restored (122), except if highly active antiretroviral therapy (HAART) is administered soon after infection (123, 124). Microbial translocation, the movement of bacteria or bacterial products through the gut lining, can occur with increased lining permeability and perturbation

of immune defenses in intestinal lumen (125). Brenchley et al. (126) found that plasma LPS levels are much higher in asymptomatic HIV-1⁺ patients or patients with AIDS as compared to healthy or acutely infected patients. Plasma LPS levels correlate positively with circulating CD8⁺CD38⁺ T cells and plasma IFN- α , and are increased during pathogenic SIV infection, but not during nonpathogenic SIV infection. LPS levels are reduced somewhat upon initiation of HAART treatment and are lower in HIV⁺ patients who are able to maintain low viremia versus those who exhibit progressive disease, demonstrating that LPS levels are linked to viral load (126). Administration of bioactive LPS in chronically SIV infected African green monkeys results in a burst of viral replication 2-4 hours post-treatment (127). These data suggest that the escape of microbial products from the intestinal tract during chronic lentiviral infection could contribute to continuous systemic immune activation.

These findings have been confirmed in a study that links increased plasma LPS to HIV-associated dementia and substance abuse by HIV-1⁺ patients (128). But another study found that plasma LPS levels did not change after HIV-1 infection in an African cohort (129). Importantly, LPS can induce Treg cell activation and proliferation, leading to enhancement of Treg cell immunosuppression (38, 130). However, the relationship between plasma LPS levels and Treg cell induction during lentiviral infection has not been explored. Additional studies will be required to determine the importance of microbial translocation in causing generalized immune

system activation during lentiviral infection, perhaps including prolonged antibiotic treatment of HIV-1⁺ patients.

d. Lentiviral-cellular interactions as a cause of immune activation and dysregulation

Non-virus-specific immune responses could be stimulated by lentiviruses themselves. In vitro evidence strongly suggests that gp120 is capable of eliciting cytokine and chemokine production after binding CD4 and coreceptors, even without productive infection or traditional viral antigen presentation (97, 131, 132). In addition, gp120-CD4 interaction has been shown to prime CD4⁺ T cells for apoptosis and promote FasL expression on T cells and macrophages, which likely leads to bystander cell death in the absence of direct infection (133, 134). Membrane bound gp120 on the surface of infected cells is able to induce cell death in non-infected cells (135, 136). IFN- α production by plasmacytoid dendritic cells has also been implicated in mediating bystander cell death, in that it induces TNF-related apoptosis-inducing ligand (TRAIL)/death receptor (DR) 5 expression by CD4⁺ T cells (88). And of course, gp120 is involved in direct apoptosis of infected cells as syncytium form (137-139). Thus, during lentiviral infection the immune system is forced to cope with ongoing cell death, which leads to replacement of T cells from the thymus, and immune activation, which leads to increased naïve and memory T cell turnover. NF κ B and NFATc are directly involved in T cell activation signaling pathways. Activation of NF κ B and NFATc leads to lentiviral LTR transcription in infected cells, which results in production of new virions (140-142). The attempt to reconstitute lost

immune effector cells also leads to the generation of new target cells (103, 106). The debilitating effects of lentiviral infection on the immune system are therefore linked to the continuous generation of new target cells and new virions which is in turn linked to exhaustive T cell activation and immune dysregulation.

C. Regulatory T cells

1. Overview

Treg cells were hypothesized to exist in the 1970s, but when researchers were not able to phenotypically identify a Treg cell population or characterize mechanisms of Treg cell suppression, the theory that T cells could negatively regulate the immune response fell out of favor among immunologists. But in 1995, the Sakaguchi laboratory published that CD25 expression is constitutive on immunosuppressive T cells (143). Since their renewed appearance in the literature, Treg cells have received much attention from researchers. Treg cells have been shown in both in vitro and in vivo experiments to be capable of repressing immune responses through a variety of mechanisms which act directly on antigen presenting cells or effector cells. Mechanisms of regulation employed by Treg cells have been reviewed recently [see refs. (144-146)] and will not be covered in detail here.

2. The Transcription Factor FOXP3

a. Expression in naturally occurring, thymically derived Treg cells

The most well characterized molecule associated with Treg cell development and function is the transcription factor FOXP3. FOXP3 is associated with CD25 expression as early as the double positive stage in thymic lymphocytes (26, 147). Thymic Treg cell development as a distinct lineage depends on the presence of FOXP3 (26, 30); however, a heterogenic FOXP3 allele knock-out mouse model has shown that some of the properties of murine Treg cells are not dependent on FOXP3, including expression of CD25, paucity of cytokine production, and in vitro anergy (148). In contrast, suppressor function and in vivo proliferative ability were shown to be dependent on FOXP3 (148), and these results are confirmed in a report by Lin and colleagues (149). FOXP3 is proposed to be a stabilizing element that locks into place gene regulation that supports the Treg cell phenotype, which is first directed through T cell receptor (TCR) activation, CD28 costimulation, or some other unidentified signal in the thymus (148, 150, 151).

b. Expression during Treg cell conversion

It is worth noting that most of the experiments done to characterize the consequences of lymphocyte FOXP3 expression have been performed using mouse models. In humans and cats, FOXP3 is not an exclusive identifier of Treg cells, as is the case in mice (152-157). FOXP3 is transiently expressed at low levels upon conventional CD4⁺ T cell activation, with no corresponding suppressor function, much like CD25 (153, 155, 157). It is possible for conventional CD4⁺ T cells to

permanently adopt a Treg cell phenotype extra-thymically, but this requires sustained FOXP3 expression (157, 158). It is probable that both scenarios are present during typical immune reactions to infection, and that new antigen-specific Treg cells are converted hand in hand with the development of immunity. Indeed, it has been shown that human CD4⁺CD25⁻CD45RO⁺ memory T cells and CD4⁺CD25⁺CD45RO⁺ Treg cells are related based on shared TCR V β family populations between the cell subsets (159-162). Likewise, murine memory T cells and Treg cells share substantial CDR3 sequence homology in the TCR^{mini} mouse model, which is characterized by limited TCR diversity, (163, 164) or after experimental immunization with pigeon cytochrome *c* in wildtype mice (165). These studies estimate that up to an 80% overlap exists between FOXP3⁺ Treg cell and FOXP3⁻ memory T cell TCR repertoires.

3. Treg Cell Activity during Infection

a. Adaptive Treg cells

Treg cells that are generated through conversion in the periphery rather than being generated de novo in the thymus are called induced or adaptive Treg cells. Adaptive Treg cells are indistinguishable from naturally occurring Treg cells because both populations express the same phenotypic markers and perform the same function in regulating the immune system. In vitro studies using human and murine cells have shown that exposing CD4⁺CD25⁻ T cells to TCR stimulation (29, 158, 166, 167) and/or TGF- β (29, 168-171), or IFN- γ (172) can induce FOXP3 expression and

immunosuppressive activity. In vivo studies in mice have shown that prolonged exposure to antigen and/or TCR costimulation can induce Treg cells (27, 173-175).

The presence of IL-2 is required for maintenance of the Treg cell population. The production of IL-2 during immune responses would support the survival of induced Treg cells (175, 176). Perhaps during the resolution of an immune response, decreased levels of IL-2 would promote apoptosis in Treg cells, which are more prone to apoptosis than conventional T cells due to their highly differentiated state, high Fas expression, and low Bcl-2 expression (161, 177, 178). As a result, normal immunity could proceed uninhibited during secondary exposure to antigen because relevant Treg cells would be absent until the conversion of a new round of antigen specific Treg cells. However, it is important to note that during chronic infection and continual exposure to antigen, the pool of antigen specific Treg cells could be maintained or replenished indefinitely and could prematurely inhibit needed antigen specific immune responses.

b. Treg cell proliferation in vivo

Another event that may play a key role in Treg cell influence during immune responses is expansion of the pool of naturally occurring or adaptive Treg cells. Treg cells exhibit a doubling rate of 8 days in healthy human peripheral blood mononuclear cells (PBMCs) as compared to a doubling rate of 24 days for CD4⁺ memory T cells (161). Treg cells are more frequent and express more Ki67 as a measure of proliferation in the PBMCs of acute myeloid leukemia patients (179). Infection of mice with herpes simplex virus induces Treg cell expansion in draining

lymph nodes (13). Expanded Treg cells were better able than Treg cells from naïve mice to suppress herpes virus-specific responses (13). Other studies in mice have confirmed antigen-induced Treg cell expansion in vivo (180, 181).

In vitro studies have determined that Treg cell expansion in vitro is at least partially dependent on IL-2 and does not cause loss of function (38, 182, 183). In fact, several studies point to evidence that Treg cell suppression is enhanced after exposure to antigens and subsequent expansion (13, 162, 181, 182). Antons et al. (162) have described enhancement of suppressive activity in the CD4⁺CD25⁺CD45RA⁺ naïve Treg cell subset upon expansion in the presence of IL-2. Treg cell induction and expansion during infection may explain the recent finding that, contrary to previous thought, FOXP3⁺ Treg cells are not enriched in expression of TCRs specific for self antigens. Instead, a study by Pacholczyk and colleagues shows that self-reactive T cells are scarce in both Treg cell and conventional CD4⁺ T cell populations (163).

c. Synchronous activation of Treg cells and T effector cells during infection

Recently, evidence has emerged which indicates that though Treg cells may be generated and activated during infection, their suppressive effects may not be felt until the effector immune response is waning. This topic has been recently reviewed in ref. (184). Certain cytokines produced during infection can counterbalance Treg cell suppression, including IL-2, IL-4, IL-7, IL-15, and IL-21, which bind receptors containing the common γ chain (185-189). Conventional T cells and Treg cells

express toll-like receptors (TLRs) that recognize molecular patterns displayed by pathogens (130). Ligation of TLR-2, TLR-8, or TLR-9 on either cell subset has been shown to render conventional T cells refractory to Treg cell suppression and/or temporarily disrupt Treg cell suppressive function (190-193). Some reports show evidence that Treg cells are activated and more suppressive after TLR-2, TLR-4, or TLR-5 ligation (130, 192, 194, 195). It is possible that during infection, Treg cells gather strength upon antigen exposure, but do not exert their regulatory abilities until after a potent immune response occurs.

D. The Role of Treg Cells during Lentiviral Pathogenesis

1. Treg Cell Dynamics during Lentiviral Infection

a. Treg cells in lymphoid tissue

Lentiviruses replicate in lymphoid tissue, and studies suggest that Treg cells accumulate in lymphoid tissue during lentiviral infection, resulting in higher Treg cell to conventional CD4⁺ T cell ratios at sites of viral replication (96, 196-198). This has important implications for lentiviral disease pathogenesis. During lentiviral infection, Treg cells are depleted systemically as is the total CD4⁺ T cell population (199-201). But evidence suggests that Treg cells may be depleted at a slower rate than conventional CD4⁺ T cells. Treg cells in cats are relatively resistant to apoptosis and apoptotic resistance remains unchanged when feline Treg cells are infected with FIV (202). Nilsson and colleagues found that HIV-1 gp120 engagement of CD4 promotes the survival of Treg cells by decreasing the incidence of apoptosis. In the same study,

it was shown that the presence of HIV-1 virions in culture increases the proportion of FOXP3⁺ cells to total CD4⁺ T cells over 3-5 days (96). An in vitro study conducted by Ji and Cloyd supports these data, and furthermore, asserts that HIV-1 binding to human Treg cells enhances expression of CD62L and $\alpha_4\beta_7$ and enhances Treg cell homing to lymph nodes and the duodenum in SCID mice (203). A report by Epple et al. (197) states that Treg cells make up 40% of the CD4⁺ T cell subset in the duodenum of untreated HIV-1⁺ patients, compared to 1.2% in uninfected individuals. In contrast, Chase et al. (121) found a decrease in the percentage of Treg cells in the lamina propria, but not lymph nodes, of SIV infected macaques from day 4 to 114 post-infection.

Rapid induction or accumulation of Treg cells expressing TGF- β , IL-10, and IDO in lymph nodes during acute pathogenic SIV infection has been demonstrated (201). FOXP3, IDO, and CTLA-4 mRNA is increased in CD4⁺ T cells from the lymph nodes, spleen, and colon, but not jejunum, of high viremic SIV-infected macaques (198). Increased levels of Treg cells in lymph nodes corresponded positively with induction of Ki67⁺ cells 1 to 4 weeks post-SIV infection, but not post-CMV infection, indicating that immune activation during SIV infection may drive the induction or accumulation of Treg cells (201). Treg cell induction in pathogenic SIV infection does not appear to be able to limit immune activation in macaques (201), but very early Treg cell and TGF- β induction 24 hours post-SIV infection in African green monkeys is correlated with control of immune activation (204).

b. Treg cells in the periphery

Many studies have reported either a loss (199, 205, 206), an increase (207, 208), or no change (38, 109, 121, 197, 209) in Treg cell levels in the blood of viremic chronically lentiviral-infected individuals as compared to non-viremic or uninfected controls, with no definite conclusions possible regarding Treg cell dynamics in response to lentiviral infection based on Treg cells from PBMCs. It is important to consider that analysis of peripheral Treg cell levels through percentage versus absolute number may lead to widely divergent conclusions that may not necessarily reflect Treg cell levels at sites of viral replication in the tissues.

In a comparison of Treg cell dynamics in pathogenic and nonpathogenic SIV infection, it was found that pathogenic infection increases PBMC Treg cell frequency at week 3 post-infection, with an eventual decline in PBMC and tissue Treg cell levels by week 11 post-infection (206). In contrast, nonpathogenic SIV infection did not change PBMC Treg cell frequency, though Treg cell levels in lymphoid tissue were not reported at any time from these animals. The authors hypothesize that during pathogenic SIV infection Treg cell induction may occur early enough to inappropriately prevent needed antiviral responses during the acute phase but not soon enough to prevent immune hyperactivation in the chronic phase (206).

c. Possible CD25 downregulation by Treg cells during lentiviral infection

A phenomenon of Treg cell CD25 downregulation or shedding during lentiviral infection has been proposed (96, 196). Only 19% of tonsillar FOXP3⁺ T cells in HIV-1 progressors are CD25⁺, compared with 44% in HIV nonprogressors

and 75% in uninfected individuals (96, 196). A separate study confirmed the presence of a substantial portion of FOXP3⁺CD25⁻ T cells in lymph nodes of HIV-1⁺ patients, but almost all CD25^{hi} cells express FOXP3 in these patients and FOXP3⁺CD25^{hi} T cells were potently suppressive against HIV-specific immune responses (210). Cellular expression of FOXP3 was found to correlate very well with CTLA-4 expression (86%) in tonsils of HIV-1 progressors (96, 196), but not in lymph nodes of chronically infected HIV-1⁺ patients (210). In addition, FOXP3⁺CD25⁻ T cells were found to inversely correlate with levels of CD8⁺CD38⁺ T cells in the blood of chronically infected HIV-1⁺ patients, while no obvious relationship was found between CD8⁺ T cell activation and FOXP3⁺CD25⁺ Treg cell levels. This suggests that FOXP3⁺CD25⁻ Treg cells may be partially responsible for mediating immunosuppression during HIV-1 infection, but their function has not been determined in an ex vivo coculture suppression assay. The theoretical downregulation of CD25 on Treg cells during lentiviral infection remains a controversial topic, as CD25 expression is important for IL-2 signaling and Treg cell homeostasis.

2. Direct Lentiviral Infection of Treg Cells

Treg cells and conventional CD4⁺ T cells may be differentially susceptible to lentiviral infection, but this remains a controversial topic. Feline Treg cells express CD134 and CXCR4 at higher levels than CD4⁺CD25⁻ T cells (47). Human CD4⁺CD25⁺CD45RO⁺ memory Treg cells and expanded CD4⁺CD25⁺CD45RA⁺

naïve Treg cells both express high levels of CXCR4 and CCR5 (162). Both subsets are susceptible to infection by R5-tropic HIV-1 strains, but are not more infected than CD4⁺CD25⁻CD45RO⁺ memory T cells (162, 211). Additional studies have found that Treg cells are either not more infected (200, 201) or less infected (212) by lentiviruses than CD4⁺CD25⁻ cells. However, some studies suggest that Treg cells are more productively infected than CD4⁺CD25⁻ T cells (211, 213). Feline Treg cells require a much lower activation threshold compared to CD4⁺CD25⁻ T cells in order to initiate virus production and feline Treg cells constitutively express transcription factors that are important for FIV replication (47, 213). In providing a mechanism for this observation, Holmes et al. (214) writes that transfection of FOXP3 into Jurkat cells promotes HIV-1 gene expression because FOXP3 enhances NFκB binding to HIV-1 LTR. It is possible that Treg cells, while probably not more infected on a per cell basis than other CD4⁺ cells, could serve as long-lived viral production factories and contribute substantially to viral load in lymphoid tissues.

3. Evidence for a Detrimental Role of Treg Cells during Lentiviral Pathogenesis

Treg cells are present during lentiviral infection and studies have shown no evidence of loss of function in Treg cells from HIV-1 or FIV infected hosts (96). In fact, work done by the Tompkins laboratory has shown that lymph node CD4⁺CD25^{hi} Treg cells are more suppressive during FIV infection compared to Treg cells from healthy cats (38, 215). In vitro exposure of healthy human Treg cells to HIV-1 enhances the suppressive abilities of Treg cells 2-5 fold and upregulates FOXP3

expression by Treg cells (203). A study from Kinter et al. (207) resulted in somewhat different conclusions, as no difference was evident between the general suppressive potential of human PBMC Treg cells from HIV-1⁺ patients and healthy controls. But a later study by that same group indicates that lymph node Treg cells from HIV-1 patients with higher viremia are more suppressive against HIV-specific CTL activity than Treg cells from low viremia patients, indicating that Treg cell fitness is linked to higher viremia as antiviral responses are suppressed (210). SIV infection of rhesus macaques has been shown to decrease the ability of ileum (121) or circulating (206) Treg cells to inhibit allogeneic T cell proliferation, however the same Treg cells were able to potently suppress SIV-specific IFN- γ production (206).

Many studies have shown that viral specific CD4⁺CD25⁻ or CD8⁺ T cell responses in lentiviral infected individuals are revealed when CD4⁺CD25⁺ Treg cells are removed from cell culture as measured by non-Treg cell proliferation, CTL killing of target cells, or chemokine or cytokine production in the presence of lentiviral antigens (167, 199, 206, 207, 210, 216-220). In vivo observations confirm the assertion that Treg cells could suppress needed antiviral responses, as HIV-1 and SIV infected individuals with inherently vigorous CD4⁺ Gag- or Tat-specific immune responses have lower frequencies of Treg cells in the blood (199, 201, 209). Andersson et al. (196) found that before HAART treatment, viral load positively corresponds to FOXP3 mRNA levels in tonsils. HAART treatment dramatically reduced viral load and FOXP3 mRNA in tonsils, but the reverse was found in the blood. HAART treatment increased FOXP3 mRNA in peripheral blood cells,

cautioning the use of PBMCs to assess correlations between Treg cells and disease progression markers (196). Also, when lymphoid tissue FOXP3 levels are compared between HIV and SIV disease progressor and non-progressor patients (status determined by viremia levels), non-progressors display lower FOXP3 expression in tonsils, lymph nodes, or spleen at levels on par with uninfected patients, and a high perforin to FOXP3 ratio is associated with nonprogressive disease (96, 198). These studies highlight the possibility that Treg cells are one of the causes of immune dysfunction during lentiviral infection by suppressing potentially effective antiviral responses. However, it is unclear whether removal of Treg cells in an in vivo situation would unleash effective antiviral T cell responses resulting in viral load reduction because effector responses have been shown to be impaired during lentiviral infection, although these studies did not take into account the presence of Treg cells in vivo (8, 9, 221, 222).

4. Evidence for a Protective Role of Treg Cells during Lentiviral Pathogenesis

Several studies point to a protective role of Treg cells in lentiviral infection, as high PBMC Treg cell frequencies are correlated with higher CD4⁺ T cell counts, lower T cell activation, and/or lower viremia, or vice versa (109, 206, 207, 209, 211, 212, 218). FOXP3 mRNA copy number in blood is decreased in non-HAART treated viremic patients and HAART treated patients, but higher on average in elite suppressor patients (individuals who control viral replication naturally without HAART treatment) as compared to uninfected controls (212). These data indicate

that greater Treg cell levels are associated with a lack of disease progression.

However, no significant correlation between FOXP3 copy number and CD4 count was found in this study (212).

It has been shown that HIV-1⁺ patients who possess Treg cells with strong immunosuppressive activity in vitro also possess CD4⁺CD25⁻ effector T cells that are able to produce significantly higher levels of TNF- α and IFN- γ than effector T cells from HIV-1⁺ patients with weak Treg cell activity (207). It is difficult to determine whether low viremia in HIV-1 infected patients with more prevalent Treg cell presence is due to Treg cell suppression of immune activation or some other factor mediating more effective antiviral responses.

E. Experimental Treg Cell Depletion

1. In Vitro CD25⁺ Cell Depletion

Depletion of CD25^{hi} T cells from ex vivo cell cultures is an effective method used to evaluate immune responses in the absence of Treg cells. While it is true that not all FOXP3-expressing CD4⁺ T cells also express CD25 (96, 196, 210), a great deal of immunosuppressive potential lies in the CD25^{hi} population, as incubation of CD4⁺CD25^{hi} Treg cells from lentiviral infected or uninfected hosts with CD25⁻ T cells results in immunosuppression. Likewise, depletion of CD4⁺CD25^{hi} Treg cells from ex vivo cell cultures using either magnetic activated cell sorting or fluorescence activated cell sorting reveals immune activity upon stimulation (167, 199, 206, 210, 216-219, 223, 224). One study compared the efficacy of anti-CD25 antibody coupled

to magnetic microbeads, Ontak (denileukin diftitox; diphtheria toxin conjugated to human IL-2), and anti-CD25 immunotoxin (RFT5-deglycosylated ricin A chain) in removing CD25⁺ T cells from activated PBMCs in vitro (225). Ontak was found to be far less effective than anti-CD25 magnetic beads or anti-CD25 immunotoxin in depleting CD25⁺ T cells in vitro (225).

2. In Vivo CD25⁺ Cell Depletion

a. CD25⁺ cell depletion in cancer models or cancer patients

Reagents that have been used to deplete or functionally inactivate CD25 expressing cells in vivo include anti-CD25 monoclonal antibodies (mAb), Ontak, and low dose cyclophosphamide, which targets rapidly dividing cells (224, 226-228). These three methods of in vivo CD4⁺CD25⁺ Treg cell depletion were compared in a mouse melanoma model when administered through the intraperitoneal route (229). The anti-murine CD25 mAb PC61 was found to be the most effective Treg cell depleting reagent. PC61 administration resulted in prolonged depletion of 75% of Treg cells without perturbation of B cells, CD4⁺CD25⁻ T cells, or CD8⁺ T cells, and also resulted in slower tumor growth. Cyclophosphamide depleted Treg cells but also depleted the majority of lymphocytes and CD8⁺ anti-tumor T cells, and Ontak only depleted half of the Treg cell population (229).

Taken one step further, Treg cell depletion via PC61 mAb enhances anti-melanoma immune responses when administered with antigen-pulsed dendritic cells or tumor antigen loaded particles (229-232). Anti-CD25 mAb-mediated depletion of

Treg cells enhances vaccine efficacy directed against pancreas adenocarcinoma tumors, herpes simplex virus type 1, and human papillomavirus in mouse models (233-235). Though it reduces the Treg population only by half on average in humans, Ontak has been shown to enhance the anti-tumor effects of a therapeutic dendritic cell vaccine directed against metastatic renal cell carcinoma (224). Ontak also boosted a carcinoembryonic antigen-dendritic cell vaccine in human cancer patients and allowed regression of metastases in 31% of melanoma patients enrolled in a clinical trial (236, 237). Taken together, these studies demonstrate that depletion of Treg cells in vivo is an effective method of boosting vaccine elicited immunity, especially against tumor antigens.

b. CD25⁺ cell depletion in models of pathogenic infection

Murine studies have shown that in vivo Treg cell depletion is sufficient on its own to boost immune responses to herpes simplex virus 1, malaria, *Litomosoides sigmodontis* helminth parasites, and *Trypanosoma congolense* even when a vaccine is not administered (37, 238-241). But the lack of a role for Treg cells in depressing protective immune responses has been shown during rotavirus infection, chronic *Toxoplasma gondii* infection, and acute *Pseudomonas aeruginosa* infection in mice (242-244). Depletion of Treg cells actually augments pathogenesis in coronavirus-induced acute encephalitis (245). These studies demonstrate that effects of Treg cell depletion in vivo are specific to the experimental model and infectious agent used.

c. Efficacy and limitations of in vivo CD25⁺ cell depletion using anti-CD25 monoclonal antibodies

Rat anti-mouse CD25 mAbs PC61 (IgG isotype) and 7D4 (IgM isotype) have been shown to abrogate Treg cell function by two different mechanisms, causing depletion of CD4⁺CD25⁺ Treg cells or CD25 shedding from Treg cells, respectively (246-250). Actual depletion of Treg cells by anti-CD25 mAb can be detected by observing a reduction in total CD4⁺ T cell absolute number, a reduction in CD4⁺CD25^{hi} T cell absolute number without a corresponding increase in CD4⁺CD25^{lo} T cell absolute number, a reduction in CD4⁺FOXP3⁺ T cell percent or absolute number, or a reduction of FOXP3 mRNA levels in lymphocytes.

Some limitations exist when an anti-CD25 mAb is used to deplete Treg cells in vivo, as anti-CD25 mAb does not eliminate all CD25⁺ Treg cells in any species, and some FOXP3⁺CD25^{lo} T cells remain after mAb treatment that may or may not possess immunosuppressive capability (250). Still, even without complete depletion of Treg cells, many studies have shown clinical differences in disease progression when the Treg cell to T effector cell ratio is disrupted. The transient nature of Treg cell depletion must be taken into account when analyzing the effects of anti-CD25 mAb treatment in vivo, as it is often applied to animals exposed to some type of infectious agent. For example, Couper et al. (250) have shown that the duration of Treg cell depletion is reduced when followed by acute malaria infection in mice, as an increase in peripheral CD4⁺FOXP3⁺ T cell absolute number occurs between 7-10 days post mAb treatment. This is not surprising, considering that adaptive Treg cells

are induced and proliferate following antigen exposure. Another key factor to be considered when using anti-CD25 mAb in vivo is immune status of the recipient animal. As demonstrated by Couper et al. (242), exposing animals to infectious agents within four days of anti-CD25 mAb administration results in depletion of effector T cells as well as Treg cells, because anti-CD25 mAb was still present at the time of CD25 upregulation by activated T cells.

3. Treg cell depletion or functional blockade during lentiviral infection

Two studies have examined the effects of CTLA-4 blockade in SIV-infected macaques. CTLA-4 is expressed by activated T cells and is enriched in the FOXP3⁺ T cell population. It has been proposed that Treg cells compete with effector T cell-dendritic cell interactions by ligating CD80/CD86 on dendritic cells through CTLA-4, and that Treg cells can induce indoleamine 2,3-dioxygenase (IDO) production by dendritic cells through CTLA-4 ligation (251, 252). In the first study conducted by Franchini and colleagues, anti-CTLA-4 blocking mAb was administered during ART treatment after macaques had been infected with SIV for almost two years (220). CTLA-4 blockade did not change Ki67⁺ T cell levels nor did it change viremia set-point after cessation of ART. However, proviral levels and TGF- β mRNA were decreased in lymph nodes at 6 weeks following the first treatment of CTLA-4 blockade and beginning of ART as compared to macaques treated only with ART (220). This effect may have been transient, as lymph node viral load was not quantified after cessation of ART or after the last administration of anti-CTLA-4

mAb. Tet-specific CD8⁺ T cells were more frequent in the blood of two macaques 10 weeks following the beginning of CTLA-4 blockade and initiation of ART (220).

In a second study conducted by Franchini and colleagues, macaques were given anti-CTLA-4 blocking mAb 4 days prior to SIV infection, and then given three additional treatments of anti-CTLA-4 blocking mAb three weeks apart (253). CTLA-4 blockade resulted in a faster loss of CD4⁺CCR5⁺ T cells in lymphoid tissues and higher viremia, indicating that CTLA-4 blockade exacerbates disease during acute SIV infection. In a subsequent experiment designed to evaluate the effects of CTLA-4 blockade in conjunction with ART and a therapeutic SIV vaccine on viral load, ART was started 14 weeks after SIV infection, and 8 weeks later three groups of macaques were administered either SIV vaccine three times five weeks apart, CTLA-4 blocking mAb four times five weeks apart, or both treatments (253). CTLA-4 blockade prevented the decrease in viral load temporarily afforded by ART in one group of animals and prevented a one log reduction in viremia that was observed in animals administered SIV vaccine only (253). Ligation of CTLA-4 on T cells interrupts cellular activation, therefore increased T cell activation provided by CTLA-4 blockade seems to enhance SIV pathogenesis during acute and early chronic infection (253). Treg cells have been shown to exert their immunosuppressive properties in many ways in addition to CTLA-4 binding to target cells. Therapy that results in actual systemic depletion of Treg cells during lentiviral infection would likely be more able to deduce the role of Treg cells in lentiviral pathogenesis.

In a study that attempted Treg cell depletion during chronic nonpathogenic SIV infection, SIV infected African green monkeys were administered Ontak three times two months apart for five consecutive days (127). Surprisingly, absolute numbers of CD4⁺CD25⁺ and CD4⁺FOXP3⁺ were not decreased in these animals and Treg cell function was not altered by Ontak treatment. Nevertheless, Ontak treatment induced a transient increase in Ki67⁺ and HLA-DR⁺ T cells that corresponded with a transient increase in viremia. This data reinforces the theory that immune activation increases lentiviral replication (127).

Jiang et al. (254) presented the first report of Treg cell depletion during the acute phase of lentiviral disease in an experimental HIV-1 animal model. Using a humanized *Rag2*^{-/-} *γC*^{-/-} mouse model, mice were infected with HIV-1 two days after Ontak administration (254). Viremia was reduced at day 10 post-infection when two mice were treated with Ontak compared to untreated mice. Because Treg cells in HIV-1 infected humanized mice are 2-5 times more infected than conventional CD4⁺ T cells, the authors concluded that Treg cell depletion was able to reduce overall viremia during acute HIV-1 infection by eliminating an important viral reservoir.

These studies emphasize the need for additional experiments examining the role of Treg cells in natural lentiviral infection via effective depletion of the majority of Treg cells before the acute phase or during the chronic phase of infection. The ability to observe lentiviral pathogenesis in the context of reduced Treg cell levels would be valuable in determining the role of Treg cells in regulating the complex and varied immune responses that are generated during lentiviral infection.

References

1. Troyer, J. L., J. Pecon-Slattery, M. E. Roelke, W. Johnson, S. VandeWoude, N. Vazquez-Salat, M. Brown, L. Frank, R. Woodroffe, C. Winterbach, H. Winterbach, G. Hemson, M. Bush, K. A. Alexander, E. Revilla, and S. J. O'Brien. 2005. Seroprevalence and genomic divergence of circulating strains of feline immunodeficiency virus among Felidae and Hyaenidae species. *J Virol* 79:8282-8294.
2. Olmsted, R. A., V. M. Hirsch, R. H. Purcell, and P. R. Johnson. 1989. Nucleotide sequence analysis of feline immunodeficiency virus: genome organization and relationship to other lentiviruses. *Proc Natl Acad Sci U S A* 86:8088-8092.
3. Hohdatsu, T., R. Pu, B. A. Torres, S. Trujillo, M. B. Gardner, and J. K. Yamamoto. 1993. Passive antibody protection of cats against feline immunodeficiency virus infection. *J Virol* 67:2344-2348.
4. Bucci, J. G., R. V. English, H. L. Jordan, T. A. Childers, M. B. Tompkins, and W. A. Tompkins. 1998. Mucosally transmitted feline immunodeficiency virus induces a CD8+ antiviral response that correlates with reduction of cell-associated virus. *J Infect Dis* 177:18-25.
5. Song, W., E. W. Collisson, P. M. Billingsley, and W. C. Brown. 1992. Induction of feline immunodeficiency virus-specific cytolytic T-cell responses from experimentally infected cats. *J Virol* 66:5409-5417.
6. Tompkins, M. B., M. E. Bull, J. L. Dow, J. M. Ball, E. W. Collisson, B. J. Winslow, A. P. Phadke, T. W. Vahlenkamp, and W. A. Tompkins. 2002. Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis. *J Infect Dis* 185:1077-1093.
7. Torten, M., M. Franchini, J. E. Barlough, J. W. George, E. Mozes, H. Lutz, and N. C. Pedersen. 1991. Progressive immune dysfunction in cats experimentally infected with feline immunodeficiency virus. *J Virol* 65:2225-2230.
8. Dean, G. A., J. A. Bernales, and N. C. Pedersen. 1998. Effect of feline immunodeficiency virus on cytokine response to *Listeria monocytogenes* in vivo. *Vet Immunol Immunopathol* 65:125-138.
9. Davidson, M. G., J. B. Rottman, R. V. English, M. R. Lappin, and M. B. Tompkins. 1993. Feline immunodeficiency virus predisposes cats to acute generalized toxoplasmosis. *Am J Pathol* 143:1486-1497.

10. Ackley, C. D., J. K. Yamamoto, N. Levy, N. C. Pedersen, and M. D. Cooper. 1990. Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *J Virol* 64:5652-5655.
11. Pedersen, N. C., E. W. Ho, M. L. Brown, and J. K. Yamamoto. 1987. Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science* 235:790-793.
12. Yamamoto, J. K., H. Hansen, E. W. Ho, T. Y. Morishita, T. Okuda, T. R. Sawa, R. M. Nakamura, and N. C. Pedersen. 1989. Epidemiologic and clinical aspects of feline immunodeficiency virus infection in cats from the continental United States and Canada and possible mode of transmission. *J Am Vet Med Assoc* 194:213-220.
13. Lund, J. M., L. Hsing, T. T. Pham, and A. Y. Rudensky. 2008. Coordination of early protective immunity to viral infection by regulatory T cells. *Science* 320:1220-1224.
14. Cao, X., S. F. Cai, T. A. Fehniger, J. Song, L. I. Collins, D. R. Piwnica-Worms, and T. J. Ley. 2007. Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity* 27:635-646.
15. Misra, N., J. Bayry, S. Lacroix-Desmazes, M. D. Kazatchkine, and S. V. Kaveri. 2004. Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J Immunol* 172:4676-4680.
16. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287-296.
17. Maloy, K. J., L. Salaun, R. Cahill, G. Dougan, N. J. Saunders, and F. Powrie. 2003. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 197:111-119.
18. Grossman, W. J., J. W. Verbsky, B. L. Tollefsen, C. Kemper, J. P. Atkinson, and T. J. Ley. 2004. Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells. *Blood* 104:2840-2848.
19. Kaparakis, M., K. L. Laurie, O. Wijburg, J. Pedersen, M. Pearce, I. R. van Driel, P. A. Gleeson, and R. A. Strugnell. 2006. CD4+ CD25+ regulatory T cells modulate the T-cell and antibody responses in helicobacter-infected BALB/c mice. *Infect Immun* 74:3519-3529.
20. Tran, D. Q., J. Andersson, R. Wang, H. Ramsey, D. Unutmaz, and E. M. Shevach. 2009. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on

- platelets and activated FOXP3⁺ regulatory T cells. *Proc Natl Acad Sci U S A* 106:13445-13450.
21. Wang, R., Q. Wan, L. Kozhaya, H. Fujii, and D. Unutmaz. 2008. Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. *PLoS One* 3:e2705.
 22. Liu, W., A. L. Putnam, Z. Xu-Yu, G. L. Szot, M. R. Lee, S. Zhu, P. A. Gottlieb, P. Kapranov, T. R. Gingeras, B. Fazekas de St Groth, C. Clayberger, D. M. Soper, S. F. Ziegler, and J. A. Bluestone. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4⁺ T reg cells. *J Exp Med* 203:1701-1711.
 23. Wing, K., Y. Onishi, P. Prieto-Martin, T. Yamaguchi, M. Miyara, Z. Fehervari, T. Nomura, and S. Sakaguchi. 2008. CTLA-4 control over Foxp3⁺ regulatory T cell function. *Science* 322:271-275.
 24. Nakamura, K., A. Kitani, I. Fuss, A. Pedersen, N. Harada, H. Nawata, and W. Strober. 2004. TGF-beta 1 plays an important role in the mechanism of CD4⁺CD25⁺ regulatory T cell activity in both humans and mice. *J Immunol* 172:834-842.
 25. Kohm, A. P., J. S. Williams, and S. D. Miller. 2004. Cutting edge: ligation of the glucocorticoid-induced TNF receptor enhances autoreactive CD4⁺ T cell activation and experimental autoimmune encephalomyelitis. *J Immunol* 172:4686-4690.
 26. Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22:329-341.
 27. Liang, S., P. Alard, Y. Zhao, S. Parnell, S. L. Clark, and M. M. Kosiewicz. 2005. Conversion of CD4⁺ CD25⁻ cells into CD4⁺ CD25⁺ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med* 201:127-137.
 28. Brandenburg, S., T. Takahashi, M. de la Rosa, M. Janke, G. Karsten, T. Muzzolini, Z. Orinska, S. Bulfone-Paus, and A. Scheffold. 2008. IL-2 induces in vivo suppression by CD4(+)CD25(+)Foxp3(+) regulatory T cells. *Eur J Immunol* 38:1643-1653.
 29. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4⁺CD25⁻ naive T cells to CD4⁺CD25⁺ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198:1875-1886.

30. Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.
31. Fontenot, J. D., J. L. Dooley, A. G. Farr, and A. Y. Rudensky. 2005. Developmental regulation of Foxp3 expression during ontogeny. *J Exp Med* 202:901-906.
32. Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.
33. Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks. 2002. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* 420:502-507.
34. Suffia, I. J., S. K. Reckling, C. A. Piccirillo, R. S. Goldszmid, and Y. Belkaid. 2006. Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens. *J Exp Med* 203:777-788.
35. Lundgren, A., E. Suri-Payer, K. Enarsson, A. M. Svennerholm, and B. S. Lundin. 2003. *Helicobacter pylori*-specific CD4+ CD25high regulatory T cells suppress memory T-cell responses to *H. pylori* in infected individuals. *Infect Immun* 71:1755-1762.
36. Suvas, S., A. K. Azkur, B. S. Kim, U. Kumaraguru, and B. T. Rouse. 2004. CD4+CD25+ regulatory T cells control the severity of viral immunoinflammatory lesions. *J Immunol* 172:4123-4132.
37. Hisaeda, H., Y. Maekawa, D. Iwakawa, H. Okada, K. Himeno, K. Kishihara, S. Tsukumo, and K. Yasutomo. 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. *Nat Med* 10:29-30.
38. Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins. 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol* 172:4752-4761.
39. Yamamoto, J. K., E. Sparger, E. W. Ho, P. R. Andersen, T. P. O'Connor, C. P. Mandell, L. Lowenstine, R. Munn, and N. C. Pedersen. 1988. Pathogenesis of experimentally induced feline immunodeficiency virus infection in cats. *Am J Vet Res* 49:1246-1258.

40. Matteucci, D., F. Baldinotti, P. Mazzetti, M. Pistello, P. Bandecchi, R. Ghilarducci, A. Poli, F. Tozzini, and M. Bendinelli. 1993. Detection of feline immunodeficiency virus in saliva and plasma by cultivation and polymerase chain reaction. *J Clin Microbiol* 31:494-501.
41. Jordan, H. L., J. Howard, R. K. Sellon, D. E. Wildt, W. A. Tompkins, and S. Kennedy-Stoskopf. 1996. Transmission of feline immunodeficiency virus in domestic cats via artificial insemination. *J Virol* 70:8224-8228.
42. O'Neil, L. L., M. J. Burkhard, and E. A. Hoover. 1996. Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. *J Virol* 70:2894-2901.
43. Diehl, L. J., C. K. Mathiason-DuBard, L. L. O'Neil, and E. A. Hoover. 1995. Longitudinal assessment of feline immunodeficiency virus kinetics in plasma by use of a quantitative competitive reverse transcriptase PCR. *J Virol* 69:2328-2332.
44. de Parseval, A., U. Chatterji, P. Sun, and J. H. Elder. 2004. Feline immunodeficiency virus targets activated CD4+ T cells by using CD134 as a binding receptor. *Proc Natl Acad Sci U S A* 101:13044-13049.
45. Troth, S. P., A. D. Dean, and E. A. Hoover. 2008. In vivo CXCR4 expression, lymphoid cell phenotype, and feline immunodeficiency virus infection. *Vet Immunol Immunopathol* 123:97-105.
46. Reggeti, F., C. Ackerley, and D. Bienzle. 2008. CD134 and CXCR4 expression corresponds to feline immunodeficiency virus infection of lymphocytes, macrophages and dendritic cells. *J Gen Virol* 89:277-287.
47. Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins. 2005. Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. *J Virol* 79:4965-4976.
48. Bendinelli, M., M. Pistello, S. Lombardi, A. Poli, C. Garzelli, D. Matteucci, L. Ceccherini-Nelli, G. Malvaldi, and F. Tozzini. 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin Microbiol Rev* 8:87-112.
49. Shimojima, M., T. Miyazawa, Y. Ikeda, E. L. McMonagle, H. Haining, H. Akashi, Y. Takeuchi, M. J. Hosie, and B. J. Willett. 2004. Use of CD134 as a primary receptor by the feline immunodeficiency virus. *Science* 303:1192-1195.

50. Hubner, W., G. P. McNerney, P. Chen, B. M. Dale, R. E. Gordon, F. Y. Chuang, X. D. Li, D. M. Asmuth, T. Huser, and B. K. Chen. 2009. Quantitative 3D video microscopy of HIV transfer across T cell virological synapses. *Science* 323:1743-1747.
51. McDonald, D., L. Wu, S. M. Bohks, V. N. KewalRamani, D. Unutmaz, and T. J. Hope. 2003. Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science* 300:1295-1297.
52. Jolly, C., K. Kashefi, M. Hollinshead, and Q. J. Sattentau. 2004. HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse. *J Exp Med* 199:283-293.
53. Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C. J. Miller, and A. T. Haase. 2005. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. *Nature* 434:1148-1152.
54. Mehandru, S., M. A. Poles, K. Tenner-Racz, A. Horowitz, A. Hurley, C. Hogan, D. Boden, P. Racz, and M. Markowitz. 2004. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. *J Exp Med* 200:761-770.
55. Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. *Nature* 434:1093-1097.
56. Arthos, J., C. Cicala, E. Martinelli, K. Macleod, D. Van Ryk, D. Wei, Z. Xiao, T. D. Veenstra, T. P. Conrad, R. A. Lempicki, S. McLaughlin, M. Pascuccio, R. Gopaul, J. McNally, C. C. Cruz, N. Censoplano, E. Chung, K. N. Reitano, S. Kottlil, D. J. Goode, and A. S. Fauci. 2008. HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nat Immunol* 9:301-309.
57. Hamann, A., D. P. Andrew, D. Jablonski-Westrich, B. Holzmann, and E. C. Butcher. 1994. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol* 152:3282-3293.
58. Geijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. *Cell* 100:587-597.

59. Turville, S. G., P. U. Cameron, A. Handley, G. Lin, S. Pohlmann, R. W. Doms, and A. L. Cunningham. 2002. Diversity of receptors binding HIV on dendritic cell subsets. *Nat Immunol* 3:975-983.
60. de Witte, L., M. Bobardt, U. Chatterji, G. Degeest, G. David, T. B. Geijtenbeek, and P. Gally. 2007. Syndecan-3 is a dendritic cell-specific attachment receptor for HIV-1. *Proc Natl Acad Sci U S A* 104:19464-19469.
61. Kwon, D. S., G. Gregorio, N. Bitton, W. A. Hendrickson, and D. R. Littman. 2002. DC-SIGN-mediated internalization of HIV is required for trans-enhancement of T cell infection. *Immunity* 16:135-144.
62. Karlsson, A., K. Parsmyr, E. Sandstrom, E. M. Fenyo, and J. Albert. 1994. MT-2 cell tropism as prognostic marker for disease progression in human immunodeficiency virus type 1 infection. *J Clin Microbiol* 32:364-370.
63. Connor, R. I., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau. 1997. Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. *J Exp Med* 185:621-628.
64. Koot, M., A. B. van 't Wout, N. A. Kootstra, R. E. de Goede, M. Tersmette, and H. Schuitemaker. 1996. Relation between changes in cellular load, evolution of viral phenotype, and the clonal composition of virus populations in the course of human immunodeficiency virus type 1 infection. *J Infect Dis* 173:349-354.
65. Schuitemaker, H., M. Koot, N. A. Kootstra, M. W. Dercksen, R. E. de Goede, R. P. van Steenwijk, J. M. Lange, J. K. Schattenkerk, F. Miedema, and M. Tersmette. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. *J Virol* 66:1354-1360.
66. Wodarz, D., and M. A. Nowak. 1998. The effect of different immune responses on the evolution of virulent CXCR4-tropic HIV. *Proc Biol Sci* 265:2149-2158.
67. Bunnik, E. M., E. D. Quakkelaar, A. C. van Nuenen, B. Boeser-Nunnink, and H. Schuitemaker. 2007. Increased neutralization sensitivity of recently emerged CXCR4-using human immunodeficiency virus type 1 strains compared to coexisting CCR5-using variants from the same patient. *J Virol* 81:525-531.
68. Pastore, C., A. Ramos, and D. E. Mosier. 2004. Intrinsic obstacles to human immunodeficiency virus type 1 coreceptor switching. *J Virol* 78:7565-7574.

69. Davenport, M. P., J. J. Zaunders, M. D. Hazenberg, H. Schuitemaker, and R. P. van Rij. 2002. Cell turnover and cell tropism in HIV-1 infection. *Trends Microbiol* 10:275-278.
70. Ho, S. H., L. Shek, A. Gettie, J. Blanchard, and C. Cheng-Mayer. 2005. V3 loop-determined coreceptor preference dictates the dynamics of CD4+-T-cell loss in simian-human immunodeficiency virus-infected macaques. *J Virol* 79:12296-12303.
71. Picker, L. J., S. I. Hagen, R. Lum, E. F. Reed-Inderbitzin, L. M. Daly, A. W. Sylwester, J. M. Walker, D. C. Siess, M. Piatak, Jr., C. Wang, D. B. Allison, V. C. Maino, J. D. Lifson, T. Kodama, and M. K. Axthelm. 2004. Insufficient production and tissue delivery of CD4+ memory T cells in rapidly progressive simian immunodeficiency virus infection. *J Exp Med* 200:1299-1314.
72. Howard, K. E., and M. J. Burkhard. 2007. FIV infection induces unique changes in phenotype and cellularity in the medial iliac lymph node and intestinal IEL. *AIDS Res Hum Retroviruses* 23:720-728.
73. Nishimura, Y., T. Igarashi, O. K. Donau, A. Buckler-White, C. Buckler, B. A. Lafont, R. M. Goeken, S. Goldstein, V. M. Hirsch, and M. A. Martin. 2004. Highly pathogenic SHIVs and SIVs target different CD4+ T cell subsets in rhesus monkeys, explaining their divergent clinical courses. *Proc Natl Acad Sci U S A* 101:12324-12329.
74. Lee, B., M. Sharron, L. J. Montaner, D. Weissman, and R. W. Doms. 1999. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proc Natl Acad Sci U S A* 96:5215-5220.
75. Gordon, S. N., N. R. Klatt, S. E. Bosinger, J. M. Brenchley, J. M. Milush, J. C. Engram, R. M. Dunham, M. Paiardini, S. Klucking, A. Danesh, E. A. Strobert, C. Apetrei, I. V. Pandrea, D. Kelvin, D. C. Douek, S. I. Staprans, D. L. Sadora, and G. Silvestri. 2007. Severe depletion of mucosal CD4+ T cells in AIDS-free simian immunodeficiency virus-infected sooty mangabeys. *J Immunol* 179:3026-3034.
76. Pandrea, I. V., R. Gautam, R. M. Ribeiro, J. M. Brenchley, I. F. Butler, M. Pattison, T. Rasmussen, P. A. Marx, G. Silvestri, A. A. Lackner, A. S. Perelson, D. C. Douek, R. S. Veazey, and C. Apetrei. 2007. Acute loss of intestinal CD4+ T cells is not predictive of simian immunodeficiency virus virulence. *J Immunol* 179:3035-3046.
77. Reimann, K. A., K. Tenner-Racz, P. Racz, D. C. Montefiori, Y. Yasutomi, W. Lin, B. J. Ransil, and N. L. Letvin. 1994. Immunopathogenic events in acute infection of

- rhesus monkeys with simian immunodeficiency virus of macaques. *J Virol* 68:2362-2370.
78. Inoshima, Y., Y. Ikeda, M. Kohmoto, M. R. Pecoraro, M. Shimojima, Y. Shimojima, G. Inada, Y. Kawaguchi, K. Tomonaga, T. Miyazawa, and T. Mikami. 1996. Persistence of high virus neutralizing antibody titers in cats experimentally infected with feline immunodeficiency virus. *J Vet Med Sci* 58:925-927.
 79. Tozzini, F., D. Matteucci, P. Bandecchi, F. Baldinotti, K. Siebelink, A. Osterhaus, and M. Bendinelli. 1993. Neutralizing antibodies in cats infected with feline immunodeficiency virus. *J Clin Microbiol* 31:1626-1629.
 80. Pu, R., S. Okada, E. R. Little, B. Xu, W. V. Stoffs, and J. K. Yamamoto. 1995. Protection of neonatal kittens against feline immunodeficiency virus infection with passive maternal antiviral antibodies. *Aids* 9:235-242.
 81. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghrayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283:857-860.
 82. Koup, R. A., J. T. Safrit, Y. Cao, C. A. Andrews, G. McLeod, W. Borkowsky, C. Farthing, and D. D. Ho. 1994. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *J Virol* 68:4650-4655.
 83. Beatty, J. A., B. J. Willett, E. A. Gault, and O. Jarrett. 1996. A longitudinal study of feline immunodeficiency virus-specific cytotoxic T lymphocytes in experimentally infected cats, using antigen-specific induction. *J Virol* 70:6199-6206.
 84. Betts, M. R., D. R. Ambrozak, D. C. Douek, S. Bonhoeffer, J. M. Brenchley, J. P. Casazza, R. A. Koup, and L. J. Picker. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. *J Virol* 75:11983-11991.
 85. Reynolds, M. R., E. Rakasz, P. J. Skinner, C. White, K. Abel, Z. M. Ma, L. Compton, G. Napoe, N. Wilson, C. J. Miller, A. Haase, and D. I. Watkins. 2005. CD8+ T-lymphocyte response to major immunodominant epitopes after vaginal exposure to simian immunodeficiency virus: too late and too little. *J Virol* 79:9228-9235.
 86. Mandl, J. N., A. P. Barry, T. H. Vanderford, N. Kozyr, R. Chavan, S. Klucking, F. J. Barrat, R. L. Coffman, S. I. Staprans, and M. B. Feinberg. 2008. Divergent TLR7 and

- TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. *Nat Med* 14:1077-1087.
87. Herbeuval, J. P., A. W. Hardy, A. Boasso, S. A. Anderson, M. J. Dolan, M. Dy, and G. M. Shearer. 2005. Regulation of TNF-related apoptosis-inducing ligand on primary CD4+ T cells by HIV-1: role of type I IFN-producing plasmacytoid dendritic cells. *Proc Natl Acad Sci U S A* 102:13974-13979.
 88. Herbeuval, J. P., J. Nilsson, A. Boasso, A. W. Hardy, M. J. Kruhlak, S. A. Anderson, M. J. Dolan, M. Dy, J. Andersson, and G. M. Shearer. 2006. Differential expression of IFN-alpha and TRAIL/DR5 in lymphoid tissue of progressor versus nonprogressor HIV-1-infected patients. *Proc Natl Acad Sci U S A* 103:7000-7005.
 89. Pereira, L. E., R. P. Johnson, and A. A. Ansari. 2008. Sooty mangabeys and rhesus macaques exhibit significant divergent natural killer cell responses during both acute and chronic phases of SIV infection. *Cell Immunol* 254:10-19.
 90. Meythaler, M., A. Martinot, Z. Wang, S. Pryputniewicz, M. Kasheta, B. Ling, P. A. Marx, S. O'Neil, and A. Kaur. 2009. Differential CD4+ T-lymphocyte apoptosis and bystander T-cell activation in rhesus macaques and sooty mangabeys during acute simian immunodeficiency virus infection. *J Virol* 83:572-583.
 91. Emilie, D., M. Peuchmaur, M. C. Maillot, M. C. Crevon, N. Brousse, J. F. Delfraissy, J. Dormont, and P. Galanaud. 1990. Production of interleukins in human immunodeficiency virus-1-replicating lymph nodes. *J Clin Invest* 86:148-159.
 92. Birx, D. L., R. R. Redfield, K. Tencer, A. Fowler, D. S. Burke, and G. Tosato. 1990. Induction of interleukin-6 during human immunodeficiency virus infection. *Blood* 76:2303-2310.
 93. Lawrence, C. E., J. J. Callanan, B. J. Willett, and O. Jarrett. 1995. Cytokine production by cats infected with feline immunodeficiency virus: a longitudinal study. *Immunology* 85:568-574.
 94. Molina, J. M., D. T. Scadden, R. Byrn, C. A. Dinarello, and J. E. Groopman. 1989. Production of tumor necrosis factor alpha and interleukin 1 beta by monocytic cells infected with human immunodeficiency virus. *J Clin Invest* 84:733-737.
 95. Than, S., R. Hu, N. Oyaizu, J. Romano, X. Wang, S. Sheikh, and S. Pahwa. 1997. Cytokine pattern in relation to disease progression in human immunodeficiency virus-infected children. *J Infect Dis* 175:47-56.

96. Nilsson, J., A. Boasso, P. A. Velilla, R. Zhang, M. Vaccari, G. Franchini, G. M. Shearer, J. Andersson, and C. Chougnnet. 2006. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 108:3808-3817.
97. Lee, C., Q. H. Liu, B. Tomkowicz, Y. Yi, B. D. Freedman, and R. G. Collman. 2003. Macrophage activation through CCR5- and CXCR4-mediated gp120-elicited signaling pathways. *J Leukoc Biol* 74:676-682.
98. Martinez-Maza, O., E. Crabb, R. T. Mitsuyasu, J. L. Fahey, and J. V. Giorgi. 1987. Infection with the human immunodeficiency virus (HIV) is associated with an in vivo increase in B lymphocyte activation and immaturity. *J Immunol* 138:3720-3724.
99. Flynn, J. N., C. A. Cannon, C. E. Lawrence, and O. Jarrett. 1994. Polyclonal B-cell activation in cats infected with feline immunodeficiency virus. *Immunology* 81:626-630.
100. Kottlilil, S., K. Shin, J. O. Jackson, K. N. Reitano, M. A. O'Shea, J. Yang, C. W. Hallahan, R. Lempicki, J. Arthos, and A. S. Fauci. 2006. Innate immune dysfunction in HIV infection: effect of HIV envelope-NK cell interactions. *J Immunol* 176:1107-1114.
101. Mahalingam, M., M. Peakman, E. T. Davies, A. Pozniak, T. J. McManus, and D. Vergani. 1993. T cell activation and disease severity in HIV infection. *Clin Exp Immunol* 93:337-343.
102. Gebhard, D. H., J. L. Dow, T. A. Childers, J. I. Alvelo, M. B. Tompkins, and W. A. Tompkins. 1999. Progressive expansion of an L-selectin-negative CD8 cell with anti-feline immunodeficiency virus (FIV) suppressor function in the circulation of FIV-infected cats. *J Infect Dis* 180:1503-1513.
103. Hazenberg, M. D., J. W. Stuart, S. A. Otto, J. C. Borleffs, C. A. Boucher, R. J. de Boer, F. Miedema, and D. Hamann. 2000. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: a longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). *Blood* 95:249-255.
104. Lempicki, R. A., J. A. Kovacs, M. W. Baseler, J. W. Adelsberger, R. L. Dewar, V. Natarajan, M. C. Bosche, J. A. Metcalf, R. A. Stevens, L. A. Lambert, W. G. Alvord, M. A. Polis, R. T. Davey, D. S. Dimitrov, and H. C. Lane. 2000. Impact of HIV-1 infection and highly active antiretroviral therapy on the kinetics of CD4+ and CD8+ T cell turnover in HIV-infected patients. *Proc Natl Acad Sci U S A* 97:13778-13783.

105. Finkel, T. H., G. Tudor-Williams, N. K. Banda, M. F. Cotton, T. Curiel, C. Monks, T. W. Baba, R. M. Ruprecht, and A. Kupfer. 1995. Apoptosis occurs predominantly in bystander cells and not in productively infected cells of HIV- and SIV-infected lymph nodes. *Nat Med* 1:129-134.
106. De Boer, R. J., H. Mohri, D. D. Ho, and A. S. Perelson. 2003. Turnover rates of B cells, T cells, and NK cells in simian immunodeficiency virus-infected and uninfected rhesus macaques. *J Immunol* 170:2479-2487.
107. Lund, F. E., D. A. Cockayne, T. D. Randall, N. Solvason, F. Schuber, and M. C. Howard. 1998. CD38: a new paradigm in lymphocyte activation and signal transduction. *Immunol Rev* 161:79-93.
108. Giorgi, J. V., Z. Liu, L. E. Hultin, W. G. Cumberland, K. Hennessey, and R. Detels. 1993. Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. *J Acquir Immune Defic Syndr* 6:904-912.
109. Cao, W., B. D. Jamieson, L. E. Hultin, P. M. Hultin, and R. Detels. 2009. Regulatory T cell expansion and immune activation during untreated HIV type 1 infection are associated with disease progression. *AIDS Res Hum Retroviruses* 25:183-191.
110. Deeks, S. G., C. M. Kitchen, L. Liu, H. Guo, R. Gascon, A. B. Narvaez, P. Hunt, J. N. Martin, J. O. Kahn, J. Levy, M. S. McGrath, and F. M. Hecht. 2004. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* 104:942-947.
111. Liu, Z., W. G. Cumberland, L. E. Hultin, H. E. Prince, R. Detels, and J. V. Giorgi. 1997. Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol* 16:83-92.
112. Levacher, M., F. Hulstaert, S. Tallet, S. Ullery, J. J. Pocidallo, and B. A. Bach. 1992. The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. *Clin Exp Immunol* 90:376-382.
113. Douek, D. C., J. M. Brenchley, M. R. Betts, D. R. Ambrozak, B. J. Hill, Y. Okamoto, J. P. Casazza, J. Kuruppu, K. Kunstman, S. Wolinsky, Z. Grossman, M. Dybul, A. Oxenius, D. A. Price, M. Connors, and R. A. Koup. 2002. HIV preferentially infects HIV-specific CD4+ T cells. *Nature* 417:95-98.

114. Kovacs, J. A., R. A. Lempicki, I. A. Sidorov, J. W. Adelsberger, B. Herpin, J. A. Metcalf, I. Sereti, M. A. Polis, R. T. Davey, J. Tavel, J. Falloon, R. Stevens, L. Lambert, R. Dewar, D. J. Schwartzentruber, M. R. Anver, M. W. Baseler, H. Masur, D. S. Dimitrov, and H. C. Lane. 2001. Identification of dynamically distinct subpopulations of T lymphocytes that are differentially affected by HIV. *J Exp Med* 194:1731-1741.
115. McCune, J. M., M. B. Hanley, D. Cesar, R. Halvorsen, R. Hoh, D. Schmidt, E. Wieder, S. Deeks, S. Siler, R. Neese, and M. Hellerstein. 2000. Factors influencing T-cell turnover in HIV-1-seropositive patients. *J Clin Invest* 105:R1-8.
116. Davey, R. T., Jr., N. Bhat, C. Yoder, T. W. Chun, J. A. Metcalf, R. Dewar, V. Natarajan, R. A. Lempicki, J. W. Adelsberger, K. D. Miller, J. A. Kovacs, M. A. Polis, R. E. Walker, J. Falloon, H. Masur, D. Gee, M. Baseler, D. S. Dimitrov, A. S. Fauci, and H. C. Lane. 1999. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proc Natl Acad Sci U S A* 96:15109-15114.
117. Meyaard, L., S. A. Otto, R. R. Jonker, M. J. Mijster, R. P. Keet, and F. Miedema. 1992. Programmed death of T cells in HIV-1 infection. *Science* 257:217-219.
118. Gougeon, M. L., H. Lecoecur, A. Dulioust, M. G. Enouf, M. Crouvoiser, C. Goujard, T. Debord, and L. Montagnier. 1996. Programmed cell death in peripheral lymphocytes from HIV-infected persons: increased susceptibility to apoptosis of CD4 and CD8 T cells correlates with lymphocyte activation and with disease progression. *J Immunol* 156:3509-3520.
119. Paillot, R., S. Richard, F. Bloas, F. Piras, H. Poulet, S. Brunet, C. Andreoni, and V. Juillard. 2005. Toward a detailed characterization of feline immunodeficiency virus-specific T cell immune responses and mediated immune disorders. *Vet Immunol Immunopathol* 106:1-14.
120. Veazey, R. S., M. DeMaria, L. V. Chalifoux, D. E. Shvetz, D. R. Pauley, H. L. Knight, M. Rosenzweig, R. P. Johnson, R. C. Desrosiers, and A. A. Lackner. 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. *Science* 280:427-431.
121. Chase, A. J., A. R. Sedaghat, J. R. German, L. Gama, M. C. Zink, J. E. Clements, and R. F. Siliciano. 2007. Severe depletion of CD4+ CD25+ regulatory T cells from the intestinal lamina propria but not peripheral blood or lymph nodes during acute simian immunodeficiency virus infection. *J Virol* 81:12748-12757.

122. Brenchley, J. M., T. W. Schacker, L. E. Ruff, D. A. Price, J. H. Taylor, G. J. Beilman, P. L. Nguyen, A. Khoruts, M. Larson, A. T. Haase, and D. C. Douek. 2004. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med* 200:749-759.
123. Guadalupe, M., E. Reay, S. Sankaran, T. Prindiville, J. Flamm, A. McNeil, and S. Dandekar. 2003. Severe CD4+ T-cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J Virol* 77:11708-11717.
124. George, M. D., E. Reay, S. Sankaran, and S. Dandekar. 2005. Early antiretroviral therapy for simian immunodeficiency virus infection leads to mucosal CD4+ T-cell restoration and enhanced gene expression regulating mucosal repair and regeneration. *J Virol* 79:2709-2719.
125. Swank, G. M., and E. A. Deitch. 1996. Role of the gut in multiple organ failure: bacterial translocation and permeability changes. *World J Surg* 20:411-417.
126. Brenchley, J. M., D. A. Price, T. W. Schacker, T. E. Asher, G. Silvestri, S. Rao, Z. Kazzaz, E. Bornstein, O. Lambotte, D. Altmann, B. R. Blazar, B. Rodriguez, L. Teixeira-Johnson, A. Landay, J. N. Martin, F. M. Hecht, L. J. Picker, M. M. Lederman, S. G. Deeks, and D. C. Douek. 2006. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat Med* 12:1365-1371.
127. Pandrea, I., C. Coleman, T. Gaufin, R. Gautam, M. Pattison, C. Monjure, A. A. Lackner, and C. Apetrei. 2008. Experimentally-induced immune activation in natural hosts of SIVs results in significant increases in viral replication. In *Keystone Symposia HIV Pathogenesis*, Banff, Canada.
128. Ancuta, P., A. Kamat, K. J. Kunstman, E. Y. Kim, P. Autissier, A. Wurcel, T. Zaman, D. Stone, M. Mefford, S. Morgello, E. J. Singer, S. M. Wolinsky, and D. Gabuzda. 2008. Microbial translocation is associated with increased monocyte activation and dementia in AIDS patients. *PLoS One* 3:e2516.
129. Redd, A. D., D. Dabitao, J. H. Bream, B. Charvat, O. Laeyendecker, N. Kiwanuka, T. Lutalo, G. Kigozi, A. A. Tobian, J. Gamiel, J. D. Neal, A. E. Oliver, J. B. Margolick, N. Sewankambo, S. J. Reynolds, M. J. Wawer, D. Serwadda, R. H. Gray, and T. C. Quinn. 2009. Microbial translocation, the innate cytokine response, and HIV-1 disease progression in Africa. *Proc Natl Acad Sci U S A* 106:6718-6723.
130. Caramalho, I., T. Lopes-Carvalho, D. Ostler, S. Zelenay, M. Haury, and J. Demengeot. 2003. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* 197:403-411.

131. Merrill, J. E., Y. Koyanagi, and I. S. Chen. 1989. Interleukin-1 and tumor necrosis factor alpha can be induced from mononuclear phagocytes by human immunodeficiency virus type 1 binding to the CD4 receptor. *J Virol* 63:4404-4408.
132. Cicala, C., J. Arthos, S. M. Selig, G. Dennis, Jr., D. A. Hosack, D. Van Ryk, M. L. Spangler, T. D. Steenbeke, P. Khazanie, N. Gupta, J. Yang, M. Daucher, R. A. Lempicki, and A. S. Fauci. 2002. HIV envelope induces a cascade of cell signals in non-proliferating target cells that favor virus replication. *Proc Natl Acad Sci U S A* 99:9380-9385.
133. Banda, N. K., J. Bernier, D. K. Kurahara, R. Kurrle, N. Haigwood, R. P. Sekaly, and T. H. Finkel. 1992. Crosslinking CD4 by human immunodeficiency virus gp120 primes T cells for activation-induced apoptosis. *J Exp Med* 176:1099-1106.
134. Oyaizu, N., Y. Adachi, F. Hashimoto, T. W. McCloskey, N. Hosaka, N. Kayagaki, H. Yagita, and S. Pahwa. 1997. Monocytes express Fas ligand upon CD4 cross-linking and induce CD4+ T cells apoptosis: a possible mechanism of bystander cell death in HIV infection. *J Immunol* 158:2456-2463.
135. Blanco, J., J. Barretina, B. Clotet, and J. A. Este. 2004. R5 HIV gp120-mediated cellular contacts induce the death of single CCR5-expressing CD4 T cells by a gp41-dependent mechanism. *J Leukoc Biol* 76:804-811.
136. Roggero, R., V. Robert-Hebmann, S. Harrington, J. Roland, L. Vergne, S. Jaleco, C. Devaux, and M. Biard-Piechaczyk. 2001. Binding of human immunodeficiency virus type 1 gp120 to CXCR4 induces mitochondrial transmembrane depolarization and cytochrome c-mediated apoptosis independently of Fas signaling. *J Virol* 75:7637-7650.
137. Blaak, H., A. B. van't Wout, M. Brouwer, B. Hooibrink, E. Hovenkamp, and H. Schuitemaker. 2000. In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline. *Proc Natl Acad Sci U S A* 97:1269-1274.
138. Sylwester, A., S. Murphy, D. Shutt, and D. R. Soll. 1997. HIV-induced T cell syncytia are self-perpetuating and the primary cause of T cell death in culture. *J Immunol* 158:3996-4007.
139. Andreau, K., J. L. Perfettini, M. Castedo, D. Metivier, V. Scott, G. Pierron, and G. Kroemer. 2004. Contagious apoptosis facilitated by the HIV-1 envelope: fusion-induced cell-to-cell transmission of a lethal signal. *J Cell Sci* 117:5643-5653.

140. Nabel, G., and D. Baltimore. 1987. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature* 326:711-713.
141. Kawakami, K., C. Scheidereit, and R. G. Roeder. 1988. Identification and purification of a human immunoglobulin-enhancer-binding protein (NF-kappa B) that activates transcription from a human immunodeficiency virus type 1 promoter in vitro. *Proc Natl Acad Sci U S A* 85:4700-4704.
142. Kinoshita, S., L. Su, M. Amano, L. A. Timmerman, H. Kaneshima, and G. P. Nolan. 1997. The T cell activation factor NF-ATc positively regulates HIV-1 replication and gene expression in T cells. *Immunity* 6:235-244.
143. Sakaguchi, S., N. Sakaguchi, M. Asano, M. Itoh, and M. Toda. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155:1151-1164.
144. Tang, Q., and J. A. Bluestone. 2008. The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat Immunol* 9:239-244.
145. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.
146. Vignali, D. A., L. W. Collison, and C. J. Workman. 2008. How regulatory T cells work. *Nat Rev Immunol* 8:523-532.
147. Cabarocas, J., C. Cassan, F. Magnusson, E. Piaggio, L. Mars, J. Derbinski, B. Kyewski, D. A. Gross, B. L. Salomon, K. Khazaie, A. Saoudi, and R. S. Liblau. 2006. Foxp3+ CD25+ regulatory T cells specific for a neo-self-antigen develop at the double-positive thymic stage. *Proc Natl Acad Sci U S A* 103:8453-8458.
148. Gavin, M. A., J. P. Rasmussen, J. D. Fontenot, V. Vasta, V. C. Manganiello, J. A. Beavo, and A. Y. Rudensky. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445:771-775.
149. Lin, W., D. Haribhai, L. M. Relland, N. Truong, M. R. Carlson, C. B. Williams, and T. A. Chatila. 2007. Regulatory T cell development in the absence of functional Foxp3. *Nat Immunol* 8:359-368.
150. Zheng, Y., S. Z. Josefowicz, A. Kas, T. T. Chu, M. A. Gavin, and A. Y. Rudensky. 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445:936-940.

151. Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat Immunol* 6:152-162.
152. Lankford, S., C. Petty, A. Lavoy, S. Reckling, W. Tompkins, and G. A. Dean. 2007. Cloning of feline FOXP3 and detection of expression in CD4+CD25+ regulatory T cells. *Vet Immunol Immunopathol*.
153. Allan, S. E., S. Q. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 19:345-354.
154. Morgan, M. E., J. H. van Bilsen, A. M. Bakker, B. Heemskerk, M. W. Schilham, F. C. Hartgers, B. G. Elferink, L. van der Zanden, R. R. de Vries, T. W. Huizinga, T. H. Ottenhoff, and R. E. Toes. 2005. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* 66:13-20.
155. Gavin, M. A., T. R. Torgerson, E. Houston, P. DeRoos, W. Y. Ho, A. Stray-Pedersen, E. L. Ocheltree, P. D. Greenberg, H. D. Ochs, and A. Y. Rudensky. 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 103:6659-6664.
156. Roncador, G., P. J. Brown, L. Maestre, S. Hue, J. L. Martinez-Torrecuadrada, K. L. Ling, S. Pratap, C. Toms, B. C. Fox, V. Cerundolo, F. Powrie, and A. H. Banham. 2005. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* 35:1681-1691.
157. Wang, J., A. Ioan-Facsinay, E. I. van der Voort, T. W. Huizinga, and R. E. Toes. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 37:129-138.
158. Walker, M. R., B. D. Carson, G. T. Nepom, S. F. Ziegler, and J. H. Buckner. 2005. De novo generation of antigen-specific CD4+CD25+ regulatory T cells from human CD4+CD25- cells. *Proc Natl Acad Sci U S A* 102:4103-4108.
159. Taams, L. S., M. Vukmanovic-Stejic, J. Smith, P. J. Dunne, J. M. Fletcher, F. J. Plunkett, S. B. Ebeling, G. Lombardi, M. H. Rustin, J. W. Bijlsma, F. P. Lafeber, M. Salmon, and A. N. Akbar. 2002. Antigen-specific T cell suppression by human CD4+CD25+ regulatory T cells. *Eur J Immunol* 32:1621-1630.

160. Kasow, K. A., X. Chen, J. Knowles, D. Wichlan, R. Handgretinger, and J. M. Riberdy. 2004. Human CD4+CD25+ regulatory T cells share equally complex and comparable repertoires with CD4+CD25- counterparts. *J Immunol* 172:6123-6128.
161. Vukmanovic-Stejic, M., Y. Zhang, J. E. Cook, J. M. Fletcher, A. McQuaid, J. E. Masters, M. H. Rustin, L. S. Taams, P. C. Beverley, D. C. Macallan, and A. N. Akbar. 2006. Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* 116:2423-2433.
162. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *J Immunol* 180:764-773.
163. Pacholczyk, R., J. Kern, N. Singh, M. Iwashima, P. Kraj, and L. Ignatowicz. 2007. Nonspecific antigens are the cognate specificities of Foxp3+ regulatory T cells. *Immunity* 27:493-504.
164. Wong, J., R. Obst, M. Correia-Neves, G. Losyev, D. Mathis, and C. Benoist. 2007. Adaptation of TCR repertoires to self-peptides in regulatory and nonregulatory CD4+ T cells. *J Immunol* 178:7032-7041.
165. Hayashi, Y., S. Tsukumo, H. Shiota, K. Kishihara, and K. Yasutomo. 2004. Antigen-specific T cell repertoire modification of CD4+CD25+ regulatory T cells. *J Immunol* 172:5240-5248.
166. Walker, M. R., D. J. Kasprowicz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+CD25- T cells. *J Clin Invest* 112:1437-1443.
167. Aandahl, E. M., J. Michaelsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon. 2004. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78:2454-2459.
168. Fantini, M. C., C. Becker, G. Monteleone, F. Pallone, P. R. Galle, and M. F. Neurath. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172:5149-5153.
169. Zheng, S. G., J. H. Wang, M. N. Koss, F. Quismorio, Jr., J. D. Gray, and D. A. Horwitz. 2004. CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol* 172:1531-1539.

170. Zheng, S. G., J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz. 2004. Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 172:5213-5221.
171. Petty, C. S., M. B. Tompkins, and W. A. Tompkins. 2008. Transforming growth factor-beta/transforming growth factor-betaRII signaling may regulate CD4+CD25+ T-regulatory cell homeostasis and suppressor function in feline AIDS lentivirus infection. *J Acquir Immune Defic Syndr* 47:148-160.
172. Wang, Z., J. Hong, W. Sun, G. Xu, N. Li, X. Chen, A. Liu, L. Xu, B. Sun, and J. Z. Zhang. 2006. Role of IFN-gamma in induction of Foxp3 and conversion of CD4+CD25- T cells to CD4+ Tregs. *J Clin Invest* 116:2434-2441.
173. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6:1219-1227.
174. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199:1401-1408.
175. Knoechel, B., J. Lohr, E. Kahn, J. A. Bluestone, and A. K. Abbas. 2005. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med* 202:1375-1386.
176. Fontenot, J. D., J. P. Rasmussen, M. A. Gavin, and A. Y. Rudensky. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6:1142-1151.
177. Taams, L. S., J. Smith, M. H. Rustin, M. Salmon, L. W. Poulter, and A. N. Akbar. 2001. Human anergic/suppressive CD4(+)/CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol* 31:1122-1131.
178. Fritzsching, B., N. Oberle, N. Eberhardt, S. Quick, J. Haas, B. Wildemann, P. H. Krammer, and E. Suri-Payer. 2005. In contrast to effector T cells, CD4+CD25+FoxP3+ regulatory T cells are highly susceptible to CD95 ligand- but not to TCR-mediated cell death. *J Immunol* 175:32-36.
179. Wang, X., J. Zheng, J. Liu, J. Yao, Y. He, X. Li, J. Yu, J. Yang, Z. Liu, and S. Huang. 2005. Increased population of CD4(+)/CD25(high), regulatory T cells with their higher apoptotic and proliferating status in peripheral blood of acute myeloid leukemia patients. *Eur J Haematol* 75:468-476.

180. Walker, L. S., A. Chodos, M. Eggena, H. Dooms, and A. K. Abbas. 2003. Antigen-dependent proliferation of CD4⁺ CD25⁺ regulatory T cells in vivo. *J Exp Med* 198:249-258.
181. Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* 100:8886-8891.
182. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional CD25⁺ CD4⁺ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198:235-247.
183. Levings, M. K., R. Sangregorio, and M. G. Roncarolo. 2001. Human cd25(+)cd4(+) t regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J Exp Med* 193:1295-1302.
184. Walker, L. S. 2009. Regulatory T cells overturned: the effectors fight back. *Immunology* 126:466-474.
185. van Amelsfort, J. M., J. A. van Roon, M. Noordegraaf, K. M. Jacobs, J. W. Bijlsma, F. P. Lafeber, and L. S. Taams. 2007. Proinflammatory mediator-induced reversal of CD4⁺,CD25⁺ regulatory T cell-mediated suppression in rheumatoid arthritis. *Arthritis Rheum* 56:732-742.
186. Clough, L. E., C. J. Wang, E. M. Schmidt, G. Booth, T. Z. Hou, G. A. Ryan, and L. S. Walker. 2008. Release from regulatory T cell-mediated suppression during the onset of tissue-specific autoimmunity is associated with elevated IL-21. *J Immunol* 180:5393-5401.
187. Pace, L., S. Rizzo, C. Palombi, F. Brombacher, and G. Doria. 2006. Cutting edge: IL-4-induced protection of CD4⁺CD25⁻ Th cells from CD4⁺CD25⁺ regulatory T cell-mediated suppression. *J Immunol* 176:3900-3904.
188. Ruprecht, C. R., M. Gattorno, F. Ferlito, A. Gregorio, A. Martini, A. Lanzavecchia, and F. Sallusto. 2005. Coexpression of CD25 and CD27 identifies FoxP3⁺ regulatory T cells in inflamed synovia. *J Exp Med* 201:1793-1803.
189. Peluso, I., M. C. Fantini, D. Fina, R. Caruso, M. Boirivant, T. T. MacDonald, F. Pallone, and G. Monteleone. 2007. IL-21 counteracts the regulatory T cell-mediated suppression of human CD4⁺ T lymphocytes. *J Immunol* 178:732-739.

190. Liu, H., M. Komai-Koma, D. Xu, and F. Y. Liew. 2006. Toll-like receptor 2 signaling modulates the functions of CD4⁺ CD25⁺ regulatory T cells. *Proc Natl Acad Sci U S A* 103:7048-7053.
191. LaRosa, D. F., A. E. Gelman, A. H. Rahman, J. Zhang, L. A. Turka, and P. T. Walsh. 2007. CpG DNA inhibits CD4⁺CD25⁺ Treg suppression through direct MyD88-dependent costimulation of effector CD4⁺ T cells. *Immunol Lett* 108:183-188.
192. Suttmuller, R. P., M. H. den Brok, M. Kramer, E. J. Bennink, L. W. Toonen, B. J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema. 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J Clin Invest* 116:485-494.
193. Peng, G., Z. Guo, Y. Kiniwa, K. S. Voo, W. Peng, T. Fu, D. Y. Wang, Y. Li, H. Y. Wang, and R. F. Wang. 2005. Toll-like receptor 8-mediated reversal of CD4⁺ regulatory T cell function. *Science* 309:1380-1384.
194. Netea, M. G., R. Suttmuller, C. Hermann, C. A. Van der Graaf, J. W. Van der Meer, J. H. van Krieken, T. Hartung, G. Adema, and B. J. Kullberg. 2004. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J Immunol* 172:3712-3718.
195. Crellin, N. K., R. V. Garcia, O. Hadisfar, S. E. Allan, T. S. Steiner, and M. K. Levings. 2005. Human CD4⁺ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4⁺CD25⁺ T regulatory cells. *J Immunol* 175:8051-8059.
196. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* 174:3143-3147.
197. Epple, H. J., C. Loddenkemper, D. Kunkel, H. Troger, J. Maul, V. Moos, E. Berg, R. Ullrich, J. D. Schulzke, H. Stein, R. Duchmann, M. Zeitz, and T. Schneider. 2006. Mucosal but not peripheral FOXP3⁺ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 108:3072-3078.
198. Boasso, A., M. Vaccari, A. Hryniewicz, D. Fuchs, J. Nacs, V. Cecchinato, J. Andersson, G. Franchini, G. M. Shearer, and C. Chougnet. 2007. Regulatory T-cell markers, indoleamine 2,3-dioxygenase, and virus levels in spleen and gut during progressive simian immunodeficiency virus infection. *J Virol* 81:11593-11603.

199. Baker, C. A., R. Clark, F. Ventura, N. G. Jones, D. Guzman, D. R. Bangsberg, and H. Cao. 2007. Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection. *Clin Exp Immunol* 147:533-539.
200. Dunham, R. M., B. Cervasi, J. M. Brenchley, H. Albrecht, A. Weintrob, B. Sumpter, J. Engram, S. Gordon, N. R. Klatt, I. Frank, D. L. Sodora, D. C. Douek, M. Paiardini, and G. Silvestri. 2008. CD127 and CD25 expression defines CD4+ T cell subsets that are differentially depleted during HIV infection. *J Immunol* 180:5582-5592.
201. Ansari, A. A., L. E. Pereira, A. E. Mayne, N. Onlamoon, K. Pattanapanyasat, K. Mori, and F. Villinger. 2007. The role of disease stage, plasma viral load and regulatory T cells (Tregs) on autoantibody production in SIV-infected non-human primates. *J Autoimmun* 28:152-159.
202. Joshi, A., T. W. Vahlenkamp, H. Garg, W. A. Tompkins, and M. B. Tompkins. 2004. Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. *Virology* 321:307-322.
203. Ji, J., and M. W. Cloyd. 2009. HIV-1 binding to CD4 on CD4+CD25+ regulatory T cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: an additional mechanism of immunosuppression. *Int Immunol* 21:283-294.
204. Kornfeld, C., M. J. Ploquin, I. Pandrea, A. Faye, R. Onanga, C. Apetrei, V. Poaty-Mavoungou, P. Rouquet, J. Estaquier, L. Mortara, J. F. Desoutter, C. Butor, R. Le Grand, P. Roques, F. Simon, F. Barre-Sinoussi, O. M. Diop, and M. C. Muller-Trutwin. 2005. Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J Clin Invest* 115:1082-1091.
205. Apoil, P. A., B. Puissant, F. Roubinet, M. Abbal, P. Massip, and A. Blancher. 2005. FOXP3 mRNA levels are decreased in peripheral blood CD4+ lymphocytes from HIV-positive patients. *J Acquir Immune Defic Syndr* 39:381-385.
206. Pereira, L. E., F. Villinger, N. Onlamoon, P. Bryan, A. Cardona, K. Pattanapanyasat, K. Mori, S. Hagen, L. Picker, and A. A. Ansari. 2007. Simian immunodeficiency virus (SIV) infection influences the level and function of regulatory T cells in SIV-infected rhesus macaques but not SIV-infected sooty mangabeys. *J Virol* 81:4445-4456.
207. Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci. 2004. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected

- individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* 200:331-343.
208. Ndhlovu, L. C., C. P. Loo, G. Spotts, D. F. Nixon, and F. M. Hecht. 2008. FOXP3 expressing CD127^{lo} CD4⁺ T cells inversely correlate with CD38⁺ CD8⁺ T cell activation levels in primary HIV-1 infection. *J Leukoc Biol* 83:254-262.
 209. Tenorio, A. R., J. Martinson, D. Pollard, L. Baum, and A. Landay. 2008. The relationship of T-regulatory cell subsets to disease stage, immune activation, and pathogen-specific immunity in HIV infection. *J Acquir Immune Defic Syndr* 48:577-580.
 210. Kinter, A., J. McNally, L. Riggin, R. Jackson, G. Roby, and A. S. Fauci. 2007. Suppression of HIV-specific T cell activity by lymph node CD25⁺ regulatory T cells from HIV-infected individuals. *Proc Natl Acad Sci U S A* 104:3390-3395.
 211. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol* 2:E198.
 212. Chase, A. J., H. C. Yang, H. Zhang, J. N. Blankson, and R. F. Siliciano. 2008. Preservation of FoxP3⁺ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4⁺ T-cell activation. *J Virol* 82:8307-8315.
 213. Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins. 2005. Different thresholds of T cell activation regulate FIV infection of CD4⁺CD25⁺ and CD4⁺CD25⁻ cells. *Virology* 335:212-221.
 214. Holmes, D., G. Knudsen, S. Mackey-Cushman, and L. Su. 2007. FoxP3 enhances HIV-1 gene expression by modulating NFkappaB occupancy at the long terminal repeat in human T cells. *J Biol Chem* 282:15973-15980.
 215. Mexas, A. M., J. E. Fogle, W. A. Tompkins, and M. B. Tompkins. 2008. CD4(+)CD25(+) regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol*.
 216. Weiss, L., V. Donkova-Petrini, L. Caccavelli, M. Balbo, C. Carbonneil, and Y. Levy. 2004. Human immunodeficiency virus-driven expansion of CD4⁺CD25⁺ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* 104:3249-3256.

217. Kinter, A. L., R. Horak, M. Sion, L. Riggan, J. McNally, Y. Lin, R. Jackson, A. O'Shea, G. Roby, C. Kovacs, M. Connors, S. A. Migueles, and A. S. Fauci. 2007. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses* 23:438-450.
218. Eggena, M. P., B. Barugahare, N. Jones, M. Okello, S. Mutalya, C. Kityo, P. Mugenyi, and H. Cao. 2005. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol* 174:4407-4414.
219. Legrand, F. A., D. F. Nixon, C. P. Loo, E. Ono, J. M. Chapman, M. Miyamoto, R. S. Diaz, A. M. Santos, R. C. Succi, J. Abadi, M. G. Rosenberg, M. I. de Moraes-Pinto, and E. G. Kallas. 2006. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. *PLoS ONE* 1:e102.
220. Hryniewicz, A., A. Boasso, Y. Edghill-Smith, M. Vaccari, D. Fuchs, D. Venzon, J. Nacsa, M. R. Betts, W. P. Tsai, J. M. Heraud, B. Beer, D. Blanset, C. Chougnet, I. Lowy, G. M. Shearer, and G. Franchini. 2006. CTLA-4 blockade decreases TGF-beta,IDO, and viral RNA expression in tissues of SIVmac251-infected macaques. *Blood* 108:3834-3842.
221. Rosenberg, E. S., J. M. Billingsley, A. M. Caliendo, S. L. Boswell, P. E. Sax, S. A. Kalams, and B. D. Walker. 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278:1447-1450.
222. Arrode, G., J. S. Finke, H. Zebroski, F. P. Siegal, and R. M. Steinman. 2005. CD8+ T cells from most HIV-1-infected patients, even when challenged with mature dendritic cells, lack functional recall memory to HIV gag but not other viruses. *Eur J Immunol* 35:159-170.
223. Smithberg, S. R., J. E. Fogle, A. M. Mexas, S. K. Reckling, S. M. Lankford, M. B. Tompkins, and G. A. Dean. 2008. In vivo depletion of CD4(+)CD25(+) regulatory T cells in cats. *J Immunol Methods* 329:81-91.
224. Dannull, J., Z. Su, D. Rizzieri, B. K. Yang, D. Coleman, D. Yancey, A. Zhang, P. Dahm, N. Chao, E. Gilboa, and J. Vieweg. 2005. Enhancement of vaccine-mediated antitumor immunity in cancer patients after depletion of regulatory T cells. *J Clin Invest* 115:3623-3633.
225. Vaclavkova, P., Y. Cao, L. K. Wu, J. Michalek, and E. S. Vitetta. 2006. A comparison of an anti-CD25 immunotoxin, Ontak and anti-CD25 microbeads for

- their ability to deplete alloreactive T cells in vitro. *Bone Marrow Transplant* 37:559-567.
226. Ghiringhelli, F., N. Larmonier, E. Schmitt, A. Parcellier, D. Cathelin, C. Garrido, B. Chauffert, E. Solary, B. Bonnotte, and F. Martin. 2004. CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative. *Eur J Immunol* 34:336-344.
 227. Lutsiak, M. E., R. T. Semnani, R. De Pascalis, S. V. Kashmiri, J. Schlom, and H. Sabzevari. 2005. Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide. *Blood* 105:2862-2868.
 228. Mahnke, K., K. Schonfeld, S. Fondel, S. Ring, S. Karakhanova, K. Wiedemeyer, T. Bedke, T. S. Johnson, V. Storn, S. Schallenberg, and A. H. Enk. 2007. Depletion of CD4+CD25+ human regulatory T cells in vivo: kinetics of Treg depletion and alterations in immune functions in vivo and in vitro. *Int J Cancer* 120:2723-2733.
 229. Matsushita, N., S. A. Pilon-Thomas, L. M. Martin, and A. I. Riker. 2008. Comparative methodologies of regulatory T cell depletion in a murine melanoma model. *J Immunol Methods*.
 230. Goforth, R., A. K. Salem, X. Zhu, S. Miles, X. Q. Zhang, J. H. Lee, and A. D. Sandler. 2009. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. *Cancer Immunol Immunother* 58:517-530.
 231. Grauer, O. M., R. P. Suttmuller, W. van Maren, J. F. Jacobs, E. Bennink, L. W. Toonen, S. Nierkens, and G. J. Adema. 2008. Elimination of regulatory T cells is essential for an effective vaccination with tumor lysate-pulsed dendritic cells in a murine glioma model. *Int J Cancer* 122:1794-1802.
 232. Prasad, S. J., K. J. Farrand, S. A. Matthews, J. H. Chang, R. S. McHugh, and F. Ronchese. 2005. Dendritic cells loaded with stressed tumor cells elicit long-lasting protective tumor immunity in mice depleted of CD4+CD25+ regulatory T cells. *J Immunol* 174:90-98.
 233. Toka, F. N., S. Suvas, and B. T. Rouse. 2004. CD4+ CD25+ T cells regulate vaccine-generated primary and memory CD8+ T-cell responses against herpes simplex virus type 1. *J Virol* 78:13082-13089.
 234. Chuang, C. M., T. Hoory, A. Monie, A. Wu, M. C. Wang, and C. F. Hung. 2009. Enhancing therapeutic HPV DNA vaccine potency through depletion of CD4+CD25+ T regulatory cells. *Vaccine* 27:684-689.

235. Viehl, C. T., T. T. Moore, U. K. Liyanage, D. M. Frey, J. P. Ehlers, T. J. Eberlein, P. S. Goedegebuure, and D. C. Linehan. 2006. Depletion of CD4+CD25+ regulatory T cells promotes a tumor-specific immune response in pancreas cancer-bearing mice. *Ann Surg Oncol* 13:1252-1258.
236. Morse, M. A., A. C. Hobeika, T. Osada, D. Serra, D. Niedzwiecki, H. K. Lysterly, and T. M. Clay. 2008. Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood* 112:610-618.
237. Rasku, M. A., A. L. Clem, S. Telang, B. Taft, K. Gettings, H. Gragg, D. Cramer, S. C. Lear, K. M. McMasters, D. M. Miller, and J. Chesney. 2008. Transient T cell depletion causes regression of melanoma metastases. *J Transl Med* 6:12.
238. Suvas, S., U. Kumaraguru, C. D. Pack, S. Lee, and B. T. Rouse. 2003. CD4+CD25+ T cells regulate virus-specific primary and memory CD8+ T cell responses. *J Exp Med* 198:889-901.
239. Chen, G., J. Liu, Q. H. Wang, Y. Wu, H. Feng, W. Zheng, S. Y. Guo, D. M. Li, J. C. Wang, and Y. M. Cao. 2009. Effects of CD4(+)CD25(+)Foxp3(+)regulatory T cells on early Plasmodium yoelii 17XL infection in BALB/c mice. *Parasitology* 136:1107-1120.
240. Taylor, M. D., N. van der Werf, A. Harris, A. L. Graham, O. Bain, J. E. Allen, and R. M. Maizels. 2009. Early recruitment of natural CD4+ Foxp3+ Treg cells by infective larvae determines the outcome of filarial infection. *Eur J Immunol* 39:192-206.
241. Namangala, B., N. Yokoyama, Y. Ikehara, O. Taguchi, K. Tsujimura, C. Sugimoto, and N. Inoue. 2008. Effect of CD4+CD25+ T cell-depletion on acute lethal infection of mice with Trypanosoma congolense. *J Vet Med Sci* 70:751-759.
242. Couper, K. N., P. A. Lanthier, G. Perona-Wright, L. W. Kummer, W. Chen, S. T. Smiley, M. Mohrs, and L. L. Johnson. 2009. Anti-CD25 antibody-mediated depletion of effector T cell populations enhances susceptibility of mice to acute but not chronic Toxoplasma gondii infection. *J Immunol* 182:3985-3994.
243. Carrigan, S. O., Y. J. Yang, T. Issekutz, N. Forward, D. Hoskin, B. Johnston, and T. J. Lin. 2009. Depletion of natural CD4+CD25+ T regulatory cells with anti-CD25 antibody does not change the course of Pseudomonas aeruginosa-induced acute lung infection in mice. *Immunobiology* 214:211-222.
244. Kim, B., N. Feng, C. F. Narvaez, X. S. He, S. K. Eo, C. W. Lim, and H. B. Greenberg. 2008. The influence of CD4+ CD25+ Foxp3+ regulatory T cells on the immune response to rotavirus infection. *Vaccine* 26:5601-5611.

245. Anghelina, D., J. Zhao, K. Trandem, and S. Perlman. 2009. Role of regulatory T cells in coronavirus-induced acute encephalitis. *Virology* 385:358-367.
246. Stephens, L. A., D. Gray, and S. M. Anderton. 2005. CD4+CD25+ regulatory T cells limit the risk of autoimmune disease arising from T cell receptor crossreactivity. *Proc Natl Acad Sci U S A* 102:17418-17423.
247. Stephens, L. A., and S. M. Anderton. 2006. Comment on "Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells". *J Immunol* 177:2036; author reply 2037-2038.
248. Zelenay, S., and J. Demengeot. 2006. Comment on "Cutting edge: anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells". *J Immunol* 177:2036-2037; author reply 2037-2038.
249. Kohm, A. P., J. S. McMahon, J. R. Podojil, W. S. Begolka, M. DeGutes, D. J. Kasproicz, S. F. Ziegler, and S. D. Miller. 2006. Cutting Edge: Anti-CD25 monoclonal antibody injection results in the functional inactivation, not depletion, of CD4+CD25+ T regulatory cells. *J Immunol* 176:3301-3305.
250. Couper, K. N., D. G. Blount, J. B. de Souza, I. Suffia, Y. Belkaid, and E. M. Riley. 2007. Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice. *J Immunol* 178:4136-4146.
251. Tang, Q., J. Y. Adams, A. J. Tooley, M. Bi, B. T. Fife, P. Serra, P. Santamaria, R. M. Locksley, M. F. Krummel, and J. A. Bluestone. 2006. Visualizing regulatory T cell control of autoimmune responses in nonobese diabetic mice. *Nat Immunol* 7:83-92.
252. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat Immunol* 3:1097-1101.
253. Cecchinato, V., E. Trynieszewska, Z. M. Ma, M. Vaccari, A. Boasso, W. P. Tsai, C. Petrovas, D. Fuchs, J. M. Heraud, D. Venzon, G. M. Shearer, R. A. Koup, I. Lowy, C. J. Miller, and G. Franchini. 2008. Immune activation driven by CTLA-4 blockade augments viral replication at mucosal sites in simian immunodeficiency virus infection. *J Immunol* 180:5439-5447.
254. Jiang, Q., L. Zhang, R. Wang, J. Jeffrey, M. L. Washburn, D. Brouwer, S. Barbour, G. I. Kovalev, D. Unutmaz, and L. Su. 2008. FoxP3+CD4+ regulatory T cells play an important role in acute HIV-1 infection in humanized Rag2^{-/-}gammaC^{-/-} mice in vivo. *Blood* 112:2858-2868.

In Vivo Depletion of CD4⁺CD25⁺ Regulatory T cells in Cats

Published in the Journal of Immunological Methods

S. Rochelle Smithberg^a, Jonathan E. Fogle^a, Angela M. Mexas^b, Stacie K. Reckling^a,

Susan M. Lankford^a, Mary B. Tompkins^b, and Gregg A. Dean^a

^aCenter for Comparative Medicine and Translational Research and Department of Molecular Biomedical Sciences, ^bDepartment of Population Health and Pathophysiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina, USA.

The data reported in figures 1, 2, and 3 were collected by JEF, AMM, and SKR.

Abstract

To establish a characterized model of regulatory T cell (Treg) depletion in the cat we assessed the kinetics of depletion and rebound in peripheral and central lymphoid compartments after treatment with anti-CD25 antibody as determined by cell surface markers and FOXP3 mRNA expression. An 82% decrease in circulating CD4⁺CD25⁺ Tregs was observed by day 11 after treatment. CD4⁺CD25⁺ cells were also reduced in the thymus (69%), secondary lymphoid tissues (66%), and gut (67%). Although CD4⁺CD25⁺ cells rebound by day 35 post-treatment, FOXP3 levels remain depressed suggesting anti-CD25 antibody treatment has a sustainable diminutive effect on the Treg population. To determine whether CD25⁺ Treg depletion strategies also deplete activated CD25⁺ effector cells, cats were immunized with feline immunodeficiency virus (FIV) p24-GST recombinant protein, allowing them to develop a measurable memory response, prior to depletion with anti-CD25 antibody. Anti-FIV p24-GST effector cell activity in peripheral blood after depletion was sustained as determined by antigen-specific T cell proliferation and humoral responses against FIV p24-GST with an ELISA for antigen-specific feline IgG. Furthermore, development of an anti-mouse response in Treg-depleted cats was similar to control levels indicating the retained capacity to respond to a novel antigen. We conclude that despite alterations in CD25⁺ cell levels during depletion, the feline immune system remains functional. We demonstrate here a model for the study of disease pathogenesis in the context of reduced numbers of immunosuppressive CD4⁺CD25⁺ Tregs throughout the feline immune system.

1. Introduction

CD4⁺CD25⁺ regulatory T cells (Tregs) have been shown to suppress antigen-specific CD4⁺ and CD8⁺ T cell responses against neoplasms, allografts, and a broad spectrum of infectious agents. Activation of Tregs in response to infectious agents can be a double-edged sword. While they can be important in reducing the magnitude of the immune response to pathogens, preventing potentially harmful immunopathology, the presence of Treg cells has also been shown to prevent complete clearance of certain pathogens.

CD4⁺CD25⁺ Tregs were recently described in the cat and were demonstrated to be chronically activated in feline immunodeficiency virus (FIV)-positive cats (Vahlenkamp et al., 2004). Analysis of these cells from both normal and FIV-infected cats demonstrated that they have the salient characteristics of CD4⁺ Tregs in humans and rodents, as they constitute about 5-10% of the peripheral T cell population, are arrested in the G0/G1 stage of the cell cycle, do not proliferate in response to mitogen, and are relatively resistant to activation-induced programmed cell death. When activated *in vitro* with LPS, CD4⁺CD25⁺ T cells from uninfected cats are able to suppress the proliferative response of Con A-stimulated CD4⁺CD25⁻ T cells. Interestingly, freshly isolated, unstimulated CD4⁺CD25⁺ T cells from FIV-infected cats significantly inhibit proliferation of Con A-stimulated CD4⁺CD25⁻ T cells, suggesting that these cells are activated *in vivo* as a result of the chronic FIV infection. As activated Tregs are non-antigen specific in their suppressive function, it is possible that these cells could in turn suppress or anergize CD4⁺ T helper cell responses to a variety of antigens including FIV antigen and thereby contribute to the acquired immunodeficiency syndrome (AIDS) that is characteristic of this infection. Similar observations have recently been

described in HIV-1 infected people (Aandahl et al., 2004; Weiss et al., 2004). Currently, it is unknown whether Treg-mediated immunosuppression undermines a successful anti-viral T cell response or beneficially limits a destructive cycle of inflammation and viral replication. This question cannot be addressed in human subjects but rather requires a well-characterized animal model such as FIV and a method for depletion of CD4⁺CD25⁺ Tregs.

Antibody depletion of cells *in vivo* has become a commonly employed method to determine the significance of a cell population in a particular process and recently to treat a number of neoplastic and immune-mediated diseases. A feline CD25-specific monoclonal antibody (9F23) is available and has been used extensively in studies of feline Tregs (Vahlenkamp et al., 2004; Joshi et al., 2005). 9F23 is of the IgG2a isotype and can therefore potentially support antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). In the present study we report the ability of 9F23 to deplete CD25⁺ cells *in vivo*.

2. Materials and Methods

2.1. Animals

To determine the effect of 9F23 on circulating CD25⁺ T cells and the most effective route of monoclonal antibody (mAb) administration, twenty specific-pathogen-free (SPF) cats purchased from Liberty Labs (Liberty, NY) were divided into four groups of five cats each. At the time of euthanasia cats were about nineteen months old. To determine the extent of CD25⁺ cell depletion in the tissues, eight cats were divided into two groups of four cats each. Data from a separate study conducted by K. Howard provided comparative normal values for

lymphoid compartment cell subsets in five additional untreated SPF cats. Cats were housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University in conditions approved of by the Institutional Animal Care and Use Committee. Animals were anesthetized with Telazol[®] administered i.v. and/or i.m. (Fort Dodge Animal Health, Overland, KS) during sample collection and euthanized with sodium pentobarbital administered i.v. (Vortech Pharmaceuticals, Dearborn, MI). As indicated, some cats were immunized two or three times i.p. with 200 µg FIV p24-GST fusion protein (Reid et al., 1991) and 0.5 ml MPL[®] + TDM adjuvant (Sigma-Aldrich, St. Louis, MO) per dose.

2.2. CD25+ cell depletion strategy

The hybridoma clone 9F23 produces a mouse mAb against feline CD25 and was generously provided by M. Honda (National Institutes of Health, Tokyo, Japan) (Ohno et al., 1992). Mouse anti-yellow fever antigen (YFA) mAb hybridoma (CRL-1689, ATCC, Manassas, VA) was also obtained to produce an IgG2a kappa light chain antibody as an isotype control. Antibody-producing hybridomas were grown in serum-free medium (Gibco, Grand Island, NY) and antibodies were protein A purified using endotoxin-free buffers. For some studies, purified antibodies were produced and certified endotoxin-free and mycoplasma-free by Leinco, Inc. (St. Louis, MO). To determine the best dosing strategy, two groups of cats received 3 mg/kg of mouse anti-feline CD25 or the anti-YFA mAb i.v. on days 0, 3, and 7. The third group of cats received 9 mg/kg of anti-CD25 mAb i.p. in one dose on day 0. Sham-treated cats in the fourth group received PBS i.v. on days 0, 3, and 7. Thereafter all

cats received 9 mg/kg of anti-CD25 mAb i.p. in one dose on day 0.

2.3. Sample collection and processing

Whole blood was collected via jugular venipuncture into Vacutainer™ tubes (Becton-Dickinson, Franklin Lakes, NJ) containing ACD on days 0, 3, 7, 9, 11, 14, 21, 28, and 35 after treatment. Centrifugation of blood over Histopaque (Sigma-Aldrich, St. Louis, MO) density gradient at 400xg for 30 min at 25°C yielded isolated PBMCs. Cells were washed once in 1x PBS and once in PBMC medium (RPMI 1640 medium supplemented with 15% FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM β-mercaptoethanol) before resuspension in PBMC medium for cell counting by trypan blue dye exclusion. Serum for enzyme-linked immunosorbent assays (ELISA) was collected in Vacutainer™ tubes without additive, centrifuged at 1300xg for 10 min at 25°C and aliquots were frozen at -80°C. Plasma for complete blood counts and leukocyte differentials was collected in Vacutainer™ tubes containing EDTA. Complete blood counts were performed using a VetScan HMT hematology system (Abaxis, Union City, CA). Peripheral LN biopsies were performed on anesthetized cats and one popliteal LN was surgically excised per time point. Sectioned LN were disassociated over a #40 wire mesh screen and cell suspensions were centrifuged at 400xg for 10 min at 25°C. Pellets were washed twice in PBMC medium and filtered through 150 µm nylon mesh (Sefar, Freibach, Austria) before counting single-cell suspensions. Viability was routinely >90%. At necropsy, mesenteric, retropharyngeal, and medial iliac lymph nodes as well as spleen, thymus, and distal small intestine were harvested and processed as previously described (Dean and Pedersen, 1998;

Howard et al., 2005). Cell aliquots were frozen in RLT buffer (Qiagen, Valencia, CA) supplemented with 1% β -mercaptoethanol for subsequent RNA isolation with the RNeasy Mini Kit (Qiagen).

2.4. Antibodies

Mouse anti-feline pan T cell mAb (1.572) (Tompkins et al., 1990) and mouse anti-feline CD25 (9F23) were conjugated to FITC using standard protocols. Mouse anti-feline CD4 (30A) and mouse anti-feline CD8 α (3.357) (Tompkins et al., 1990) were conjugated to FITC using standard protocols, to PE using Alexa Fluor[®] 647 mAb labeling kit (Invitrogen, Eugene, OR), or to biotin using Molecular Probes[™] Mini-biotin-XX labeling kit (Invitrogen). Streptavidin-PerCP (BD Pharmingen, San Diego, CA) or Streptavidin-APC (Southern Biotech, Birmingham, AL) were used as secondary fluorochromes. Mouse anti-feline CD4-PE (3-4F4, Southern Biotech, Birmingham, AL), mouse anti-canine CD21 (CA2-1D6, Serotec, Raleigh, NC), and goat anti-mouse IgG2a-FITC (R19-15, BD Pharmingen, San Diego, CA) were also used in flow cytometric analysis.

2.5. Flow cytometric staining and analysis

At least 5×10^5 freshly isolated cells were stained for phenotypic analysis in 1x PBS and incubated with directly conjugated antibodies for 15 min at 4°C. Cells were washed 2 times with cold PBS and centrifuged at 600xg for 5 min at 10°C. This procedure was repeated as necessary for complete staining with streptavidin-APC or streptavidin-PerCP. To determine whether CD25+ cells were bound by 9F23 in vivo, PBMC were incubated with goat anti-

mouse IgG2a-FITC only. To determine the total number of CD25+ cells, cells were first incubated with an excess of unconjugated 9F23, washed as described, and then stained with goat anti-mouse IgG2a-FITC. Samples were fixed using 2% paraformaldehyde or analyzed immediately on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) or LSR II flow cytometer (BD Biosciences) using CellQuest or FACSDiva software (BD Biosciences), respectively.

2.6. *FOXP3* real-time RT-PCR

The mRNA from cats that received i.p. 9F23 injections was isolated from samples frozen in RLT buffer and 1% β -mercaptoethanol using the RNeasy Mini Kit (Qiagen). One-step real-time RT-PCR assays were performed on a Bio-Rad MyiQ™ PCR Detection System using TaqMan® EZ RT-PCR kit (Applied Biosystems) containing *rTth* DNA polymerase and feline FOXP3- and HPRT-specific primers and dual-labeled probes (Table 1). Primer pairs were designed to span an exon junction and the resulting amplicons were confirmed by sequencing. *In vitro* transcribed FOXP3 and HPRT RNAs were used to generate standard curves for absolute quantitation. 200 ng lymphocyte RNA was used in each assay. All samples were run in duplicate. FOXP3 RT-PCR cycling conditions were 60°C for 30 min, 95°C for 5 min, followed by 45 cycles of 95°C for 20 s and 60.5°C for 1 min. HPRT cycling conditions were 60°C for 30 min, 95°C for 5 min, followed by 45 cycles of 95°C for 20 s and 57°C for 1 min.

2.7. ELISAs

For the FIV p24 antibody ELISA, Immulon-2HB plates (Dynex Technologies, Chantilly, VA) were coated with 1.0 $\mu\text{g/ml}$ p24-GST fusion protein and the assay was performed as previously described (Staats et al., 1996). For the mouse antibody ELISA, Maxisorp plates (Nunc, Rochester, NY) were coated with 5 $\mu\text{g/ml}$ mouse anti-feline CD25 in bicarbonate coating buffer (0.5 M NaHCO_3 , pH 8.6) at 4°C overnight. Plates were blocked with 200 $\mu\text{l/well}$ blocking buffer (0.5 M NaHCO_3 , 5% nonfat dry milk, pH 8.6) for two hours at room temperature. Serum samples were diluted in ELISA sample diluent (5% nonfat dry milk, 5% goat serum, 0.05% Tween 20, and 0.1% Kathon in 1x PBS) and added to blocked plates at 100 $\mu\text{l/well}$. Plates were incubated for two hours at 37°C and then washed four times with wash buffer (0.5% Tween 20 in 1x PBS). Antibody was identified with goat anti-cat IgG-horseradish peroxidase (HRP) (Bethyl Labs, Montgomery, TX) diluted 1:20,000 and incubated for one hour at room temperature. Plates were washed four times with a 5 min incubation between the third and fourth wash before the addition of tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD) for 10 min. The reaction was stopped with 1 N H_2SO_4 before reading the optical density at 450 nm on a Tecan Sunrise absorbance reader (Zurich, Switzerland). A threefold higher optical density of tested sample over preinoculation samples determined positive antibody titers. Differences in post-treatment endpoint titers were determined by t-test using a 0.05 level of significance (SAS, Cary, NC).

2.8. Anti-FIV p24-GST T cell proliferation

Freshly isolated lymphocytes from cats were washed in 1x PBS by centrifugation at 600xg for 5 min. Cells were resuspended to 2.5×10^5 cells/ml in 1.25 μ M carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Eugene, OR) diluted in 1x PBS and stained in the dark at room temperature for 5 min. Staining was quenched by washing cells in cold PBMC medium. Cells were plated in a polystyrene 96-well round-bottom plate (Costar, Corning, NY) at 1×10^5 cells/well in with either 2 μ g/ml concanavalin A (ConA) (Sigma-Aldrich, St. Louis, MO) or 100 μ g/ml p24-GST fusion protein. Untreated cells were also plated as a background control. Cells were incubated for four days at 37°C in 5% CO₂, then washed in 1xPBS containing 10% FBS and analyzed on an LSR II flow cytometer with FACSDiva software.

2.9. IFA for Anti-nuclear antibody (ANA)

Day 21 serum samples from five cats treated by i.p. anti-CD25 administration were evaluated for anti-nuclear antibody activity. Positive control serum was obtained from the NCSU-CVM clinical immunology laboratory. The control and experimental serum samples were stored at -80°C. Ten-fold serial dilutions (1:20 to 1:200) of control serum and each sample were prepared using PBS as a diluent. Ten μ l of 1:20 dilution from the control and from each sample was placed on ANA Hep-2 substrate slides® (Immuno Concepts, Sacramento, CA). Slides were incubated at 37°C for fifteen minutes. The slides were then washed three times with PBS. FITC conjugated anti-feline IgG (Cappel, West Chester, PA) was then applied to each sample and incubated at 37°C for fifteen minutes. The slides were again washed three

times with PBS. Slides were mounted with a coverslip and evaluated on a Zeiss IM 35 fluorescence microscope (Jena, Germany). All five samples were negative (less than 1:20) for ANA activity.

2.10. Coculture suppression assay

Mesenteric and retropharyngeal LN from cats were stained with anti-feline CD4-PE (3-4F4) and anti-feline CD25-FITC (9F23). CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were purified on a MoFlo Cell Sorter (Dako, Fort Collins, CO). Target CD4⁺CD25⁻ cells were washed in 1xPBS and stained with 1.25 μM CFSE for 5 min in the dark. Target cells were added to a 96-well round-bottom plate at 5x10⁴ cells/well and were incubated alone or with suppressor cells. Autologous CD4⁺CD25⁺ cells were added to CD4⁺CD25⁻ cells to yield suppressor:target cell ratios from 0.016:1 to 0.5:1, or were incubated alone. Cells were incubated for 4 days at 37°C with or without 2 μg/ml ConA, then washed in 1xPBS containing 10% FBS and analyzed on an LSR II flow cytometer with FACSDiva software. Suppression of proliferation was calculated as (% proliferation of CD4⁺CD25⁻ cells alone - % proliferation of CD4⁺CD25⁻ cells in coculture) / % proliferation of CD4⁺CD25⁻ cells alone.

3. Results

3.1. Depletion of CD4⁺CD25⁺ T cells with anti-feline CD25 monoclonal antibody 9F23

Our first objective was to determine whether treatment of cats with anti-CD25 monoclonal antibody 9F23 would result in the depletion of CD4⁺CD25⁺ cells. Two routes of antibody administration were compared. In the first, three treatments of antibody were given

intravenously at a dose of 3 mg/kg at day 0, 3 and 7. In the second strategy, 9 mg/kg of antibody was given intraperitoneally at day 0 only. Control cats were given an equivalent dose of isotype control antibody intravenously at days 0, 3 and 7. Depletion of CD4⁺CD25⁺ cells from peripheral blood was observed in animals receiving 9F23 antibody by both routes of administration. But depletion was more dramatic and consistent in the group given antibody by the i.p. route with a significant decrease seen at day 7, 11, 14 and 28 (P<0.05, t-test) as compared to day 0. The nadir occurred at day 11 with an 82% depletion of CD4⁺CD25⁺ T cells (Fig. 1A). As reflected by the decrease in absolute CD4⁺ T cell number in the group given antibody by the i.p. route, CD4⁺CD25⁺ cells were depleted and not functionally inactivated by 9F23 (Fig. 1B). All animals receiving 9F23 i.v. were depleted of CD4⁺CD25⁺ cells, however, due to variability in the degree of depletion and the kinetics within the group there was no statistical significance. Based on these results, the i.p. route of antibody administration was selected for further studies. The isotype control antibody did not cause changes in CD4⁺CD25⁺ cell frequency in peripheral blood as calculated by flow cytometry and lymphocyte differentials.

3.2. 9F23 depletes CD25⁺ B cells and CD8⁺ T cells

In addition to high expression levels on regulatory T cells, CD25 is also expressed on activated T and B cells. Therefore, we evaluated the effect of CD25⁺ cell depletion on CD8⁺CD25⁺ and CD21⁺CD25⁺ cells as well as CD4⁺CD25⁺ cells. Indeed, all three populations were depleted in peripheral blood by treatment with 9F23. Figure 2 shows the kinetics of depletion and rebound of CD4⁺, CD8⁺, and B cell populations expressing CD25 in

cats treated intraperitoneally. Just as depletion of the CD4⁺CD25⁺ population was mirrored by a corresponding decrease in total CD4⁺ T cells, total CD8⁺ and CD21⁺ absolute cell number was reduced surrounding the nadir of depletion in the group receiving 9F23 intraperitoneally (Fig. 1C and 1D). B cells and CD8⁺ T cells rebounded to pre-treatment levels by day 14 and exceed pre-treatment numbers at day 21. This is presumably due to the antigenicity of the mouse antibody and the ensuing activation of responding B cells and CD8⁺ T cells.

3.3. Depletion is limited by induction of anti-mouse antibody response

As would be expected, a similar anti-mouse antibody response was induced in all cats receiving either 9F23 or the YFA isotype control antibody (Fig. 3 and data not shown). We explored the dynamics of CD25⁺ cell depletion and anti-mouse antibody induction by additionally assessing the number of peripheral blood lymphocytes that had bound 9F23 directly ex vivo by simply labeling cells with an anti-mouse FITC-conjugated antibody. All three parameters are overlaid in Figure 3 demonstrating CD25⁺ cell depletion occurred when cells were coated with 9F23, but once an anti-mouse antibody response was mounted, CD25⁺ cells rebounded.

3.4. Depletion of CD4⁺CD25⁺ T cells and FOXP3⁺ cells in tissues

We observed a significant reduction of circulating CD4⁺CD25⁺ cells in cats given 9F23 intraperitoneally. Thus, we employed this strategy to further investigate the extent of depletion of CD4⁺CD25⁺ cells from lymphoid tissues. As before, a significant reduction in

the percentage of CD4⁺CD25⁺ cells in the PBMC population (67%) was observed by day 11 as compared to cats not treated with 9F23 (Fig. 4). Similar levels of depletion were noted in the thymus (69%) and in secondary lymphoid compartments including the iliac lymph node (61%), mesenteric lymph node (59%), retropharyngeal lymph node (65%), and spleen (79%). In the gut, levels of CD4⁺CD25⁺ lamina propria lymphocytes (62%) as well as intraepithelial lymphocytes (71%) were reduced until day 35 (Figure 4). All tissues except PBMCs and intestinal lymphocytes exhibited an increase in CD4⁺CD25⁺ cell percentages by day 35 after depletion.

The transcription factor FOXP3 is the most definitive known Treg marker. We compared levels of FOXP3 mRNA in tissues of untreated cats to tissues of CD25⁺ cell-depleted cats and observed a trend of FOXP3 mRNA reduction within each lymphoid compartment investigated; however, statistical significance could not be achieved due to variability in the control group (Fig. 5). In secondary lymphoid tissues FOXP3 levels did not rebound by day 35 to the same degree as CD4⁺CD25⁺ cells, suggesting the increase in CD4⁺CD25⁺ cell percentage is most likely due to activation of helper T cells in response to mouse antibody rather than a repopulation of FOXP3⁺ Tregs. In peripheral blood FOXP3 levels were reduced even further by day 35, suggesting 9F23 may have a sustained diminutive effect on the actual Treg population.

3.5. Proliferation of effector T cells is sustained during CD25⁺ cell depletion

One of the main goals of our study was to determine if anti-CD25 antibody altered immune function in treated cats. To do this, we tracked the effects of 9F23 treatment on an established memory response in our depletion model. Cats were first immunized with recombinant FIV p24-GST (200 µg/dose) and MPL[®]-TDM adjuvant given i.p. either two or three times at 3 week intervals. The development of a functional memory response to p24-GST was detected by CFSE T cell proliferation assay before cats were treated with 9F23 (data not shown). At each of the time points assessed, FIV p24-GST-specific proliferation was detected in PBMCs from at least 50% of cats tested, with an average of 79% of cats positively responding to the antigen over the course of the study, showing maintenance of effector T cell function even as CD25⁺ cells were depleted (Table 2). IgG titers to FIV p24-GST were also monitored and showed minimal fluctuation after Treg depletion as compared to titers before treatment with antibody (Fig. 6).

3.6. CD4⁺CD25⁺ T cells are functionally consistent with Treg at day 35 after Treg depletion

In order to characterize the rebound of CD4⁺CD25⁺ T cells in secondary lymphoid organs by day 35 after depletion, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated from mesenteric and retropharyngeal LN by FACS and cocultured in a functional suppression assay. Because of the small number of CD4⁺CD25⁺ T cells in lymph nodes on day 11 after depletion, no attempt was made to isolate this cell subset at this time point. CD4⁺CD25⁺ Tregs were cultured in a two-fold series of 1:2 to 1:64 ratios with CFSE-stained CD4⁺CD25⁻ cells.

CD4⁺CD25⁺ cells remained stained with low levels of residual anti-CD25-FITC from FACS isolation and were not included in the proliferating population (Fig. 7A). CD4⁺CD25⁺ cells displayed a Treg functional phenotype by inhibiting CD4⁺CD25⁻ ConA-induced proliferation at a suppressor:target cell ratio as low as 0.31:1 (Fig. 7B).

3.7. No evidence of autoimmunity after transient CD4⁺CD25⁺ depletion

Because a main function of Tregs is to suppress responses to autoantigens, depletion of Tregs may result in autoimmune disease. Following Treg depletion, we were unable to document anti-nuclear antibody activity in any of cats administered 9F23 intraperitoneally. In all probability Tregs remaining after depletion prevented inappropriate autoimmune activity.

4. Discussion

Numerous experimental approaches have been used to assess the role of regulatory T cells in normal and pathologic immune responses. Beyond phenotypic analysis and ex vivo functional assays, the in vivo depletion of CD25⁺ Tregs has been particularly informative in various mouse models of neoplasia, transplantation, autoimmune disease, and infectious disease. The present studies report a method for the depletion of CD25⁺ Tregs in cats using a feline-specific monoclonal antibody against CD25.

Administration of a single dose of anti-CD25 monoclonal antibody (9F23) by the intraperitoneal route consistently resulted in the depletion of CD4⁺CD25⁺ Tregs in blood and tissues. Clone 9F23 is of the IgG2a isotype, and can potentially support antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. We believe that depletion of

CD4⁺CD25⁺ cells occurred after binding by 9F23 since CD4⁺ absolute cell numbers declined concurrent with the nadir of CD4⁺CD25⁺ cell numbers (Fig. 1A and 1B). Furthermore, relative FOXP3 mRNA levels also declined in tissues as CD4⁺CD25⁺ cells were depleted (Fig. 5). The reduction in circulating CD4⁺CD25⁺ cells was most pronounced at day 11 post-depletion (82%) in the blood and remained significantly low until day 28. CD4⁺CD25⁺ cells in lymphoid inductive sites and the thymus were also significantly diminished by day 11, later rebounding by day 35. In lymphoid effector sites CD4⁺CD25⁺ cells were minimally reduced on day 11 but significantly decreased at day 35. The reasons for the unique depletion kinetics in this compartment are not clear. Perhaps this is related to differential distribution of the anti-CD25 antibody to tissues initially and the subsequent trafficking of Tregs thereafter. Although poorly understood, it is clear that there are tissue-specific mechanisms for Treg migration and retention (Annacker et al., 2005; Wei et al., 2006).

There are two concerns regarding the use of anti-CD25 monoclonal antibodies to deplete Tregs. First, not all Tregs are CD25⁺, however, we have shown in the cat that, similar to human and mouse, the majority of FOXP3⁺ cells (>70%) are also CD25⁺ (unpublished observations). Currently, FOXP3 expression is the most specific marker for Tregs (Banham et al., 2006). However, there are no means to selectively deplete FOXP3⁺ cells due to the intranuclear localization of this transcription factor. Given the limitations of *in vivo* antibody-depletion strategies, CD25 remains the best target available to eliminate Tregs.

The second concern with CD25⁺ cell depletion is that other cells in addition to Tregs express CD25 and depletion of these cells may negatively impact pre-existing and novel

immune responses. CD25 is expressed by a subpopulation of natural killer cells and dendritic cells (Malyguine et al., 1996; Yrlid et al., 2006) and it is also transiently expressed by activated T and B cells (Taniguchi and Minami, 1993; Brisslert et al., 2006), while resting memory T cells and mature B cells express little to no CD25. The very low frequency of CD25⁺ dendritic cells and natural killer cells in circulation limited observation of these populations in the present studies; however, depletion of CD8⁺CD25⁺ T cells and CD21⁺CD25⁺ B cells with kinetics similar to the Treg population was observed. Despite this, we established that cats depleted of CD25⁺ cells are capable of mounting an effective primary humoral response to antigen similar to untreated cats as indicated by robust anti-mouse antibody production, as well as maintaining IgG titers after a secondary response to FIV p24-GST. Furthermore, the cellular memory response was also intact after depletion. Cats immunized with recombinant FIV p24-GST maintained their T-cell mediated memory function after depletion as measured by antigen-specific proliferation.

In addition to a significant, but not complete, depletion of CD4⁺CD25⁺ cells in lymphoid tissues, we also observed a profound and more prolonged reduction of FOXP3 expression in blood, secondary lymphoid tissue, and intestinal lymphocyte populations. The CD4⁺CD25⁺ T cell population rebounded to some degree by day 35 after depletion in all compartments analyzed except the blood and intestine. Surprisingly, FOXP3 levels did not rebound, remaining low through the terminal time point at day 35. Because FOXP3 remains a more reliable Treg marker than surface CD4 and CD25 coexpression, we conclude that removal of Tregs in our depletion strategy is sustained longer than suggested by CD4⁺CD25⁺ cells percentages, extending to at least day 35 post-depletion.

Total removal of Tregs allows the development of destructive autoimmune disease in the murine model (Fontenot et al., 2003). We were able to isolate Tregs at day 35 after depletion and analyzed them for functional activity. CD4⁺CD25⁺ cells inhibited mitogen-induced proliferation of autologous CD4⁺CD25⁻ T cells at a Treg: target cell ratio as low as 1:32. As a possible measure of the development of auto-immune disease, we observed that cats tested negative for antinuclear antibody activity following CD25⁺ cell depletion. We suggest that small amounts of Tregs remaining after depletion are adequate to suppress autoreactive responses and that fully functional Tregs are included, at least in part, in the CD4⁺CD25⁺ cell population present after depletion treatment. Other redundant regulatory mechanisms are also in place to prevent autoimmunity (Prud'homme, 2004).

Immunosuppressive activity by Tregs during lentiviral infections such as HIV, SIV and FIV has been hypothesized to be both helpful and detrimental to the affected individual. On one hand, it has been proposed that increased Treg activity contributes to slowed disease progression by limiting a destructive cycle of inflammation and viral replication (Kinter et al., 2004). On the other hand, data suggests the suppressive effects of Tregs inhibit an otherwise capable antiviral response (Aandahl et al., 2004; Weiss et al., 2004; Estes et al., 2006). Indeed, whether Tregs are beneficial or detrimental may also depend on the stage of infection and the general status of an individual's immune system. The depletion of Tregs in FIV-infected cats has great potential to shed light on the role of Tregs during various stages of infection, and is most practical considering the lack of a murine experimental model of natural lentivirus infection. Tregs are activated during FIV infection and are demonstrably immunosuppressive (Vahlenkamp et al., 2004). Feline Tregs are also preferentially infected

by FIV and may serve as a reservoir for virus replication (Joshi et al., 2005), therefore removal of these cells could allow substantial alterations in disease progression. We propose to use the presently described methods to deplete Tregs from chronically and acutely FIV-infected cats and determine effects on antiviral immune response and viral load.

Acknowledgments

We acknowledge Dr. Kristina Howard and Dr. Sushila Nordone for their helpful advice and assistance in data analysis. We also thank Dr. Howard for her contribution of untreated control group data. We thank Alora LaVoy, Deb Anderson, Janet Dow, Erin Egan, Linda English, and Glicerio Ignacio for their excellent technical assistance. This work was supported by NIH grants R21 AI065223 (GAD) and R01 AI038177 (MBT). Rochelle Smithberg is supported by the Ruth L. Kirschstein National Research Service Awards NIH Training Grant T32 GM008776 as a part of North Carolina State University's Biotechnology Training Program. Jonathan Fogle is supported in part by the Fort Dodge Immunology Fellowship at the North Carolina State University College of Veterinary Medicine.

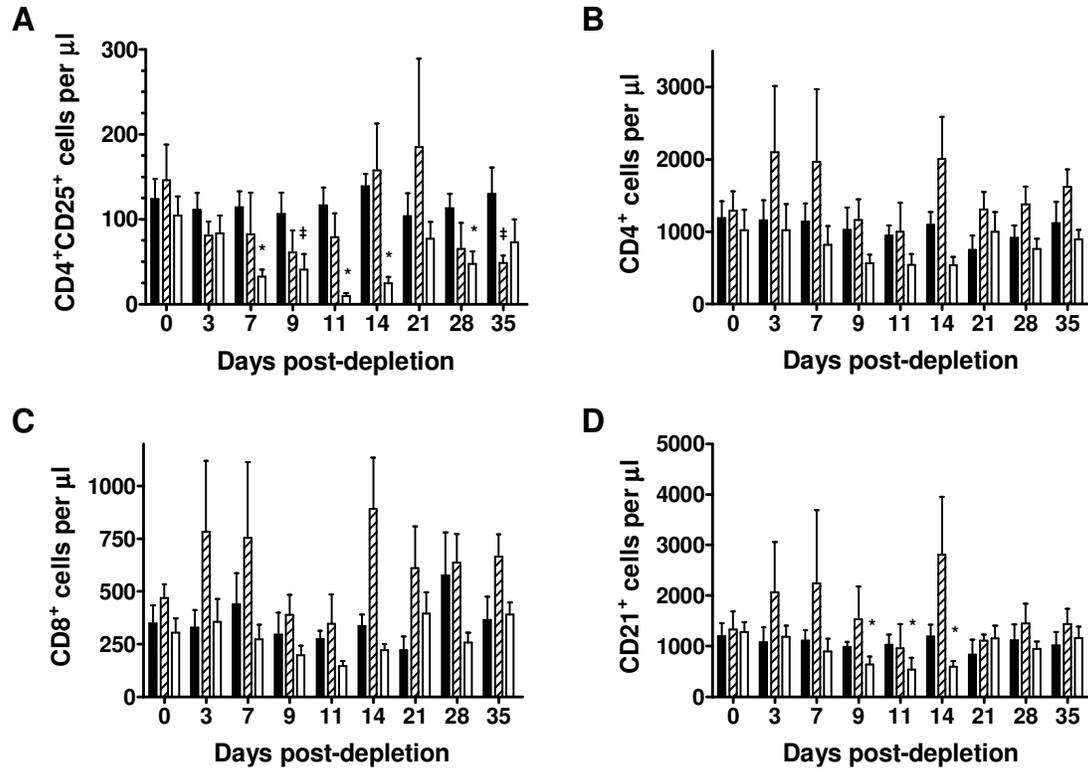


Figure 1. In vivo depletion of circulating cells with mAb 9F23. Cats were treated with isotype control antibody (closed bars, n=5), or anti-CD25 mAb (9F23) at 3 mg/kg intravenously at day 0, 3, and 7 (hatched bars, n=4), or were given 9 mg/kg 9F23 intraperitoneally at day 0 (open bars, n=5). Percent CD4⁺CD25⁺ (A), CD4⁺ (B), CD8⁺ (C), or CD21⁺ (D) cells was determined by flow cytometry and multiplied by the absolute number of peripheral blood lymphocytes. Data is shown as absolute number of PBMCs. * indicates p<0.05 and ‡ indicates p<0.06 by t-test compared to day 0 values for each treatment group. Bars are SEM.

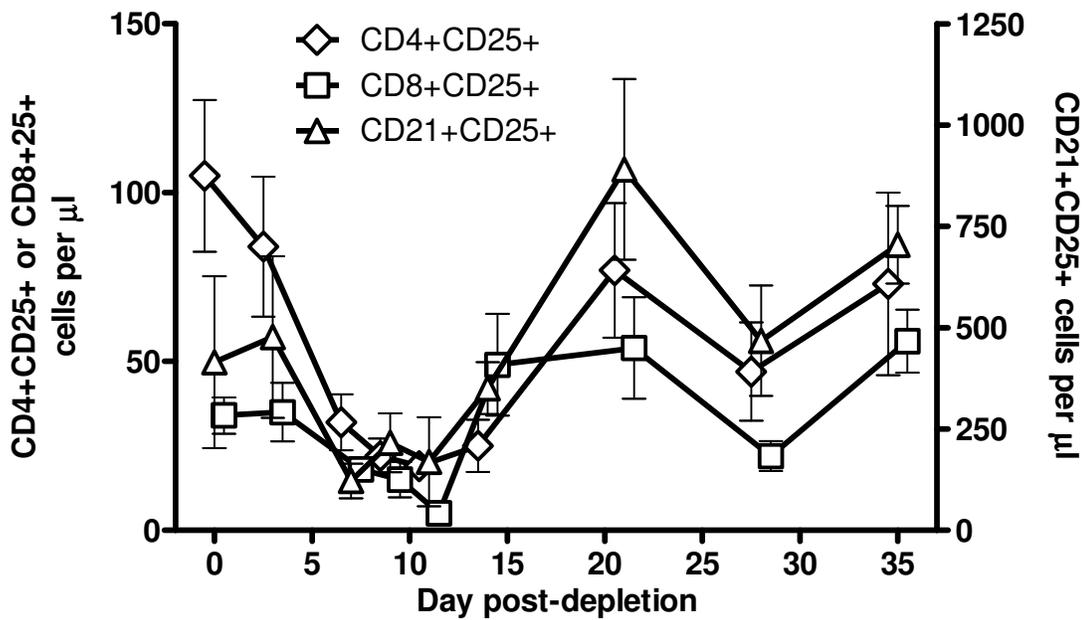


Figure 2. CD25⁺ B cells and T cell subpopulations are depleted by 9F23. Lymphocyte subpopulations assessed by flow cytometry are shown for cats (n=5) treated with 9F23 by the i.p. route. Absolute numbers of CD4⁺CD25⁺ T cells and CD8⁺CD25⁺ T cells are graphed to the primary Y-axis, CD21⁺CD25⁺ cells are graphed to the secondary Y-axis. Bars are SEM.

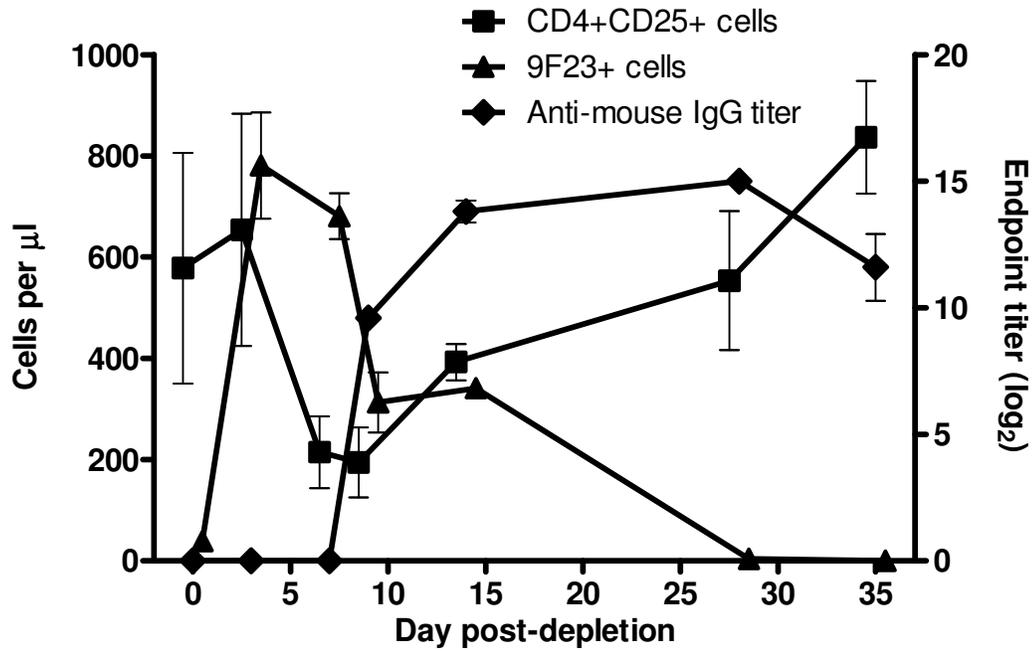


Figure 3. Anti-mouse antibody response precedes rebound in CD25⁺ cells. The absolute number of CD25⁺ cells and 9F23-coated cells detected with an anti-mouse IgG2a secondary antibody are shown relative to the primary Y-axis for cats receiving 9F23 by i.p. route (n=5). The log₂ anti-mouse IgG mean endpoint titer in serum of cats receiving 9F23 by i.p. route (n=5) is shown relative to the secondary Y-axis. Bars are SEM.

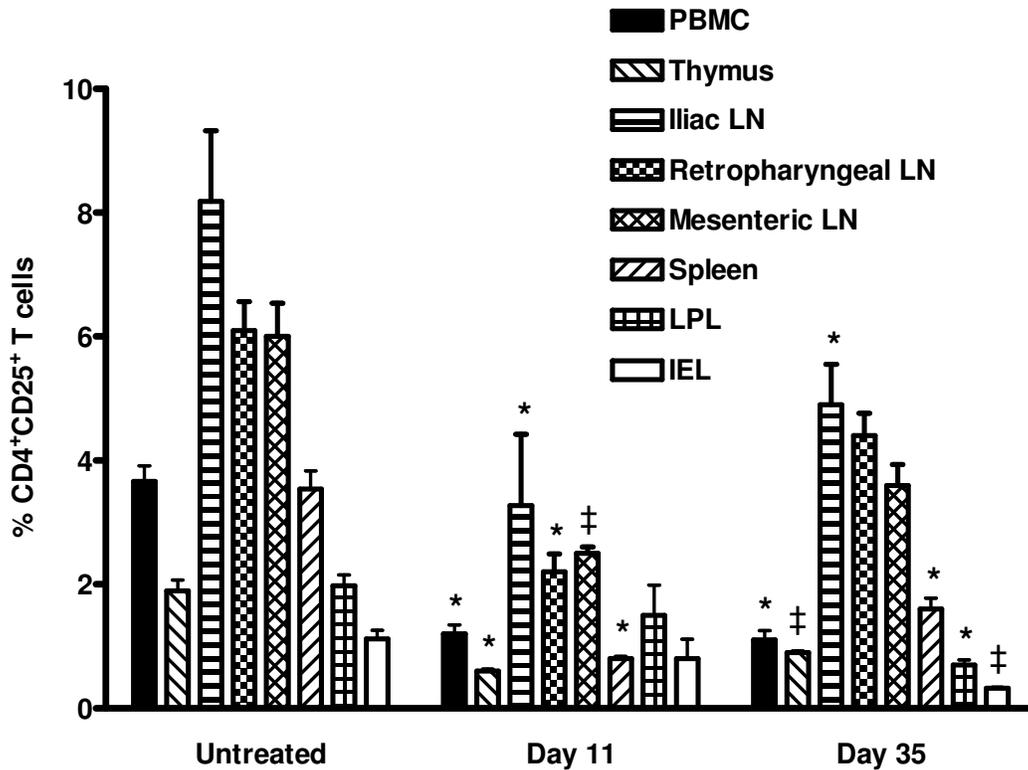


Figure 4. In vivo depletion of CD4⁺CD25⁺ cells from lymphoid tissues. Cats were treated with 9F23 i.p. at day 0 after immunization against FIV p24-GST (200 μ g administered i.p. 2-3 times). Four cats were sacrificed on day 11 and four cats were sacrificed on day 35 after administration of 9F23. Untreated control cats (n=5) did not receive mAb. Percent CD4⁺CD25⁺ cells in circulation, primary lymphoid tissue, secondary lymphoid tissue, and the gastrointestinal tract was determined by flow cytometry and data is shown as percent CD4⁺CD25⁺ cells of total lymphocyte population. * indicates P<0.05 and ‡ indicates p=0.06 by t-test compared to untreated group. Bars are SEM.

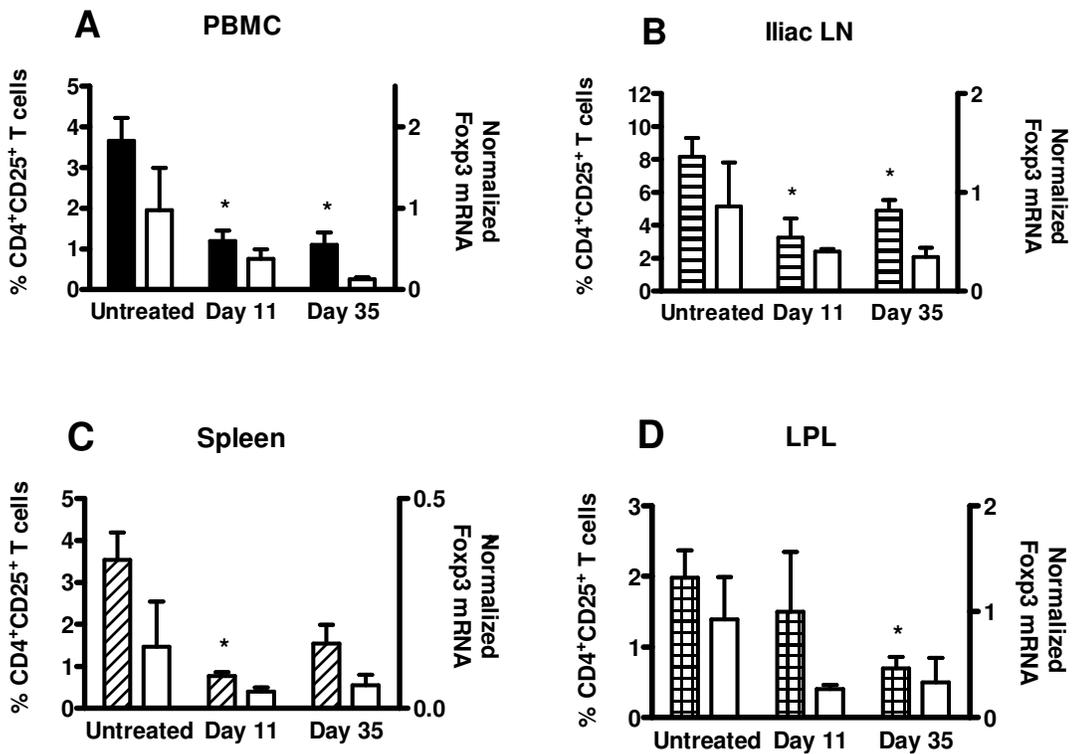


Figure 5. In vivo reduction of CD4⁺CD25⁺ cells and FOXP3 by 9F23 in lymphoid tissues. Cats immunized against FIV p24-GST were treated with 9F23 i.p. on day 0. FOXP3:HPRT mRNA relative fluorescent units (open bars) was determined by quantitative real-time PCR and is shown relative to the primary Y-axis. Percent CD4⁺CD25⁺ cells in PBMC (A; closed bars), iliac LN (B; horizontal-striped bars), spleen (C; hatched bars), and LPL (D; cross-hatched bars) was determined by flow cytometry and is shown relative to the primary Y-axis. * indicates $p < 0.05$ by t-test compared to untreated group. Bars are SEM.

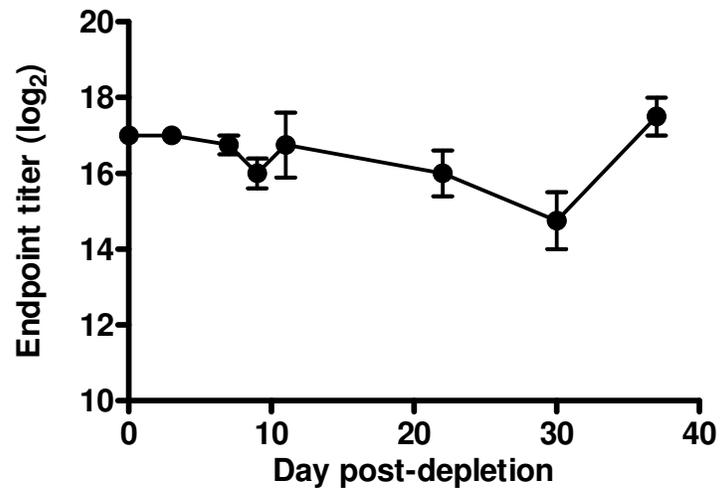


Figure 6. Maintenance of anti-FIV p24-GST IgG titers after administration of 9F23. Cats (n=8) were immunized with FIV p24-GST and treated with 9F23 i.p. on day 0. FIV p24-GST feline IgG ELISAs were performed on serum samples. Bars are SEM.

Figure 7. Suppression of CD25⁻ T cell proliferation by CD25⁺ peripheral lymph node T cells. FACS purified CD4⁺CD25⁻ T cells from the mesenteric and retropharyngeal lymph nodes of four cats sacrificed 35 days after 9F23 mAb i.p. administration were labeled with CFSE and cocultured with FACS purified autologous CD4⁺CD25⁺ T cells in the presence of ConA (2 µg/ml) for four days. CD4⁺CD25⁺ T cells were also labeled with CFSE and incubated alone in the presence of ConA for four days. Histograms of T cell proliferation from representative cocultures are shown (A). The top left and top middle histograms show proliferation of ConA-stimulated CD4⁺CD25⁻ and CD4⁺CD25⁺ cells, respectively. The top right histogram demonstrates residual FITC present on FACS purified CD4⁺CD25⁺ cells. The middle and bottom rows of histograms show the level of proliferation of ConA-stimulated CD4⁺CD25⁻ cells cocultured with decreasing numbers of CD4⁺CD25⁺ cells (Treg:Teff). The percent of cells that were proliferating are indicated on each histogram. Suppression of proliferation was calculated as (% proliferation of CD4⁺CD25⁻ cells alone - % proliferation of CD4⁺CD25⁻ cells in coculture) / % proliferation of CD4⁺CD25⁻ cells alone (B). Bars are SEM.

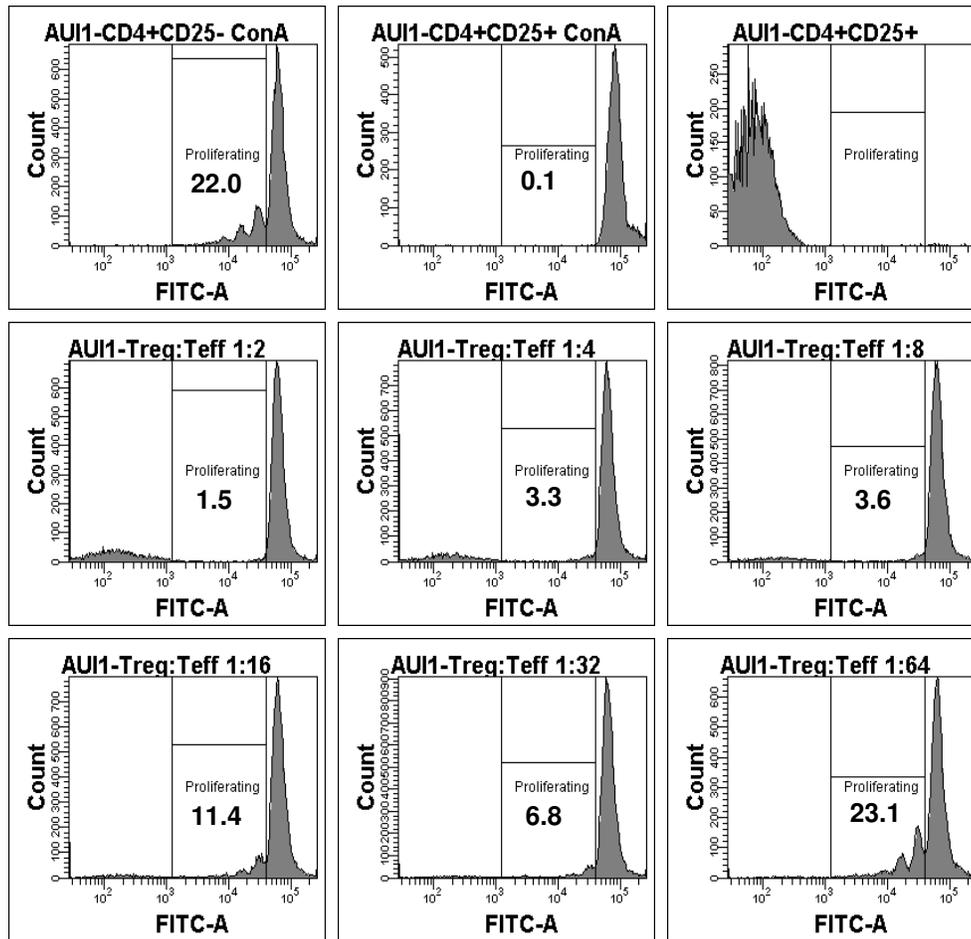
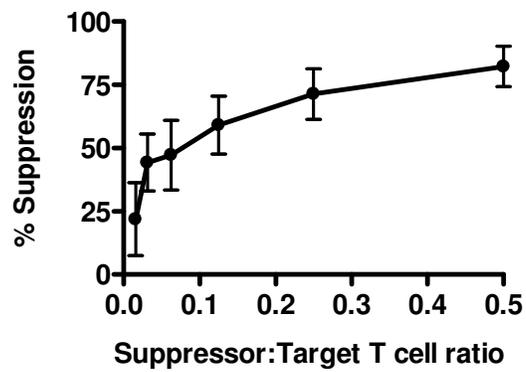
A**B**

Table I. Feline-specific primer and probe sequences for real-time RT-PCR.

	Forward	Reverse	Probe
FOXP3	GCCTGCCACCTGGAATCAAC ^a	GTGTGCTGGGGCTGGGA	CAGTGCTGGCTCCCTGGACACCA ^b
HPRT	TTACGCTGAGGATTGGAAAAGGT	GCCACCCATCTCCTTCAACAC	CTCGGGCAAGACGCTCGGTCTGTC

^a All sequences listed 5' - to -3'.

^b Probes labeled at 5' - with 6-FAMTM and -3' with Black Hole QuencherTM1

Table II. Maintenance of anti-FIV p24-GST T cell proliferation after administration of 9F23.

Day	Positive responders / N
0	6 / 7
3	3 / 3
7	5 / 6
9	5 / 6
11	3 / 4
22	2 / 4
28-31	3 / 4

References

- Aandahl, E.M., Michaelsson, J., Moretto, W.J., Hecht, F.M. and Nixon, D.F. (2004) Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78, 2454-9.
- Annacker, O., Coombes, J.L., Malmstrom, V., Uhlig, H.H., Bourne, T., Johansson-Lindbom, B., Agace, W.W., Parker, C.M. and Powrie, F. (2005) Essential role for CD103 in the T cell-mediated regulation of experimental colitis. *J Exp Med* 202, 1051-61.
- Banham, A.H., Powrie, F.M. and Suri-Payer, E. (2006) FOXP3+ regulatory T cells: Current controversies and future perspectives. *Eur J Immunol* 36, 2832-6.
- Brisslert, M., Bokarewa, M., Larsson, P., Wing, K., Collins, L.V. and Tarkowski, A. (2006) Phenotypic and functional characterization of human CD25+ B cells. *Immunology* 117, 548-57.
- Dean, G.A. and Pedersen, N.C. (1998) Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. *J Virol* 72, 9436-40.
- Estes, J.D., Li, Q., Reynolds, M.R., Wietgreffe, S., Duan, L., Schacker, T., Picker, L.J., Watkins, D.I., Lifson, J.D., Reilly, C., Carlis, J. and Haase, A.T. (2006) Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection. *J Infect Dis* 193, 703-12.
- Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y. (2003) Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4, 330-6.
- Howard, K.E., Fisher, I.L., Dean, G.A. and Jo Burkhard, M. (2005) Methodology for isolation and phenotypic characterization of feline small intestinal leukocytes. *J Immunol Methods* 302, 36-53.
- Joshi, A., Garg, H., Tompkins, M.B. and Tompkins, W.A. (2005) Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. *J Virol* 79, 4965-76.

- Kinter, A.L., Hennessey, M., Bell, A., Kern, S., Lin, Y., Daucher, M., Planta, M., McGlaughlin, M., Jackson, R., Ziegler, S.F. and Fauci, A.S. (2004) CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* 200, 331-43.
- Malyguine, A.M., Saadi, S., Platt, J.L. and Dawson, J.R. (1996) Differential expression of natural killer cell markers: human versus baboon. *Transplantation* 62, 1319-24.
- Ohno, K., Goitsuka, R., Kitamura, K., Hasegawa, A., Tokunaga, T. and Honda, M. (1992) Production of a monoclonal antibody that defines the alpha-subunit of the feline IL-2 receptor. *Hybridoma* 11, 595-605.
- Prud'homme, G.J. (2004) Altering immune tolerance therapeutically: the power of negative thinking. *J Leukoc Biol* 75, 586-99.
- Reid, G., Rigby, M.A., McDonald, M., Hosie, M.J., Neil, J.C. and Jarrett, O. (1991) Immunodiagnosis of feline immunodeficiency virus infection using recombinant viral p17 and p24. *Aids* 5, 1477-83.
- Staats, H.F., Nichols, W.G. and Palker, T.J. (1996) Mucosal immunity to HIV-1: systemic and vaginal antibody responses after intranasal immunization with the HIV-1 C4/V3 peptide T1SP10 MN(A). *J Immunol* 157, 462-72.
- Taniguchi, T. and Minami, Y. (1993) The IL-2/IL-2 receptor system: a current overview. *Cell* 73, 5-8.
- Tompkins, M.B., Gebhard, D.H., Bingham, H.R., Hamilton, M.J., Davis, W.C. and Tompkins, W.A. (1990) Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. *Vet Immunol Immunopathol* 26, 305-17.
- Vahlenkamp, T.W., Tompkins, M.B. and Tompkins, W.A. (2004) Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol* 172, 4752-61.
- Wei, S., Kryczek, I. and Zou, W. (2006) Regulatory T-cell compartmentalization and trafficking. *Blood* 108, 426-31.

Weiss, L., Donkova-Petrini, V., Caccavelli, L., Balbo, M., Carbonneil, C. and Levy, Y.
(2004) Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory
T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients.
Blood 104, 3249-56.

Yrlid, U., Milling, S.W., Miller, J.L., Cartland, S., Jenkins, C.D. and MacPherson, G.G.
(2006) Regulation of intestinal dendritic cell migration and activation by
plasmacytoid dendritic cells, TNF-alpha and type 1 IFNs after feeding a TLR7/8
ligand. *J Immunol* 176, 5205-12.

**In Vivo Depletion of Regulatory T Cells Improves Antiviral and Novel
Immune Responses in Cats Chronically Infected with Feline
Immunodeficiency Virus**

S. Rochelle Mikkelsen¹, Stacie K. Reckling¹, Erin A. Egan¹, and Gregg A. Dean¹

¹Center for Comparative Medicine and Translational Research, College of Veterinary
Medicine, North Carolina State University, Raleigh, NC, USA.

Abstract

Regulatory T (Treg) cells are activated in the periphery during immune responses to pathogens, are phenotypically characterized as CD4⁺CD25⁺FOXP3⁺, and suppress effector T cell responses in a cell-contact dependent manner or through production of cytokines. Ex vivo studies clearly demonstrate that Treg cells have the potential to suppress anti-HIV-1 T cell responses. Lentivirus-induced CD4⁺CD25⁺ Treg cells were first described and functionally characterized in feline immunodeficiency virus (FIV)-infected cats. In the present study we demonstrate that mouse anti-feline CD25 monoclonal antibody (mAb) therapy effectively depletes Treg cells in cats for up to four weeks and does not induce production of inflammatory cytokines above baseline levels nor does it exacerbate viral replication. On the contrary, significant FIV-specific immune responses are revealed in Treg cell-depleted cats as measured by interferon (IFN)- γ production. These anti-FIV effector cells exist prior to Treg cell depletion and are not expanded while Treg cells are depleted. Importantly, cats receiving the Treg cell-depleting mAb are able to produce a significantly more robust humoral response to new antigen as compared to cats receiving an isotype control mAb. We conclude that Treg cells do suppress FIV-specific immune responses in vivo. Treg cell function is enhanced during FIV infection. This allows the effect of Treg cell depletion to be enhanced during chronic FIV infection, resulting in an elevated humoral immune response to a novel antigen during Treg cell depletion. We propose that short-term in vivo Treg cell depletion during chronic lentiviral infection could provide a window of opportunity for therapeutic vaccination in individuals with controlled viral replication.

Introduction

In the face of prolonged exposure to antigen, many regulatory mechanisms are in place to restrict potential damage to the host posed by chronically activated immune responses. Regulatory T (Treg) cells are important negative regulators of activated T cells, are activated in the periphery during immune responses to pathogens, and are phenotypically characterized as CD4⁺CD25⁺FOXP3⁺ (26, 36, 54, 67). Lentivirus-induced CD4⁺CD25⁺ Treg cells were first described and functionally characterized in FIV-infected cats (34, 65). Like HIV-1, FIV is a lentivirus that causes a chronic progressive disease resulting in AIDS (9, 22, 66).

Ex vivo studies clearly demonstrate that Treg cells have the potential to suppress anti-HIV-1 T cell responses (1, 35, 36, 39, 67). Correlative epidemiologic studies present conflicting pictures of the potential role of Treg cells in vivo (11, 17, 56). On one hand, the localization of suppressive Treg cells to the lymph nodes of HIV-1-infected patients may prevent potentially beneficial HIV-1-specific cytotoxic T lymphocyte (CTL) activity in major sites of viral replication (35). Treg cells have been shown to redistribute to lymphoid tissue during HIV-1 infection where they are positioned to modulate cell-mediated immunity (3, 45). On the other hand, several investigators have proposed that HIV-1-specific immune activation in the absence of Treg cells could lead to increased viral replication and activation-induced cell death, causing the characteristic decline of CD4⁺ T helper cells observed in HIV-1 infected individuals over time (1, 23, 36). Lymph node fibrosis, thymic dysfunction, clonal exhaustion, and loss of memory T cell pools due to lasting immune activation have

adverse effects on host health and quality of antiviral immune responses (6). Treg cells, as key modulators of immune activity, are in a unique position to either subdue harmful chronic hyperactivation during lentiviral infection or prevent a potentially beneficial antiviral response. From observations made thus far, no clear resolution has been reached concerning the undoubtedly complex role of Treg cells in the face of lentiviral infection. Direct in vivo modulation of Treg cells in a relevant animal model is necessary to obtain straightforward data regarding the specific function of Treg cells and their effect on host health during lentiviral infection.

Naturally occurring, thymically derived Treg cells and adaptive Treg cells have been phenotyped using a variety of molecules in addition to CD4 and CD25, however, no exclusive Treg cell surface marker has been identified. Intranuclear expression of the forkhead box P3 (FOXP3) transcription factor is associated with Treg cell function within and outside of the CD4⁺CD25⁺ cell population, but labeling of FOXP3⁺ cells for phenotypic identification renders them unusable in live-cell assays (26). Although CD25, as well as FOXP3, is upregulated upon T cell activation and is thus not an exclusive Treg cell marker, only the CD4⁺CD25⁺ cell population has been consistently described as functionally immunosuppressive across species, and detection of CD4 and CD25 expression remains the best live-cell identifier of Treg cells (27, 43, 53).

Monoclonal antibody (mAb) depletion of cells in vivo has become a routine method to determine the significance of a particular cell population in immune processes. Specifically, CD25⁺ cell depletion using mAb therapy has been shown to boost immunity

when used in combination with a melanoma vaccine or a human papilloma virus vaccine in mouse models (18, 28). One anti-CD25 therapeutic agent, denileukin diftitox, has repeatedly been shown to be effective in treating cutaneous T cell lymphomas as well as other lymphoid malignancies (68). We have developed a method to deplete Treg cells from the cat using an anti-feline CD25 mAb (57). In a previous study, we showed that FOXP3 mRNA remained low in immune tissues for at least 4 weeks following the nadir of CD4⁺CD25⁺ cell depletion in FIV-naïve cats (57). Here, we report that Treg cell depletion reveals a much greater cell-mediated anti-FIV response than can otherwise be observed in FIV-infected cats, even in the face of immune dysfunction. Although there was concern that Treg cell depletion could lead to non-specific immune activation, proinflammatory cytokine production, or increased viral burden, no such side effects were observed. Finally, the introduction of a novel antigen induced much more robust humoral responses in FIV-infected cats depleted of Treg cells as compared to Treg cell-sufficient FIV-infected cats or FIV-naïve cats depleted of Treg cells in previous studies.

Materials and Methods

Animals, viral inoculum, and monoclonal antibody administration

A total of 26 female specific-pathogen-free (SPF) cats were purchased from Liberty Labs (Liberty, NY). Cats were housed and cared for in accordance with Association for the Assessment of Laboratory Animal Care standards and Institutional Animal Care and Use Committee guidelines. Cats were infected with the pathogenic molecular clone FIV-NCSU₁

by intravaginal and intravenous routes between 24 and 43 weeks of age (61). Mouse anti-feline CD25 hybridoma (9F23) (47), a gift from M. Honda at the National Institutes of Health in Tokyo, Japan, and mouse anti-yellow fever antigen (YFA) hybridoma (CRL-1689; ATCC, Manassas, VA) were expanded, purified, and certified endotoxin- and mycoplasma-free by Leinco, Inc. (St. Louis, MO). Two groups of cats received one injection of 9 mg/kg anti-feline CD25 mAb intraperitoneally (i.p.) at 10 to 14 months post-infection (57). Two additional groups were treated with 9 mg/kg anti-YFA mAb i.p. as a mouse IgG2a κ light chain isotype control mAb. Another group received 3 ml/kg 0.9% sodium chloride (Hospira, Lake Forest, IL) i.p. as a vehicle control.

Sample Collection and Processing

Whole blood was collected into Vacutainer tubes (BD, Franklin Lakes, NJ) containing ACD on days 0, 10, 21, 29, 42, and 60 or days 0, 10, and 23 after treatment. PBMCs were isolated by centrifugation of blood over Histopaque (Sigma, St. Louis, MO) and serum was processed as previously described (13, 58). Blood for plasma isolation, complete blood counts, and leukocyte differentials was collected in Vacutainer tubes containing EDTA. Plasma was isolated by centrifugation ($800 \times g$ for 10 min) and aliquots were frozen at -80°C . Popliteal lymph node (LN) biopsies were performed on anesthetized cats either 28 days before treatment and 29 days post-treatment or on day 0. At day 23 or 60 post-treatment, mesenteric, prescapular, retropharyngeal, and medial iliac LNs in addition to spleen and distal small intestine were harvested and processed as described previously (20,

30). In some cases, popliteal and prescapular LNs were sorted for CD4⁺CD25⁺, CD4⁺CD25⁻, CD8⁺, and CD21⁺ lymphocytes on a MoFlo cell sorter (Dako, Fort Collins, CO). Cells were used immediately in ex vivo assays or cell aliquots were frozen at -80°C for subsequent RNA and DNA isolation.

Phenotypic Analysis

At least 1×10^6 cells were labeled with the following antibodies for flow cytometric analysis. Anti-CD3 (NZM1) (46), anti-CD4 (30A) (60), and anti-CD8 α (3.357) (60) were used unconjugated to fluorochromes. CD4-PE (3-4F4) and CD5-biotin (F43) were purchased from SouthernBiotech (Birmingham, AL). CD14-PE (Tuk4; Dako) and CD21-FITC (CA2-1D6; Serotec, Raleigh, NC) were purchased. Goat anti-mouse IgG-Cy5 (Jackson ImmunoResearch, West Grove, PA) or streptavidin-PerCP (BD Biosciences, San Diego, CA) were used as secondary fluorochromes. Mouse anti-feline CD25 (9F23) was conjugated to FITC using standard protocols. Anti-CD8 α (3.357) was conjugated to PerCP using a kit from Prozyme (San Leandro, CA). Intracellular FOXP3 staining was performed with eBioscience FOXP3 staining buffers and FOXP3-PECy7 (FJK-16s; San Diego, CA) according to manufacturer's protocol, with the exception that cells remain in the permeabilization/wash buffer no longer than 30 min. For intracellular cytokine staining, cells were incubated with 1x monensin (Biolegend, San Diego, CA) for six hours, labeled for surface markers, fixed with 4% paraformaldehyde, permeabilized with BD Cytotfix/Cytoperm kit Perm/Wash buffer, and stained with IL-2-PE (MQ1-17H12; BioLegend) and tumor

necrosis factor (TNF)- α -APC (6401.1111; BD). Flow cytometric analysis was performed using an LSR II flow cytometer and FACS DIVA software (BD). At least 100,000 gated events were collected per sample.

Viral Parameters

Quantitative real-time one-step reverse transcriptase (RT)-PCR assays were performed on a Bio-Rad MyiQ™ PCR detection system (Hercules, CA). RNA was extracted from plasma using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) following manufacturer's protocol. Detection of plasma viremia in RNA samples was performed using FIV-Gag specific primers, FIVNC.491f and FIVNC.617r, and the FIV-NCSU₁-specific probe FIVNC.555p as described with minor adjustments (14). Each RNA sample was incubated with TaqMan universal PCR master mix without AmpErase® UNG (Applied Biosystems, Branchburg, NJ), 800 nM forward and reverse primers, 80 nM TaqMan probe, and 1x Multiscribe™ and RNase inhibitor mix (Applied Biosystems). Plasma viremia RT-PCR cycling conditions were as follows: 30 min at 48°C, 10 min at 95°C, and 50 cycles of a 15 sec step at 95°C followed by a 1 min step at 57.5°C. Fluorescence was recorded at the end of each annealing/extension step. A 10-fold dilution series of RNA standards provided a detection range from 10¹ to 10⁵ RNA molecules per reaction (14). Bio-Rad MyiQ™ optical system software v2.0 was used to generate a standard curve; viral RNA copies/mL plasma was subsequently calculated.

To detect proviral load in PBMC and tissue samples, quantitative PCR was performed on DNA samples extracted with the DNeasy tissue kit (Qiagen). Each amplification reaction included 0.5 µg DNA sample or standard, TaqMan universal PCR master mix (Applied Biosystems), 491f primer, 617r primer, and 555p probe as described with minor modifications (49). Real-time PCR cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 45 cycles of a 15 s step at 95°C followed by a 1 min step at 57.5°C. To normalize proviral load per 10⁶ cells, 200 nM CCR5 forward primer and 500 nM CCR5 reverse primer were used in the PCR with 200 nM TaqMan probe and 50 ng DNA sample. CCR5 primers (forward 5'-ACGTCTACCTGCTCAACCTGG-3', reverse 5'-ACCGTCTTACACATCCCATCCC-3') and probe (FAM-5'-TCCGACCTGCTCTTCCTCTTCACCCTCC-3') were designed using Beacon Designer. The limit of detection was ≤10 copies of FIV per 1 µg DNA. Standards, controls, and samples were run in duplicate for real-time PCRs.

FOXP3 mRNA Quantitation

In order to quantify FOXP3 mRNA copy number in tissue samples, RNA was extracted from tissue samples with the RNeasy mini kit (Qiagen). Real-time RT-PCR was performed using the TaqMan EZ RT-PCR kit (Applied Biosystems) and FOXP3- and hypoxanthine guanine phosphoribosyl transferase (HPRT)-specific primers and probes as described previously (57). Standards, controls, and samples were run in duplicate.

Peptides

Peptides (Synpep, Dublin, CA) synthesized from FIV Gag p24 and FIV Env protein sequences were 15 amino acids (aa) in length (24). Eight peptides from FIV p24 and nine peptides from FIV Env determined to solicit optimum IFN- γ production from FIV-exposed lymphocytes were pooled as described (19). In addition, the entire FIV p24 sequence of Gag was synthesized by JPT (Springfield, VA) consisting of 15-aa peptides overlapping by 10 aa. Peptides were reconstituted in 10% dimethyl sulfoxide (DMSO) and stored at -80°C.

Interferon- γ ELISpot

Capture and detection antibodies from the feline IFN- γ detection module (R&D Systems, Minneapolis, MN) were used with ELISPOT Immobilon-P 96-well plates (Millipore, Bedford, MA) to quantify IFN- γ -producing cells after stimulation with FIV p24 or Env optimized peptide pools as previously described (59). The protocol was modified with the use of 2.5 or 5×10^5 fresh instead of frozen cells/well.

Anti-mouse Serum Titers

Serum IgG titers against mouse antibody were determined by ELISA as previously described (57).

Statistics

Data were compared by unpaired *t*-test using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results

Anti-CD25 monoclonal antibody treatment depletes Treg cells in FIV-infected cats without disturbing the immune cell profile

We sought to verify whether Treg cells could be depleted in FIV-infected cats with the same kinetics we previously observed in FIV-naïve cats (57). Eighteen cats that had been infected with FIV for 14 months were randomly divided into three groups of six cats each and injected intraperitoneally with either anti-CD25 mAb, isotype control mAb (IgG2a, κ light chain), or vehicle control (saline). The nadir of CD4⁺CD25⁺ cell depletion occurred at day 10 after anti-CD25 mAb treatment, similar to our previous observations in FIV-naïve cats (57). Depletion was most dramatic in the CD4⁺CD25^{hi}-expressing T cell population (Figure 1A). Treg cells express CD25 at higher densities than effector cells, therefore, CD4⁺CD25^{hi} T cells represent a more pure population of Treg cells (7, 57). A 92% average reduction in the absolute number of circulating CD4⁺CD25^{hi} cells compared to day 0 levels was observed in 5 out of 6 cats receiving anti-CD25 mAb (Figure 1B). One cat treated with anti-CD25 mAb did not display decreased numbers of Treg cells at any time after antibody administration; therefore, the data for this cat was removed from all experimental assay group averages to eliminate skewing of results. CD4⁺CD25⁺ cells rebounded by day 29,

however we have previously shown that anti-CD25 mAb treatment reduces FOXP3 mRNA levels in blood and tissues up to day 35 post-treatment, suggesting that rebound CD4⁺CD25⁺ T cells at day 29 are activated CD4⁺CD25⁺ effector cells rather than Treg cells (57).

To verify the depletion of CD4⁺CD25^{hi} Treg cells from lymphoid tissues, a second study including eight FIV⁺ cats divided into two groups of four was performed. The cats were treated with either anti-CD25 mAb or isotype control mAb. At day 23 post-treatment the percentage of CD4⁺CD25^{hi} Treg cells in the lymphoid tissues of the CD25-depleted cats was on average 84% lower than in control-treated cats (Figure 1C). Other lymphocyte subsets were not significantly affected by anti-CD25 mAb, isotype control mAb, or saline treatment (data not shown). Anti-CD25 mAb administration effectively depleted CD4⁺CD25^{hi} Treg cells in FIV⁺ cats with kinetics similar to FIV-naïve cats without causing significant alterations in other lymphocyte subpopulations, including no significant impact on absolute CD4⁺ or CD8⁺ T cell, B cell, or total lymphocyte numbers (data not shown).

Viral burden is unaffected by anti-CD25 mAb treatment

Viral RNA in plasma and integrated proviral DNA in cells are established indicators of virologic status, however, FIV-NCSU₁-infected cats in the chronic stage of disease exhibit low levels of viral burden compared to the acute phase (58). It has been proposed that in HIV-1 and pathogenic SIV infection, persistent viral replication and associated T cell proliferation result in abnormal lymphoid architecture, diminished immune function and the development of AIDS (12, 29). Therefore, prolonged immune activation as a consequence of

transient Treg cell depletion would be undesirable. Indeed, CTLA-4 blockade lasting over 9 weeks during acute SIV_{mac251} infection of macaques results in elevated viral replication (15).

We asked whether Treg cell depletion over a period of 4 weeks would lead to increased viral replication due to immune hyperactivation or lead to a reduction of infected cells and reduced viremia and provirus. Plasma viremia did not change significantly on days 10, 21 or 23, 29, 42, or 60 post-treatment as compared to baseline levels (not detectable to 10,000 viral RNA molecules/ml plasma) in the three treatment groups. Likewise, no significant changes in provirus were observed in PBMC and most tissues between treatment groups (Figure 2A and data not shown). However, in the CD25 depleted group, the provirus copy number in peripheral LN was reduced at day 23 post-treatment as compared to day 0 ($p=0.06$; Figure 2B).

We then asked whether the decline in proviral load in peripheral LNs of CD25 depleted cats could be ascribed to changes within a specific lymphocyte subset. Peripheral LNs harvested on day 0 and on day 23 post-treatment were sorted for CD4⁺CD25⁻, CD4⁺CD25^{hi}, CD8⁺, and CD21⁺ lymphocytes. No significant changes in proviral load were noted between day 0 and day 23 in these lymphocyte subsets from either the CD25 depleted group or isotype control group or between groups at either time point (Figure 2C). There may have been additional cell populations responsible for the changes we observed in the whole lymph node, however, this seems unlikely.

In other lymphoid tissues harvested either 23 or 60 days post-treatment, proviral burden did not significantly differ among the treated groups (not detectable to 40,000

proviral copies/ 10^6 cells; data not shown). Overall, we observed no systemic reduction in provirus or plasma viremia but also no detrimental increase in FIV replication. It is doubtful that short-term Treg cell depletion in individuals with low viral burden would result in damaging immune hyperactivation or change the established viral set-point.

It has been proposed that Treg cells are preferentially infected during the acute phase of lentiviral infection and that Treg cells may serve as a reservoir of latent provirus or be better producers of virions than conventional $CD4^+$ T cells (4, 33, 34, 42, 48, 63). We examined whether $CD4^+CD25^{hi}$ Treg cells, the target of depletion in this study, carry more integrated provirus as a population than other lymphocyte subsets during the chronic stage of FIV infection. If so, it is possible that depletion of Treg cells could affect viral set-point. It has previously been shown that B cells are the major lymphocyte reservoir during the asymptomatic stage of FIV, but we questioned whether $CD4^+CD25^{hi}$ Treg cells were infected to a greater extent than $CD4^+CD25^-$ conventional T cells (21). As shown in Figure 2D, Treg cells did not carry more integrated provirus than $CD4^+CD25^-$ T cells, suggesting that the Treg cells depleted in this study were not a preferential reservoir of integrated provirus in the chronic phase of disease.

Immunologic responses against FIV are transiently improved after anti-CD25 mAb treatment

To assess anti-FIV T cell responses in cats depleted of Treg cells, lymphoid tissue single cell suspensions were evaluated by ELISPOT for IFN- γ production in response to

immunogenic FIV p24 and Env peptide pools. IFN- γ responses to FIV antigen stimulus were significantly greater in popliteal lymphocytes 29 days after cats received anti-CD25 mAb as compared to control groups. However, this improvement in the anti-FIV response did not persist to day 60 (Figure 3A).

We then asked if the increase in FIV-specific IFN- γ secretion in the CD25 depleted group was due to expansion of anti-FIV effector cells or if the activity of these cells was simply unmasked by in vivo Treg cell depletion. FIV-specific T cell proliferation was not increased in cells isolated from cats that received anti-CD25 mAb as compared to cells from control treated cats (data not shown). Next, an IFN- γ ELISPOT assay was performed in which CD4⁺CD25⁻ and CD8⁺ T cells were purified and segregated from Treg cells and cultured with Env peptides both before and 23 days after mAb treatment. We reasoned that if antiviral effector cell frequency increased in the absence of Treg cell influence, the number of IFN- γ secreting cells would be greater in the ELISPOT cultures at day 23 than at day 0 in the CD25 depleted group. If the effector cells are preexisting and not expanded after Treg cell depletion, then approximately the same number of IFN- γ secreting cells would be observed at day 0 and day 23. Indeed, we found that the IFN- γ -producing FIV-specific T cells were present in vivo before Treg cell depletion and were not expanded during Treg cell depletion (Figure 3B).

Anti-CD25 treatment does not induce systemic inflammatory cytokine production

In multiple experimental models involving cutaneous, pulmonary, or intestinal inflammation, Treg cells have been shown to possess anti-inflammatory capabilities (50, 55). HIV-1 disease progression to AIDS correlates highly with immune activation and inflammation (6, 29). Therefore, the possibility that Treg cell depletion in animals with ongoing chronic lentiviral infection could induce a storm of proinflammatory cytokines was addressed in our model. We analyzed CD4⁺ and CD8⁺ T cells for intracellular IL-2 and TNF- α by flow cytometry before and 23 days after mAb treatment in peripheral lymph nodes. We also quantified surface TGF- β expression by lymphocytes. No significant increase in inflammatory cytokine production in either the CD25 depleted or control group was observed over baseline levels after treatment (Figure 4A,B, data not shown). Transient Treg cell depletion in chronically FIV-infected cats does not result in increased T cell proinflammatory IL-2 or TNF- α production.

FOXP3 expression is upregulated in CD4⁺CD25⁺ cells of cats receiving isotype-control antibody, but not anti-CD25 antibody

FOXP3 is an intranuclear transcription factor associated with the suppressive phenotype of CD4⁺CD25⁺ Treg cells in mice, humans, and cats (10, 25, 38, 69). In chronically-infected FIV⁺ cats receiving isotype control mouse antibody, intranuclear FOXP3 expression was significantly upregulated in the PBMC CD4⁺CD25⁺ Treg cell population at day 10 post-treatment, while cats receiving anti-CD25 antibody or receiving no antibody had

decreased or unchanged FOXP3 expression in the Treg cell population, respectively (Figure 5). Human Treg cells have been shown to upregulate FOXP3 expression upon TCR stimulation *ex vivo* (2, 62). It is probable that this upregulation observed in the isotype control treated cats was due to transient FOXP3 expression related to Treg cell activation by the mouse antibody (27, 43). The data suggest that depletion of CD4⁺CD25⁺ cells eliminated the Treg cell population responsible for FOXP3 upregulation in response to a foreign antigen. Though percentage of FOXP3⁺ cells within the CD4⁺CD25⁺ cell population was relatively unchanged between day 0 and day 10, it is of note that the absolute number of CD4⁺CD25⁺ PBMCs in the CD25 depleted group were reduced by 76% at day 10 (data not shown), therefore, the absolute number of CD4⁺CD25⁺FOXP3⁺ cells was substantially reduced by day 10 post-treatment.

Cats treated with anti-CD25 mAb produce a more robust humoral response to a primary immune stimulus

We observed that Treg cell depletion improves the antiviral immune response to preexisting FIV infection, but we also inquired whether Treg cell depletion would improve a primary immune response if anti-CD25 mAb was administered during the first exposure to a new antigen. While anti-CD25 mAb was used in this study to deplete Treg cells, the depleting antibody as well as the isotype control mAb served as novel antigenic stimuli that allowed measurement of a developing primary humoral response against the mouse immunoglobulin. Cats depleted of Treg cells produced anti-mouse IgG titers significantly

higher than cats receiving isotype control antibody. This was true whether the serum titers were measured against the anti-CD25 mAb (Figure 6A) or the isotype control mAb (Figure 6B). In contrast, serum IgG titers against FIV p24 or FIV Env remained unchanged throughout the course of the study (data not shown). Treg cell depletion allows the induction of a more robust primary humoral immune response than would otherwise be possible during chronic lentiviral infection.

Discussion

The role of Treg cells in the pathogenesis of HIV-1 is a topic of great interest. From existing data it is difficult to predict whether Treg cells play a beneficial or a detrimental role in lentivirus infection, depending on the experimental model used, stage of disease, and the anatomical source of Treg cells. On one hand several studies have shown that CD4⁺ and CD8⁺ T cell responses are improved after the ex vivo removal of Treg cells from cultures (1, 8, 35, 67). On the other hand, studies that show a positive correlation between Treg cell frequency and disease non-progression argue that Treg suppression of inflammatory responses reduces the spread and replication of virus (3, 5, 36, 48). Indeed, it is not clear whether Treg cell frequency is increased or decreased during chronic HIV-1 infection (5, 36, 64, 67). In vivo manipulation of Treg cells using a relevant animal model can help fill the knowledge gaps left by ex vivo and in vitro studies regarding the nature of Treg cells in HIV-1 disease and determine the feasibility of therapeutic strategies to modulate Treg cell function.

In the present study we employed the well-characterized FIV/cat model to investigate the in vivo significance of Treg cell activity during the asymptomatic phase of lentivirus infection. Treg cell depletion results in an improved antiviral T cell response during the period of depletion, but the increased antiviral response did not persist after a rebound of Treg cells in lymphoid tissues. This demonstrates that CD4⁺CD25⁺ Treg cells do indeed suppress FIV-specific immune responses in vivo. We did not detect expansion of FIV-specific effector cells, though it is possible that the antiviral response could be expanded if there is sufficient viral antigen to drive the expansion. In the chronically FIV-infected cats described in this paper, viremia was very low as is typical during the asymptomatic phase of the infection and did not increase during Treg cell depletion. Without adequate viral antigenemia there is no reason to expect an expansion of cell-mediated responses during the asymptomatic phase. This conclusion is supported by the observation that anti-FIV antibody responses did not increase in Treg cell-depleted animals. This is similar to what might be expected if Treg cell depletion is attempted in HIV-1 patients that are controlling viral replication or are receiving anti-retroviral therapy. Thus, if Treg cell depletion is to be considered as part of an attempt to improve immune responses in HIV-1 infected people, it would most likely be necessary to add a therapeutic vaccine to the strategy. A window of Treg cell depletion would provide an opportunity to boost antiviral T and B cell responses. In this regard the present study offers encouraging results.

The use of a mouse monoclonal antibody to deplete Treg cells in cats limits the number of times a cat can be treated before a robust anti-mouse antibody response is

generated. However, this foreign protein provided the opportunity to assess a novel immune response in Treg cell depleted and control antibody treated animals. A subset of CD4⁺CD25⁺CD69⁻ Treg cells has been shown to be capable of suppressing B cell class switching and antibody production by interfering with T cell help in germinal centers of lymph nodes or by interacting directly with B cells in germinal centers (40, 41). It has previously been shown that cats chronically infected with FIV and exposed to either a variety of infectious agents or antigens develop delayed and blunted humoral immune responses as compared to FIV-naïve cats (51, 52). However, the robust anti-mouse mAb humoral response in Treg cell-depleted animals demonstrates the potent suppressive influence Treg cells can have on the humoral arm of the immune system and the capacity of the immune system to respond even during a state of CD4 depletion and immune dysfunction. Presumably, this could be extended to cell-mediated responses but this was not directly evaluated in this study. Perhaps equally important is that Treg cells from cats chronically infected with FIV that receive a non-depleting isotype control mAb rapidly upregulate FOXP3 protein. In FIV-naïve cats from a previous study, no such Treg cell induction was observed when cats were identically treated with the isotype control mAb (57). Treg cells increase FOXP3 expression during acute FIV and SIV infection, indicating an increase in suppressive function of these cells (24, 37, 42). It has also been reported that FIV infection leads to increased Treg cell activation (42, 65). Our data support the notion that FIV infection results in elevated Treg cell activity under steady-state conditions that may prime for heightened suppressive Treg cell responses to novel antigens and contribute to impaired

immune responses. The dramatic difference in antibody response to a novel antigen between groups receiving Treg cell depleting mAb versus isotype control mAb is likely a combination of removing Treg cell suppression on one hand and excessive Treg cell induction on the other hand.

There is concern that Treg cell depletion, whether in the course of HIV-1 disease pathogenesis or via treatment strategies, could exacerbate the vicious cycle of immune activation, viral replication and CD4⁺ T cell depletion. Whether this scenario could play out likely depends on the viral set-point, the immune set-point, the duration and extent of Treg cell depletion, and the degree to which Treg cells represent a viral reservoir. This study suggests that a low viral set-point will not be altered if the immune system is functional in the absence of Treg cells. Again, if Treg cell depletion were to be attempted in humans with HIV-1, as part of the patient selection process it would be prudent to demonstrate that an increased CTL response is possible with Treg cell depletion in vitro. From the present study we can conclude that under the conditions of low viral set-point and a functional cell-mediated immune response, Treg cell depletion via anti-CD25 mAb treatment is unlikely to lead to increased plasma viremia or induce proinflammatory cytokines. In support of this conclusion, Hryniewicz et al. (31) treated chronically SIV_{mac251}-infected macaques with a CTLA-4-blocking mAb in order to interfere with one mechanism of Treg cell suppression in vivo. The study showed that CTLA-4 blockade did not increase viremia after cessation of ART treatment and even decreased provirus in lymph nodes as compared to control treated animals (31). And while high percentages of FOXP3⁺ PBMCs have been associated with

decreased T cell activation, increased CD4⁺ cell numbers, and decreased HIV-1 provirus, a short duration of Treg cell depletion did not cause problematic immune activation leading to viral replication, even though the mAb treatment induced a measurable immune response (16, 44). Whether administration of a therapeutic vaccine during Treg depletion in chronically infected animals would be able to reduce viral set-point or be confounded by activation-induced viral replication remains to be determined.

CD4⁺CD25⁺FOXP3⁺ Treg cells have been shown to express CXCR4 or CCR5 and be particularly susceptible to lentiviral infection as compared to conventional T cells both in vitro and in vivo (4, 33, 42, 48, 63). Because Treg cells have been shown to produce more FIV viral particles than conventional T cells when activated with mitogen or IL-2 in vitro, they are proposed to be a reservoir of viral production (32, 34). We show that Treg cells do not carry more provirus as compared to conventional T cells in the asymptomatic stage of FIV disease, as has been shown in HIV chronic progressors not on HAART therapy, though we did not determine their capacity to produce virus or infect other cells (16). In our model, because Treg cells do not appear to be a viral reservoir any more than conventional CD4⁺ T cells during the chronic phase, it is unlikely that Treg cell depletion would be sufficient therapy itself to reduce viral burden in the individual.

These studies demonstrate that in vivo depletion of CD4⁺CD25⁺ Treg cells unmasks antiviral T cell responses in chronically FIV-infected cats without affecting viral load positively or negatively. Short-term removal of Treg cells in HIV-infected patients who are controlling viral load could be beneficial to the patients' disease prognosis. Further

investigation into a Treg cell depletion strategy for therapeutic vaccination is justified due to the indication that transient Treg cell depletion can create a window of opportunity to induce novel immune responses during lentiviral infection.

Acknowledgements

The authors would like to thank Alora LaVoy, Rita de Cassia Simoes, and Emily Smith for their technical expertise and Dr. Kristina Howard for her assistance in this study. This work is supported by the Public Health Service grant AI065223 from the National Institute of Allergy and Infectious Diseases. Rochelle Mikkelsen is supported by the Ruth L. Kirschstein National Research Service Awards grant GM008776 as a part of North Carolina State University's Biotechnology Training Program.

Figure 1. Depletion of Treg cells by anti-CD25 mAb treatment. (A) Groups of six cats each received 9 mg/kg anti-CD25 mAb (9F23) or 9 mg/kg isotype control mAb (CRL-1689, (IgG2a, κ light chain)) i.p. on day 0. A third group of cats received 3 ml/kg saline on day 0 (n=6). Lymphocytes were labeled with anti-CD4 and anti-CD25 antibodies and analyzed by flow cytometry for Treg cell percentages. A representative dot plot of PBMCs isolated on day 0 and day 10 of one cat receiving anti-CD25 mAb treatment is shown. P1 indicates CD4⁺CD25^{hi} cells. (B) Percent CD4⁺CD25^{hi} cells within the total lymphocyte population was determined by flow cytometry on days 0, 10, 21, 29, 42, and 60 following treatment and multiplied by the absolute number of peripheral blood lymphocytes. Absolute CD4⁺CD25^{hi} cell numbers are reported relative to day 0 absolute numbers, which have been normalized to 100%. Statistical significance was calculated compared to day 0 values. Means \pm SEM are shown. (C) In a second study two groups of four cats each were treated with 9 mg/kg anti-CD25 mAb or 9 mg/kg isotype control mAb i.p. on day 0. Lymphoid tissues were harvested on day 0 or day 23. Means \pm SD are shown. Statistical significance was calculated compared to isotype control group for each tissue. * indicates $p < 0.05$.

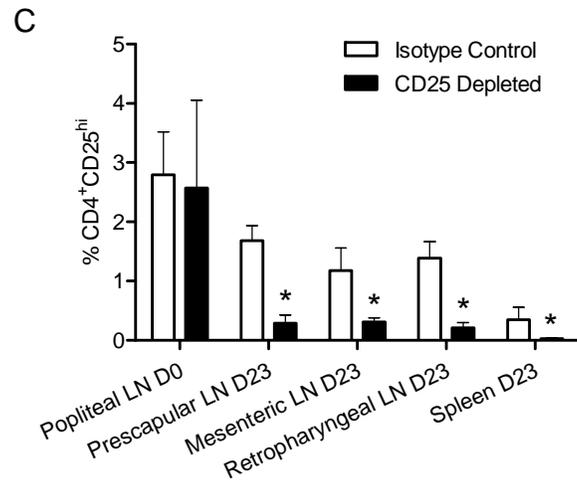
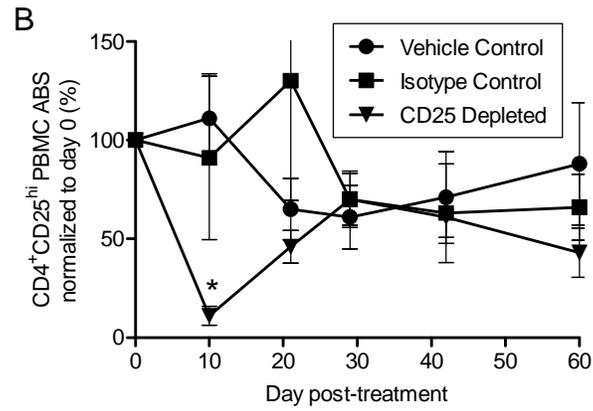
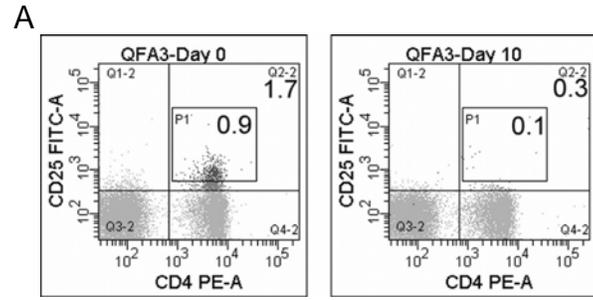
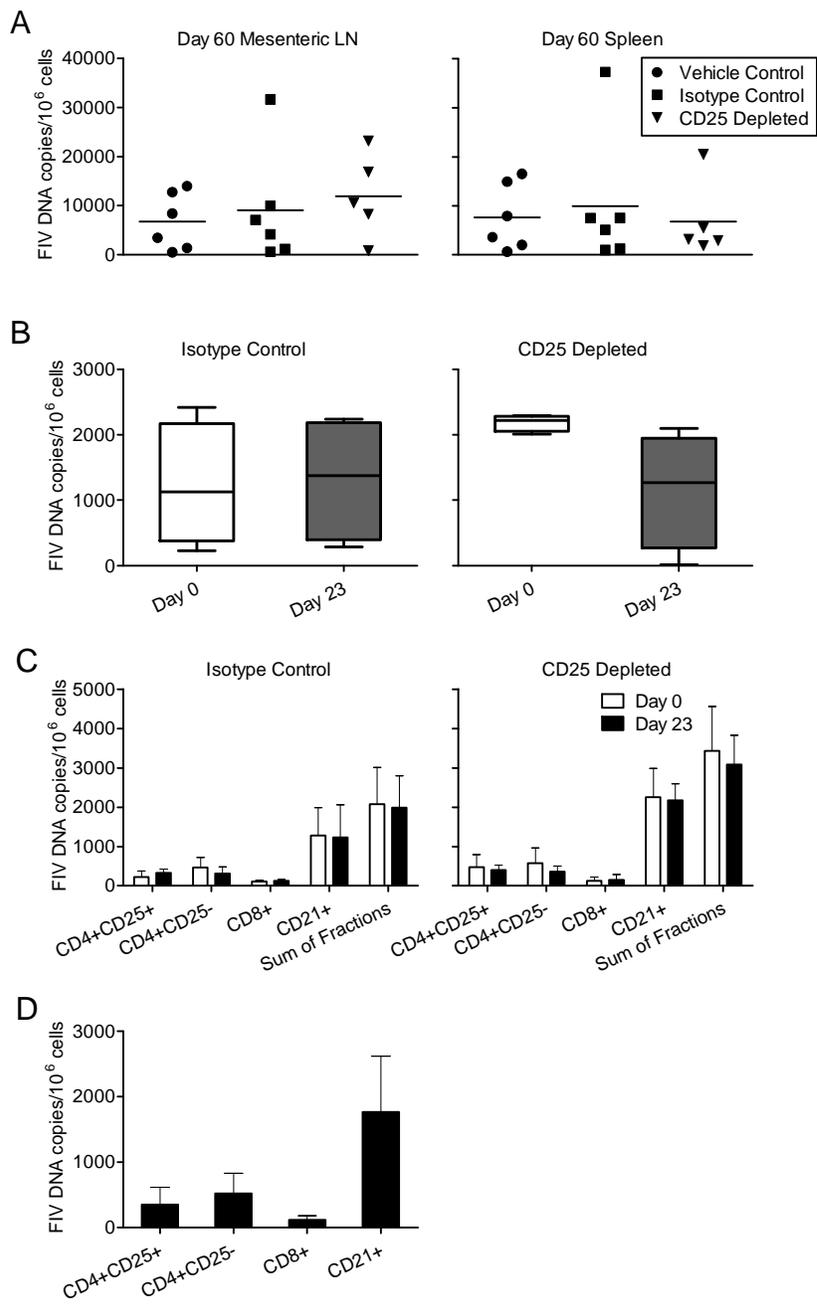


Figure 2. Viral burden is unaffected by anti-CD25 mAb treatment. To detect provirus in PBMCs and tissue samples, quantitative real-time PCR was performed on extracted DNA samples. FIV copy number was quantified relative to CCR5 copy number. (A) Provirus was detected in mesenteric lymph node and spleen on day 60 post-treatment. (B) Provirus from whole popliteal lymph nodes harvested on day 0 was compared to provirus from whole prescapular lymph nodes harvested on day 23 post-treatment for each individual cat. (C) Single cell suspensions of popliteal lymph nodes (n=4/group) harvested before treatment and prescapular lymph nodes (n=4/group) harvested 23 days after mAb treatment were sorted on a MoFlo cell sorter (Dako) for CD4⁺CD25⁻, CD4⁺CD25^{hi}, CD8⁺, and CD21⁺ lymphocytes. Provirus was quantified for each isolated lymphocyte subset. The last two bars in each graph represent the sum of the provirus carried by all four lymphocyte subsets. (D) Provirus was detected in lymphocyte subsets from popliteal lymph nodes harvested before treatment (n=8). Means \pm SD are shown.



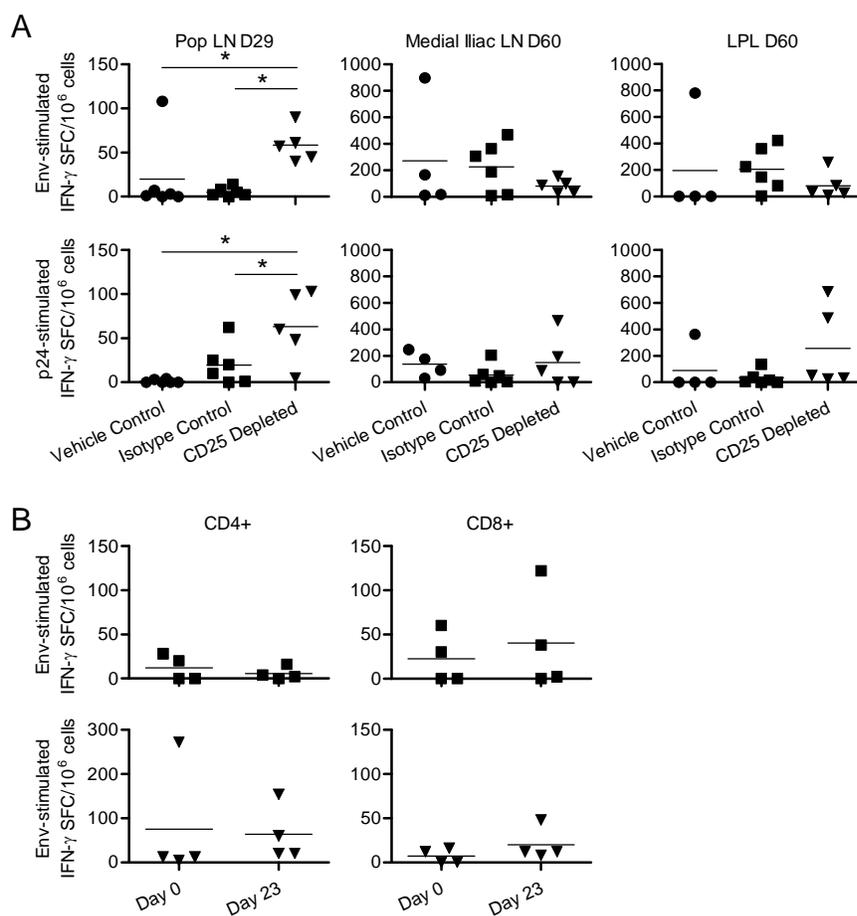


Figure 3. FIV-specific IFN- γ responses are transiently improved after anti-CD25 mAb treatment. (A) Popliteal lymph node cells, medial iliac lymph node cells, and lamina propria lymphocytes were incubated for 36 hours with 100 μ g/ml FIV Env or FIV p24 peptide pools. IFN- γ spot forming cells (SFCs) per million cells from CD25 depleted, isotype control, and vehicle control treated groups were determined by ELISpot. (B) CD4⁺CD25⁻ and CD8⁺ T cells from popliteal lymph nodes on day 0 and prescapular lymph nodes on day 23 were cultured with Env peptides in the absence of Treg cells. IFN- γ SFCs were determined by ELISPOT. ■ indicates isotype control group; ▼, CD25 depleted group. * indicates $p < 0.05$.

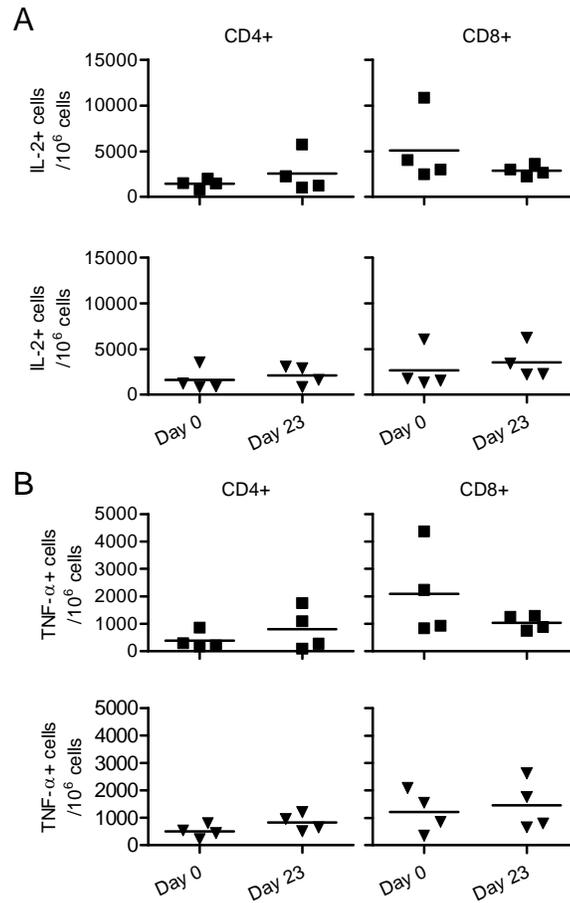


Figure 4. Anti-CD25 treatment does not induce systemic inflammatory cytokine production. Intracellular IL-2 and TNF- α staining was performed on lymphocytes after a 6 hour incubation with 1x monensin, surface phenotype staining, and fixation with 2% paraformaldehyde. The cells were permeabilized with BD Perm/Wash buffer. (A) IL-2⁺ and (B) TNF- α ⁺ cells per million CD4⁺ or CD8⁺ T cells were quantified by flow cytometry in popliteal lymph nodes before mAb treatment and prescapular lymph nodes 23 days after mAb treatment. ■ indicates isotype control group; ▼, CD25 depleted group.

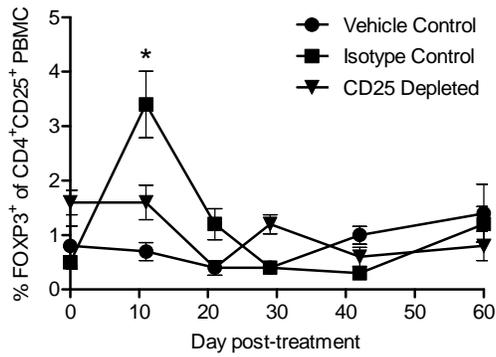


Figure 5. Intranuclear FOXP3 is upregulated in CD4⁺CD25⁺ cells of cats treated with nondepleting mouse mAb. Lymphocytes were labeled to detect surface CD4 and CD25 and intranuclear FOXP3 by flow cytometry. Percent FOXP3⁺ cells within gated CD4⁺CD25⁺ PBMCs is reported for days 0, 10, 21, 29, 42, and 60 post-treatment (n=5-6/group). Statistical significance was determined compared to day 0 values. * indicates $p < 0.05$.

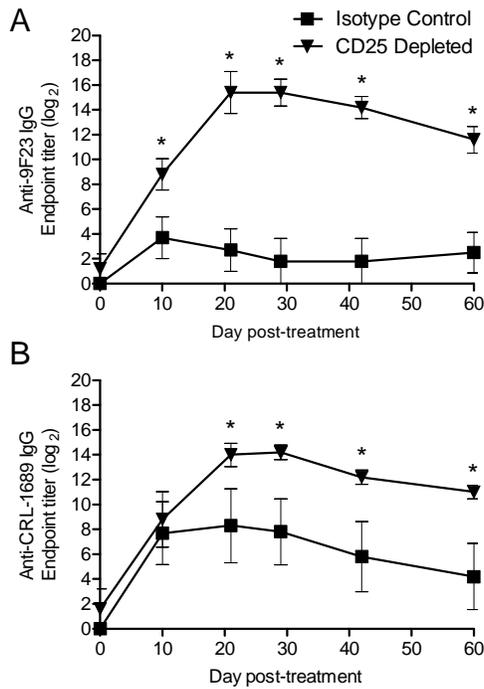


Figure 6. Cats treated with anti-CD25 mAb produce a more robust humoral response against mouse immunoglobulin. Serum was harvested on days 0, 10, 21, 29, 42, and 60 after treatment from cats treated with either anti-CD25 mAb (n=5) or isotype control mAb (n=6). ELISA plates were coated with either (A) mouse anti-feline CD25 clone 9F23 or (B) isotype control mouse anti-YFA clone CRL-1689 and then incubated with 2-fold serial dilutions of serum samples. Anti-mouse serum IgG was subsequently detected. Positive antibody titers were calculated based on a 3-fold higher optical density than negative controls. Means \pm SEM are shown. Statistical significance was determined between groups for each time point. * indicates $p < 0.05$.

References

1. **Aandahl, E. M., J. Michaelsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon.** 2004. Human CD4⁺ CD25⁺ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* **78**:2454-9.
2. **Allan, S. E., S. Q. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings.** 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* **19**:345-54.
3. **Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnnet.** 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* **174**:3143-7.
4. **Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalams, and D. Unutmaz.** 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *J Immunol* **180**:764-73.
5. **Apoil, P. A., B. Puissant, F. Roubinet, M. Abbal, P. Massip, and A. Blancher.** 2005. FOXP3 mRNA levels are decreased in peripheral blood CD4⁺ lymphocytes from HIV-positive patients. *J Acquir Immune Defic Syndr* **39**:381-5.
6. **Appay, V., and D. Sauce.** 2008. Immune activation and inflammation in HIV-1 infection: causes and consequences. *J Pathol* **214**:231-41.
7. **Baecher-Allan, C., V. Viglietta, and D. A. Hafler.** 2004. Human CD4⁺CD25⁺ regulatory T cells. *Semin Immunol* **16**:89-98.
8. **Baker, C. A., R. Clark, F. Ventura, N. G. Jones, D. Guzman, D. R. Bangsberg, and H. Cao.** 2007. Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection. *Clin Exp Immunol* **147**:533-9.
9. **Bendinelli, M., M. Pistello, S. Lombardi, A. Poli, C. Garzelli, D. Matteucci, L. Ceccherini-Nelli, G. Malvaldi, and F. Tozzini.** 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin Microbiol Rev* **8**:87-112.

10. **Bettelli, E., M. Dastrange, and M. Oukka.** 2005. Foxp3 interacts with nuclear factor of activated T cells and NF-kappa B to repress cytokine gene expression and effector functions of T helper cells. *Proc Natl Acad Sci U S A* **102**:5138-43.
11. **Boasso, A., M. Vaccari, J. Nilsson, G. M. Shearer, J. Andersson, V. Cecchinato, C. Chougnet, and G. Franchini.** 2006. Do regulatory T-cells play a role in AIDS pathogenesis? *AIDS Rev* **8**:141-7.
12. **Brenchley, J. M., D. A. Price, and D. C. Douek.** 2006. HIV disease: fallout from a mucosal catastrophe? *Nat Immunol* **7**:235-9.
13. **Burkhard, M. J., C. K. Mathiason, T. Bowdre, and E. A. Hoover.** 2001. Feline immunodeficiency virus Gag- and Env-specific immune responses after vaginal versus intravenous infection. *AIDS Res Hum Retroviruses* **17**:1767-78.
14. **Burkhard, M. J., L. Valenski, S. Leavell, G. A. Dean, and W. A. Tompkins.** 2002. Evaluation of FIV protein-expressing VEE-replicon vaccine vectors in cats. *Vaccine* **21**:258-68.
15. **Cecchinato, V., E. Trynieszewska, Z. M. Ma, M. Vaccari, A. Boasso, W. P. Tsai, C. Petrovas, D. Fuchs, J. M. Heraud, D. Venzon, G. M. Shearer, R. A. Koup, I. Lowy, C. J. Miller, and G. Franchini.** 2008. Immune activation driven by CTLA-4 blockade augments viral replication at mucosal sites in simian immunodeficiency virus infection. *J Immunol* **180**:5439-47.
16. **Chase, A. J., H. C. Yang, H. Zhang, J. N. Blankson, and R. F. Siliciano.** 2008. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation. *J Virol* **82**:8307-15.
17. **Chougnet, C. A., and G. M. Shearer.** 2007. Regulatory T cells (Treg) and HIV/AIDS: summary of the September 7-8, 2006 workshop. *AIDS Res Hum Retroviruses* **23**:945-52.
18. **Chuang, C. M., T. Hoory, A. Monie, A. Wu, M. C. Wang, and C. F. Hung.** 2009. Enhancing therapeutic HPV DNA vaccine potency through depletion of CD4+CD25+ T regulatory cells. *Vaccine* **27**:684-9.
19. **Dean, G. A., A. LaVoy, and M. J. Burkhard.** 2004. Peptide mapping of feline immunodeficiency virus by IFN-gamma ELISPOT. *Vet Immunol Immunopathol* **100**:49-59.

20. **Dean, G. A., and N. C. Pedersen.** 1998. Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. *J Virol* **72**:9436-40.
21. **Dean, G. A., G. H. Reubel, P. F. Moore, and N. C. Pedersen.** 1996. Proviral burden and infection kinetics of feline immunodeficiency virus in lymphocyte subsets of blood and lymph node. *J Virol* **70**:5165-9.
22. **Dunham, S. P.** 2006. Lessons from the cat: development of vaccines against lentiviruses. *Vet Immunol Immunopathol* **112**:67-77.
23. **Eggena, M. P., B. Barugahare, N. Jones, M. Okello, S. Mutalya, C. Kityo, P. Mugenyi, and H. Cao.** 2005. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol* **174**:4407-14.
24. **Estes, J. D., Q. Li, M. R. Reynolds, S. Wietgreffe, L. Duan, T. Schacker, L. J. Picker, D. I. Watkins, J. D. Lifson, C. Reilly, J. Carlis, and A. T. Haase.** 2006. Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection. *J Infect Dis* **193**:703-12.
25. **Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky.** 2003. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* **4**:330-6.
26. **Fontenot, J. D., J. P. Rasmussen, L. M. Williams, J. L. Dooley, A. G. Farr, and A. Y. Rudensky.** 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* **22**:329-41.
27. **Gavin, M. A., T. R. Torgerson, E. Houston, P. DeRoos, W. Y. Ho, A. Stray-Pedersen, E. L. Ocheltree, P. D. Greenberg, H. D. Ochs, and A. Y. Rudensky.** 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* **103**:6659-64.
28. **Goforth, R., A. K. Salem, X. Zhu, S. Miles, X. Q. Zhang, J. H. Lee, and A. D. Sandler.** 2009. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. *Cancer Immunol Immunother* **58**:517-30.
29. **Grossman, Z., M. Meier-Schellersheim, W. E. Paul, and L. J. Picker.** 2006. Pathogenesis of HIV infection: what the virus spares is as important as what it destroys. *Nat Med* **12**:289-95.

30. **Howard, K. E., I. L. Fisher, G. A. Dean, and M. Jo Burkhard.** 2005. Methodology for isolation and phenotypic characterization of feline small intestinal leukocytes. *J Immunol Methods* **302**:36-53.
31. **Hryniewicz, A., A. Boasso, Y. Edghill-Smith, M. Vaccari, D. Fuchs, D. Venzon, J. Nacs, M. R. Betts, W. P. Tsai, J. M. Heraud, B. Beer, D. Blanset, C. Chougnnet, I. Lowy, G. M. Shearer, and G. Franchini.** 2006. CTLA-4 blockade decreases TGF-beta,IDO, and viral RNA expression in tissues of SIVmac251-infected macaques. *Blood* **108**:3834-42.
32. **Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins.** 2005. Different thresholds of T cell activation regulate FIV infection of CD4+CD25+ and CD4+CD25- cells. *Virology* **335**:212-21.
33. **Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins.** 2005. Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. *J Virol* **79**:4965-76.
34. **Joshi, A., T. W. Vahlenkamp, H. Garg, W. A. Tompkins, and M. B. Tompkins.** 2004. Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. *Virology* **321**:307-22.
35. **Kinter, A., J. McNally, L. Riggin, R. Jackson, G. Roby, and A. S. Fauci.** 2007. Suppression of HIV-specific T cell activity by lymph node CD25+ regulatory T cells from HIV-infected individuals. *Proc Natl Acad Sci U S A* **104**:3390-5.
36. **Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci.** 2004. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* **200**:331-43.
37. **Kornfeld, C., M. J. Ploquin, I. Pandrea, A. Faye, R. Onanga, C. Apetrei, V. Poaty-Mavoungou, P. Rouquet, J. Estaquier, L. Mortara, J. F. Desoutter, C. Butor, R. Le Grand, P. Roques, F. Simon, F. Barre-Sinoussi, O. M. Diop, and M. C. Muller-Trutwin.** 2005. Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J Clin Invest* **115**:1082-91.

38. **Lankford, S., C. Petty, A. Lavoy, S. Reckling, W. Tompkins, and G. A. Dean.** 2007. Cloning of feline FOXP3 and detection of expression in CD4+CD25+ regulatory T cells. *Vet Immunol Immunopathol*.
39. **Legrand, F. A., D. F. Nixon, C. P. Loo, E. Ono, J. M. Chapman, M. Miyamoto, R. S. Diaz, A. M. Santos, R. C. Succi, J. Abadi, M. G. Rosenberg, M. I. de Moraes-Pinto, and E. G. Kallas.** 2006. Strong HIV-1-specific T cell responses in HIV-1-exposed uninfected infants and neonates revealed after regulatory T cell removal. *PLoS ONE* **1**:e102.
40. **Lim, H. W., P. Hillsamer, A. H. Banham, and C. H. Kim.** 2005. Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J Immunol* **175**:4180-3.
41. **Lim, H. W., P. Hillsamer, and C. H. Kim.** 2004. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest* **114**:1640-9.
42. **Mexas, A. M., J. E. Fogle, W. A. Tompkins, and M. B. Tompkins.** 2008. CD4(+)CD25(+) regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol*.
43. **Morgan, M. E., J. H. van Bilsen, A. M. Bakker, B. Heemskerk, M. W. Schilham, F. C. Hartgers, B. G. Elferink, L. van der Zanden, R. R. de Vries, T. W. Huizinga, T. H. Ottenhoff, and R. E. Toes.** 2005. Expression of FOXP3 mRNA is not confined to CD4+CD25+ T regulatory cells in humans. *Hum Immunol* **66**:13-20.
44. **Ndhlovu, L. C., C. P. Loo, G. Spotts, D. F. Nixon, and F. M. Hecht.** 2008. FOXP3 expressing CD127^{lo} CD4⁺ T cells inversely correlate with CD38⁺ CD8⁺ T cell activation levels in primary HIV-1 infection. *J Leukoc Biol* **83**:254-62.
45. **Nilsson, J., A. Boasso, P. A. Vella, R. Zhang, M. Vaccari, G. Franchini, G. M. Shearer, J. Andersson, and C. Chougnet.** 2006. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* **108**:3808-17.
46. **Nishimura, Y., M. Shimojima, E. Sato, Y. Izumiya, Y. Tohya, T. Mikami, and T. Miyazawa.** 2004. Downmodulation of CD3epsilon expression in CD8alpha+beta- T cells of feline immunodeficiency virus-infected cats. *J Gen Virol* **85**:2585-9.

47. **Ohno, K., R. Goitsuka, K. Kitamura, A. Hasegawa, T. Tokunaga, and M. Honda.** 1992. Production of a monoclonal antibody that defines the alpha-subunit of the feline IL-2 receptor. *Hybridoma* **11**:595-605.
48. **Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz.** 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol* **2**:E198.
49. **Pedersen, N. C., C. M. Leutenegger, J. Woo, and J. Higgins.** 2001. Virulence differences between two field isolates of feline immunodeficiency virus (FIV-APetaluma and FIV-CPGammar) in young adult specific pathogen free cats. *Vet Immunol Immunopathol* **79**:53-67.
50. **Read, S., V. Malmstrom, and F. Powrie.** 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *J Exp Med* **192**:295-302.
51. **Reubel, G. H., G. A. Dean, J. W. George, J. E. Barlough, and N. C. Pedersen.** 1994. Effects of incidental infections and immune activation on disease progression in experimentally feline immunodeficiency virus-infected cats. *J Acquir Immune Defic Syndr* **7**:1003-15.
52. **Reubel, G. H., J. W. George, J. E. Barlough, J. Higgins, C. K. Grant, and N. C. Pedersen.** 1992. Interaction of acute feline herpesvirus-1 and chronic feline immunodeficiency virus infections in experimentally infected specific pathogen free cats. *Vet Immunol Immunopathol* **35**:95-119.
53. **Roncador, G., P. J. Brown, L. Maestre, S. Hue, J. L. Martinez-Torrecuadrada, K. L. Ling, S. Pratap, C. Toms, B. C. Fox, V. Cerundolo, F. Powrie, and A. H. Banham.** 2005. Analysis of FOXP3 protein expression in human CD4+CD25+ regulatory T cells at the single-cell level. *Eur J Immunol* **35**:1681-91.
54. **Rouse, B. T., P. P. Sarangi, and S. Suvas.** 2006. Regulatory T cells in virus infections. *Immunol Rev* **212**:272-86.
55. **Sather, B. D., P. Treuting, N. Perdue, M. Miazgowicz, J. D. Fontenot, A. Y. Rudensky, and D. J. Campbell.** 2007. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. *J Exp Med* **204**:1335-47.
56. **Sempere, J. M., V. Soriano, and J. M. Benito.** 2007. T regulatory cells and HIV infection. *AIDS Rev* **9**:54-60.

57. **Smithberg, S. R., J. E. Fogle, A. M. Mexas, S. K. Reckling, S. M. Lankford, M. B. Tompkins, and G. A. Dean.** 2008. In vivo depletion of CD4(+)CD25(+) regulatory T cells in cats. *J Immunol Methods* **329**:81-91.
58. **Stevens, R., K. E. Howard, S. Nordone, M. Burkhard, and G. A. Dean.** 2004. Oral immunization with recombinant listeria monocytogenes controls virus load after vaginal challenge with feline immunodeficiency virus. *J Virol* **78**:8210-8.
59. **Stevens, R., A. Lavoy, S. Nordone, M. Burkhard, and G. A. Dean.** 2005. Pre-existing immunity to pathogenic *Listeria monocytogenes* does not prevent induction of immune responses to feline immunodeficiency virus by a novel recombinant *Listeria monocytogenes* vaccine. *Vaccine* **23**:1479-90.
60. **Tompkins, M. B., D. H. Gebhard, H. R. Bingham, M. J. Hamilton, W. C. Davis, and W. A. Tompkins.** 1990. Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. *Vet Immunol Immunopathol* **26**:305-17.
61. **Tompkins, M. B., P. D. Nelson, R. V. English, and C. Novotney.** 1991. Early events in the immunopathogenesis of feline retrovirus infections. *J Am Vet Med Assoc* **199**:1311-5.
62. **Tran, D. Q., H. Ramsey, and E. M. Shevach.** 2007. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood* **110**:2983-90.
63. **Tran, T. A., M. G. de Goer de Herve, H. Hendel-Chavez, B. Dembele, E. Le Nevot, K. Abbed, C. Pallier, C. Goujard, J. Gasnault, J. F. Delfraissy, A. M. Balazuc, and Y. Taoufik.** 2008. Resting regulatory CD4 T cells: a site of HIV persistence in patients on long-term effective antiretroviral therapy. *PLoS ONE* **3**:e3305.
64. **Tsunemi, S., T. Iwasaki, T. Imado, S. Higasa, E. Kakishita, T. Shirasaka, and H. Sano.** 2005. Relationship of CD4+CD25+ regulatory T cells to immune status in HIV-infected patients. *Aids* **19**:879-86.
65. **Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins.** 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol* **172**:4752-61.

66. **VandeWoude, S., and C. Apetrei.** 2006. Going wild: lessons from naturally occurring T-lymphotropic lentiviruses. *Clin Microbiol Rev* **19**:728-62.
67. **Weiss, L., V. Donkova-Petrini, L. Caccavelli, M. Balbo, C. Carbonneil, and Y. Levy.** 2004. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* **104**:3249-56.
68. **Wong, B. Y., S. A. Gregory, and N. H. Dang.** 2007. Denileukin diftitox as novel targeted therapy for lymphoid malignancies. *Cancer Invest* **25**:495-501.
69. **Zheng, Y., S. Z. Josefowicz, A. Kas, T. T. Chu, M. A. Gavin, and A. Y. Rudensky.** 2007. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* **445**:936-40.

**Treg Cell Depletion Prior to Acute Feline Immunodeficiency Virus
Infection Does Not Alter Disease Pathogenesis**

S. Rochelle Mikkelsen¹, Julie M. Long¹, Lin Zhang¹, and Gregg A. Dean¹

¹Center for Comparative Medicine and Translational Research, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, USA.

Abstract

Chronic HIV-1 infection is characterized by generalized immune dysfunction. Many studies suggest that CD4⁺CD25^{hi}FOXP3⁺ immunosuppressive regulatory T (Treg) cells are involved in regulation of the immune response during HIV-1 infection and could be involved in modulating immune dysregulation. Feline immunodeficiency virus (FIV) infection in cats is a useful model for HIV-1 pathogenesis, and suppressive capabilities of feline Treg cells are enhanced during FIV infection. We have previously shown that transient Treg cell depletion in vivo during chronic FIV infection unmasks FIV-specific immune responses. In this study, we aimed to determine whether Treg cell depletion using an anti-feline CD25 monoclonal antibody prior to natural FIV infection in cats could alter FIV pathogenesis, and thereby determine the role of Treg cells during acute FIV infection. We report here that Treg cell depletion prior to FIV infection does not significantly change provirus or viremia levels or CD4⁺ T cell levels in blood and lymphoid tissues during the acute phase. The effects of anti-CD25 mAb treatment appear to be truncated in cats acutely infected with FIV as compared to chronically infected cats or FIV naïve cats, as Treg cell levels were heightened in all treatment groups within two weeks following FIV infection. We propose that the influence of Treg cell suppression during FIV pathogenesis is most prominent after established infection, when Treg cells are activated and more functionally suppressive.

Introduction

Acute phase pathogenic HIV-1, simian immunodeficiency virus (SIV), and feline immunodeficiency virus (FIV) infection are characterized by robust viral proliferation that innate, cell-mediated, and humoral antiviral immune responses are eventually able to control, but not halt, within a few weeks post-infection (1-4). Chronic lentiviral infection is characterized by generalized immune dysfunction (5, 6). Data suggest that mismanagement of the immune response begins early after infection (1, 7, 8). Many studies suggest that CD4⁺CD25^{hi}FOXP3⁺ immunosuppressive regulatory T (Treg) cells are involved in regulation of the immune response during lentiviral infection and could be involved in immune dysregulation. Treg cells are susceptible to lentiviral infection and are depleted along with conventional CD4⁺ T cells during lentiviral pathogenesis (9-11). However, some studies suggest that Treg cells are depleted at a slower rate due to decreased susceptibility to apoptosis, which could lead to increased Treg cell to T effector cell ratios (12-15). Treg cell accumulation in lymphoid tissues where lentiviral replication occurs has been shown, though the degree to which Treg cell survival, Treg cell induction, or Treg cell expansion contribute to this phenomenon remains debatable (12, 16-18).

Work done in the Tompkins laboratory suggests that feline Treg cells are activated and more suppressive during the acute and chronic phases of FIV infection (19, 20). Studies with human Treg cells indicate that HIV-1 can enhance Treg cell suppressive capacity (14). Multiple studies have shown, using cells from hosts infected with HIV-1 or SIV, that the removal of Treg cells from cultured lymphocytes reveals previously suppressed viral-specific

immune responses (9, 21-25). Treg cell induction has been associated with limited anti-SIV immune responses during acute infection (26) and individuals with more robust anti-lentiviral responses possess lower Treg cell frequencies (9, 11, 27). We have previously shown that transient Treg cell depletion in vivo during chronic FIV infection unmasks FIV-specific immune responses but does not lead to a reduction in viral load, presumably due to a lack of immune stimulation when FIV antigen levels are low (Mikkelsen, SR. Ph.D. Dissertation, Chapter 3). These data suggest that Treg cells suppress appropriate antiviral immunity during lentiviral infection.

Treg cell depletion using diphtheria toxin conjugated to human IL-2 (Ontak) prior to HIV-1 infection in humanized mice has been shown by Su and colleagues to reduce plasma viremia on days 4-10 post-infection (28). This was hypothesized to occur because Treg cells serve as a major reservoir of HIV-1 infection in humanized mice (28). Some data support the hypothesis that Treg cells carry more integrated provirus or are more productive sites of viral replication than conventional CD4⁺ T cells (13, 19, 29-31). On the contrary, other studies have found that Treg cells possess either the same or lower levels of integrated lentiviral provirus as compared to conventional CD4⁺ T cells (10, 11, 32)(Mikkelsen, SR. Ph.D. Dissertation, Chapter 3). Therefore, in this study we aimed to determine whether Treg cell depletion using an anti-feline CD25 monoclonal antibody prior to natural lentiviral infection in the well characterized FIV/cat model could reduce viral load. We hypothesized that this could occur either due to removal of Treg cell immunosuppression leading to heightened antiviral responses or due to removal of cells that host lentiviral infection and reproduction.

Curtailed viral load after Treg cell depletion would indicate that Treg cells are detrimental during acute lentiviral infection.

On the other hand, several studies point to a protective role of Treg cells during lentiviral infection, as high PBMC Treg cell frequencies are correlated with high CD4⁺ T cell counts, low T cell activation, and/or low viremia (15, 22, 25, 27, 30, 32, 33). Increased immune activation during lentiviral infection, especially CD38 expression by CD8⁺ T cells, is linked to immune dysregulation and poor disease prognosis in hosts (15, 34-37). Elevated viral load and/or increased generalized immune activation after Treg cell depletion would indicate that Treg cells are protective during acute lentiviral infection.

In order to determine the role of Treg cells during acute FIV infection, we depleted Treg cells prior to FIV infection using anti-CD25 mAb treatment that has been previously characterized (38). We report here that Treg cell depletion prior to FIV infection does not significantly change viral load or CD4⁺ T cell levels in tissues. The effects of anti-CD25 mAb treatment appear to be truncated in cats acutely infected with FIV as compared to chronically infected cats or FIV naïve cats, as Treg cell levels were heightened in all treatment groups within two weeks following FIV infection. We propose that the influence of Treg cell suppression during FIV infection is most prominent after established infection, when Treg cells are activated and more functionally suppressive.

Materials and Methods

Animals, viral inoculum, and monoclonal antibody administration

A total of 24 female specific-pathogen-free (SPF) cats were purchased from Liberty Labs (Liberty, NY). Cats were housed and cared for in accordance with Association for the Assessment of Laboratory Animal Care standards and Institutional Animal Care and Use Committee guidelines. Mouse anti-feline CD25 hybridoma (9F23) (39), a gift from M. Honda at the National Institutes of Health in Tokyo, Japan, and mouse anti-yellow fever antigen (YFA) hybridoma (CRL-1689; ATCC, Manassas, VA) were grown in serum-free medium. Antibody was purified and certified endotoxin- and mycoplasma-free by Leinco, Inc. (St. Louis, MO). One group of cats received a single injection of 9 mg/kg anti-feline CD25 mAb intraperitoneally (i.p.) between 25 and 31 weeks of age and 12 days prior to infection with FIV-C36 (38). Two additional groups were treated with 9 mg/kg anti-YFA mAb i.p. as a mouse IgG2a κ light chain isotype control mAb or 3 ml/kg 0.9% sodium chloride (Hospira, Lake Forest, IL) i.p. as a vehicle control. Cats were infected with $2 \times 10^{5.2}$ TCID₅₀ units molecular clone FIV-C36 (40), a generous gift from Dr. Sue VandeWoude of Colorado State University, by intravaginal and intravenous routes 12 days after mAb or saline treatment.

Sample Collection and Processing

Whole blood was collected into Vacutainer tubes (BD, Franklin Lakes, NJ) containing ACD on days -12, 0, 7, 14, 35, and 54 post-infection (p.i.). PBMCs were isolated

by centrifugation of blood over Histopaque (Sigma, St. Louis, MO) and serum was processed as previously described (41, 42). Blood for plasma isolation, complete blood counts, and leukocyte differentials was collected in Vacutainer tubes containing EDTA. Plasma was isolated by centrifugation ($800 \times g$ for 10 min) and aliquots were frozen at -80°C for subsequent RNA isolation. Popliteal lymph node (LN) biopsies were performed on anesthetized cats on days 14 and 35 p.i. At day 54 p.i., mesenteric and retropharyngeal LNs in addition to spleen were harvested and processed as described previously (43). Cells were used immediately in ex vivo assays or cell aliquots were frozen at -80°C for subsequent DNA isolation.

Phenotypic Analysis

At least 1×10^6 cells were labeled with the following antibodies for flow cytometric analysis. Antibodies against feline CD3 (NZM1) (44), CD4 (30A) (45), CD8 α (3.357) (45), and IFN- γ (E6D4A5) (46) were purified from hybridoma supernatants in our lab. Anti-CD80 (B7.1.66) was generously provided by Dr. Mary Tompkins of North Carolina State University (47). Anti-CD1a (Fe1.5F4) was provided by Dr. Peter Moore of the University of California at Davis (48). Antibodies against CD56 and IL-2 were purchased from Biolegend (San Diego, CA). Antibodies against Ki67 and TNF- α were purchased from BD Biosciences (San Diego, CA). Antibody against B220 was purchased from Southern Biotech (Birmingham, AL). Antibody against TGF- β was purchased from R&D Systems (Minneapolis, MN). Anti-MHC II was purchased from Serotec (Raleigh, NC). Some

antibodies were conjugated to Pacific Blue, Pacific Orange, Alexa Fluor 647, or biotin using kits from Invitrogen Molecular Probes (Carlsbad, CA). Some antibodies were conjugated to PerCP or PE using kits from Prozyme (San Leandro, CA). Mouse anti-feline CD25 (9F23) was conjugated to FITC using standard protocols. Anti-mouse IgG3-APC (Jackson ImmunoResearch, West Grove, PA), anti-mouse IgG3-APC-Cy7 (Southern Biotech), and Streptavidin-Pacific Orange (Invitrogen Molecular Probes) were used as secondary antibodies. Intracellular FOXP3 staining was performed with eBioscience FOXP3 staining buffers and FOXP3-PE-Cy7 (FJK-16s; San Diego, CA) according to manufacturer's protocol, with the exception that cells remain in the permeabilization/wash buffer no longer than 30 min. For intracellular cytokine staining, cells were incubated with 1x monensin (Biolegend, San Diego, CA) for six hours, labeled for surface markers, fixed with 4% paraformaldehyde, permeabilized with BD Cytofix/Cytoperm kit Perm/Wash buffer, and stained with anti-cytokine mAbs. For intracellular Ki67 staining, cells were first labeled for surface markers, incubated with BD Cytofix/Cytoperm for 15 min, washed with BD Perm/Wash buffer, incubated with BD Cytoperm Plus for 10 min, washed, and incubated with BD Cytofix/Cytoperm for 5 min. Cells were then labeled with Ki67 Ab. Flow cytometric analysis was performed using an LSR II flow cytometer and FACS DIVA software (BD). 100,000 – 1,000,000 gated events were collected per sample.

Viral Parameters

Quantitative real-time one-step reverse transcriptase (RT)-PCR assays to quantify viremia were performed on a Bio-Rad MyiQ™ PCR detection system (Hercules, CA). RNA was extracted from plasma using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA) following manufacturer's protocol. Detection of plasma viremia in RNA samples was performed using FIV-C *gag* specific primers 704f and 756r and probe 727p (49). Each RNA sample was incubated with TaqMan RT-PCR Mix (Applied Biosystems, Branchburg, NJ), TaqMan RT Enzyme Mix (Applied Biosystems), 400 nM forward and reverse primers, and 80 nM TaqMan probe. Plasma viremia RT-PCR cycling conditions were as follows: 30 min at 48°C, 10 min at 95°C, and 50 cycles of a 15 sec step at 95°C followed by a 1 min step at 55.5°C. Fluorescence was recorded at the end of each annealing/extension step. RNA standards were generated by in vitro transcription of a pGEM-T Easy plasmid (Promega, Madison, WI) encoding FIV clade C *gag* using the T7 MAXIscript kit (Applied Biosystems) according to manufacturer's instructions. A 10-fold dilution series of RNA standards provided a detection range from 10¹ to 10⁵ RNA molecules per reaction. Bio-Rad MyiQ™ optical system software v2.0 was used to generate a standard curve; viral RNA copies/mL plasma was subsequently calculated.

To detect proviral load in PBMC and tissue samples, quantitative PCR was performed on DNA samples extracted with the DNeasy blood and tissue kit (Qiagen). Each amplification reaction included 0.5 µg DNA sample, TaqMan universal PCR master mix (Applied Biosystems), 400 nM 704f and 756r primers, and 40 nM 727p probe. Real-time

PCR cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, and 45 cycles of a 15 s step at 95°C followed by a 1 min step at 55.5°C. To normalize proviral load per 10⁶ cells, a 10-fold dilution series of a pGEM-T Easy plasmid encoding FIV C *gag* was used to provide a detection range of 10¹ to 10⁵ copies. A quantitative PCR was performed to detect CCR5 copies in 50 ng DNA sample as previously described (Mikkelsen, SR. Ph.D. Dissertation, Chapter 3). The limit of detection was ≤10 copies of FIV per 1 μg DNA. Standards, controls, and samples were run in duplicate for real-time PCRs.

Peptides

The entire FIV p24 protein sequence was synthesized by JPT (Springfield, VA) consisting of 15-aa peptides overlapping by 10 aa. Peptides were reconstituted in 10% dimethyl sulfoxide (DMSO) and stored at -80°C.

Interferon-γ ELISpot

Capture and detection antibodies from the feline IFN-γ detection module (R&D Systems) were used with ELISPOT Immobilon-P 96-well plates (Millipore, Bedford, MA) to quantify IFN-γ-producing cells after stimulation with FIV p24 peptides as previously described (50). The protocol was modified with the use of 2.5 or 5 × 10⁵ fresh instead of frozen cells/well.

IgG Titers Determined by ELISA

Serum IgG titers against mouse antibody were determined by ELISA as previously described (38). For the FIV p24 antibody ELISA, Immulon-2HB plates (Dynex Technologies, Chantilly, VA) were coated with 1.0 µg/ml p24-GST fusion protein and the assay was performed as previously described (51). For the FIV gp95 antibody ELISA, Grenier Lumitrac 600 high-binding plates (Monroe, NC) were coated with 2.5 µg/ml gp95-Fc antigen (52) diluted in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.5). Plates were blocked with 200 µl/well blocking buffer (3% nonfat dry milk, 5% goat serum, 0.1% Kathon in carbonate buffer) for 2 hours at room temperature. Plates were washed four times with wash buffer (0.5% Tween 20 in 1x PBS). Serum samples were diluted in complete sample diluent (2.5% human serum, 1% bovine serum albumin, 1% nonfat dry milk, 0.05% Tween 20, 0.1% Kathon in 1x PBS) and added to blocked plates at 100 µl/well. Plates were incubated for three hours at 37°C and then washed four times with wash buffer. Antibody was identified with goat anti-cat IgG-horseradish peroxidase (HRP) (Bethyl Labs, Montgomery, TX) diluted 1:80,000 in complete sample diluent and incubated for one hour at room temperature. Plates were washed five times. 100 µl/well Pierce SuperSignal ELISA Femto chemiluminescent substrate (Rockford, IL) was added to plates. Plates were read on a Perkin Elmer Wallac Victor³ 1420 Multilabel Counter (Waltham, MA) 2 minutes after addition of substrate. A threefold higher optical density of tested sample over pre-infection samples determined positive antibody titers.

Statistics

Comparisons between post-treatment or post-infection data to baseline values within groups were made using unpaired *t*-tests. Comparisons between treatment groups were made using 1-way ANOVA with Tukey's post-test. Statistics were calculated using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Results

Anti-CD25 monoclonal antibody treatment depletes Treg cells in cats prior to FIV infection.

Twenty-four SPF cats were divided into three groups of eight cats each, with siblings distributed across the groups. Cats received anti-CD25 mAb, isotype control mAb, or saline intraperitoneally on day -12 relative to date of infection with FIV-C36. FIV-C36 is a highly pathogenic molecular clone of an FIV clade C isolate (40). Acute FIV-C36 infection is characterized by very high peak viremia and substantial CD4⁺ T cell loss, leading to early symptoms of immunodeficiency (40, 53). Cats received $2 \times 10^{5.2}$ TCID₅₀ units FIV-C36 intravaginally and intravenously at the nadir of CD4⁺CD25^{hi} cell depletion on day 0. Expression of CD25 and FOXP3 were used to identify Treg cells. Treg cells express CD25 more densely than conventional T cells, and the transcription factor FOXP3 is associated with Treg cell function. However, it is important to note that both CD25 and FOXP3 are upregulated upon lymphocyte activation (54-59). Anti-CD25 mAb administration resulted in a 78% average reduction in circulating CD4⁺CD25^{hi} T cells and a 50% average reduction in

CD4⁺CD25⁺FOXP3⁺ T cells by day 0 (Figure 1A, B). In the isotype control and the vehicle control groups peripheral CD4⁺CD25^{hi} cells were depleted to a degree by day 14 post-infection (p.i.) (Figure 1A). This corresponded with peak viremia and depletion of total CD4⁺ T cells as a result of pathogenic FIV infection (Figure 2, 4A). In all groups CD4⁺CD25^{hi} cells exceeded baseline levels by day 54 p.i. (Figure 1A).

In the CD25 depleted group, peripheral CD4⁺CD25⁺FOXP3⁺ cells exceeded baseline levels by day 7 p.i. (Figure 1B). CD4⁺CD25⁺FOXP3⁺ cells rebounded more quickly and to a higher degree after CD25 depletion in acutely infected cats as compared to FIV naïve or chronically infected FIV⁺ cats (Figure 1B) (38) (Mikkelsen, SR. Ph.D. Dissertation, Chapter 3). During chronic antigen presentation, it is possible for antigen-specific Treg cells to be induced from non-Treg cells (60-62). Control group cats in addition to CD25 depleted cats exhibited increased absolute numbers of CD4⁺CD25⁺FOXP3⁺ cells by day 14 p.i. (Figure 1B). This effect is presumably due to T cell activation or Treg cell conversion and/or proliferation in response to FIV infection. CD4⁺CD25⁺FOXP3⁺ cell levels were not reduced in popliteal lymph nodes at day 14 or day 35 p.i. in the CD25 depleted group as compared to the control groups (data not shown). We were not able to determine changes in Treg cell levels in tissues after FIV infection, as lymph nodes were not biopsied prior to infection. TGF-β has been associated with Treg cell function in cats (19, 63). CD4⁺CD25⁺TGF-β⁺ T cell levels were elevated in the blood as compared to baseline levels by day 35 p.i. in all three treatment groups (data not shown). These data indicate increased peripheral Treg cell presence in all cats during acute FIV-C36 infection regardless of treatment.

It has been proposed that FOXP3⁺ Treg cells shed the IL-2 receptor, CD25, during HIV-1 infection (12, 16). We observed no evidence of progressive CD25 shedding by Treg cells during FIV infection, as 50-70% of FOXP3⁺ cells were CD4⁺CD25⁺ in lymph nodes and spleen from days 14-54 p.i., while only 12-15% of FOXP3⁺ cells were CD4⁺CD25⁻ (data not shown). This validates the use of anti-CD25 mAb in targeting Treg cells during FIV infection.

CD25 depletion does not significantly alter viral load during acute FIV infection

It has been hypothesized that Treg cells are either more frequently infected by latent lentivirus (19, 28) or are more efficient virion producers when infected as compared to conventional T cells (13, 29-31). Treg cell depletion using Ontak prior to HIV-1 infection in humanized mice has been shown to reduce plasma viremia on days 4-10 p.i. (28). It is therefore possible that reduced Treg cell levels at the onset of lentiviral infection could result in reduced viremia or provirus levels during acute infection. It is also possible that Treg cell depletion could reduce viral load due to heightened antiviral responses, or that Treg cell depletion could increase viral load due to elevated immune activation and viral replication.

We found that Treg cell depletion at the onset of infection does not cause significant changes in viremia or proviral load. A trend of higher viremia and PBMC proviral load was found in the CD25 depleted group as compared to the control groups on days 14 and 35 p.i. (Figure 2, 3). Proviral load was significantly higher in the mesenteric lymph nodes of CD25 depleted cats as compared to vehicle control cats, but not isotype control cats, on day 54 p.i.

(Figure 3). Proviral load in the retropharyngeal lymph nodes of CD25 depleted cats tended to be higher than in retropharyngeal lymph nodes of control cats on day 54 p.i. (Figure 3). However, in the popliteal lymph nodes on days 14 and 35 p.i., and in the spleen and PBMC on day 54 p.i., proviral load was not higher in the CD25 depleted group (Figure 3). Both viremia and proviral levels reach set-point by day 54 p.i. in all cats (Figure 2, 3). Our data indicate that anti-CD25 mAb treatment prior to FIV-C36 infection does not reduce viral load, but may in fact slightly augment viral replication during acute infection. In addition, viral load in the isotype treated group was generally slightly higher than in the vehicle control group in lymphoid tissues and in the periphery. It is possible that immune activation due to the presence of murine mAb predisposed cats to slightly heightened viral replication.

Lymphocyte dynamics and immune cell activation during acute FIV infection are not altered by anti-CD25 mAb treatment

Acute pathogenic FIV infection is characterized by a dramatic decline in peripheral CD4⁺ T cell counts with a slight decline in CD8⁺ T cells developing around or directly following the occurrence of peak viremia (49). CD4⁺ T cell counts rebound to a degree but remain lower than pre-infection levels as they gradually decline throughout the course of the disease. Cytotoxic CD8⁺ T cells eventually expand to higher than baseline levels as they respond to infection (49, 64). We observed characteristic CD4⁺ and CD8⁺ T cell dynamics during acute FIV-C36 infection (Figure 4A, B). In the control group cats, B cells were decreased to a degree on day 14 p.i., but cats in the CD25 depleted group did not experience

a similar transient B cell decline (Figure 4C). Natural killer (NK) cells were decreased in all three groups on day 14 p.i., and an increase in NK cell absolute numbers was observed on day 35 p.i. (Figure 4D). No significant differences were observed in T cell, B cell, or NK cell dynamics between treatment groups in PBMC or in lymphoid tissues (Figure 4; data not shown). There were also no differences in CD4:CD8 T cell ratios in tissues, or cell density or cellularity in the lymph nodes between the groups (data not shown).

Treg cells have been shown to suppress lentiviral-specific T cell proliferation *ex vivo* (24, 33). We asked whether Treg cell depletion *in vivo* could augment T cell proliferation after FIV infection. T cell proliferation in the periphery was determined via Ki67 expression. Heightened CD4⁺ T cell proliferation was observed in all treatment groups on day 14 p.i. that corresponded with the nadir of CD4⁺ T cell depletion due to FIV infection and peak viremia (Figure 5A). CD8⁺ T cell proliferation was maximal at day 35 p.i. in all three treatment groups (Figure 5B). Peripheral Ki67⁺ B cell and NK cell levels did not change significantly after FIV infection and were not significantly different between the groups (data not shown). Ki67 expression by lymphocytes in tissues was not significantly different between the groups (data not shown).

CD1a⁺ myeloid dendritic cell (DC) levels and CD80 expression by DCs in lymphoid tissues were not significantly different between groups (data not shown). Interestingly however, MHC II expression by tissue DCs was consistently higher in mAb treated groups as compared to the vehicle control group as early as day 14 p.i. (Figure 5C). This suggests that more DCs were activated in mAb treated groups as compared to the vehicle control group.

Cytokine-expressing CD8⁺ T cell levels are lower after anti-CD25 mAb treatment on day 35 post-FIV infection

Intracellular expression of cytokines TNF- α , IL-2, and IFN- γ was determined in popliteal lymph node CD4⁺ and CD8⁺ T cells during acute FIV infection in control treated and CD25 depleted cats. No significant differences in cytokine expression by T cells were found between the treatment groups at day 14 p.i. (Figure 6). In contrast, CD8⁺TNF- α ⁺ levels were significantly lower in the CD25 depleted group as compared to the vehicle control group at day 35 p.i., and there was a trend of lower IL-2 and IFN- γ expression by CD8⁺ T cells in the CD25 depleted group as well. Cytokine expression by CD4⁺ T cells at day 35 p.i. was not significantly different between the groups (Figure 6). By day 54 p.i. no differences were observed in T cell cytokine expression between treatment groups (data not shown). Likewise, no differences were found in T cell surface TGF- β expression between treatment groups in lymphoid tissues (data not shown).

Anti-FIV specific responses are slightly depressed in the anti-CD25 mAb treated group

We have previously shown that FIV-specific IFN- γ secreting cells are revealed during transient Treg cell depletion in cats chronically infected with FIV (Mikkelsen, SR. Ph.D. Dissertation, Chapter 3). We asked whether CD25 depletion before FIV infection could have the same effect on FIV-specific immune responses. Lymphocytes were stimulated with FIV p24 peptides in an IFN- γ ELISPOT assay. No significant differences were found between the groups; however, there was a trend of fewer FIV-specific IFN- γ secreting cells in the

CD25 depleted group as compared to the control groups in tissues analyzed on days 14, 35, and 54 p.i. (Figure 7).

Neutralizing antibodies have been shown to play a role in controlling FIV replication. Anti-FIV gp95 IgG titers were not significantly different between the groups during acute FIV infection; however, anti-FIV p24 IgG titers were significantly higher in the vehicle control group as compared to the CD25 depleted group on days 7 and 14 p.i. (Figure 8). These data may explain why viral load was slightly higher in the CD25 depleted group at certain time points during acute infection.

A trend of higher humoral anti-murine responses occurs in CD25 mAb treated cats

Previously, we observed that CD25 depletion with a murine mAb in cats chronically infected with FIV results in the development of more robust anti-murine IgG titers as compared to cats treated with a murine isotype control mAb (Mikkelsen, SR. Ph.D. Dissertation, Chapter 3). We sought to determine whether differences in anti-mouse antibody titers exist between the two mAb treated groups during acute FIV infection. We found that anti-murine IgG titers tended to be higher in the CD25 depleted group starting at day 7 p.i., but these differences were not significant (Figure 9). This result is found in contrast to anti-FIV IgG titers in the CD25 depleted group relative to the control groups shown in Figure 8. It is possible that the induction of Treg cells after FIV infection in the CD25 depleted group abrogated more robust anti-FIV humoral responses, but because

murine mAbs were administered 12 days before infection, anti-murine mAb responses developed earlier and more in-frame with Treg cell depletion.

Discussion

Positive and negative correlations between Treg cell frequency and indicators of HIV-1 disease progression, including antiviral responses, immune activation, CD4⁺ T cell levels, and viral load have implicated Treg cells as regulators of the immune response to HIV-1 infection. Most of these studies have been conducted using data from chronically infected individuals. In cats chronically infected with FIV, Treg cells have been shown to possess enhanced suppressive capacity (20). A study in acutely infected FIV⁺ cats indicates that Treg cells are activated very early during FIV infection, exhibiting increased FOXP3 and TGF- β expression and increased immunosuppressive capabilities (19). Therefore, we sought to determine whether Treg cell depletion prior to FIV infection could alter FIV pathogenesis. We found that anti-CD25 mAb treatment prior to FIV infection did not significantly change disease progression in terms of viral load or CD4⁺ T cell destruction. We did however, observe evidence of peripheral Treg cell expansion and/or induction soon after FIV infection in CD25 depleted and control group cats. Importantly, we found that the feline immune system is able to mount antiviral responses and control viral replication in the presence of Treg cells in control treated cats.

Naïve CD4⁺CD25⁺FOXP3⁺CD45RO⁻ Treg cells have been described by Antons and colleagues (65). Naïve Treg cells are present in human adult blood and exhibit increased

suppressive capacity and CD45RO and CTLA-4 expression after expansion (65). An anti-CD45RO mAb is not available for use with feline cells, but Tompkins and colleagues did observe an upregulation of CTLA-4 expression on Treg cells in chronically infected FIV+ cats (20). Several studies in mice have shown that Treg cell expansion due to antigen exposure in vivo leads to enhanced Treg cell suppression (60, 66, 67). Both naïve human Treg cells and feline Treg cells from SPF cats acquire increased suppressive capacity after TCR stimulation or in vivo FIV infection, respectively (19, 20, 65). We observed increased absolute numbers of CD4⁺CD25⁺FOXP3⁺ and CD4⁺CD25⁺TGF-β⁺ T cells in the periphery after FIV infection even in the CD25 depleted group. It seems reasonable to hypothesize that expansion and activation of naïve Treg cells occurred in all treatment groups, as Treg cell depletion was incomplete in the CD25 depleted group. In support of this hypothesis, Treg cells have been shown to accumulate alongside Ki67⁺ cells during acute SIV infection, just as we observed during this study (26). In addition, we cannot exclude the possibility that Treg cells were induced from non-Treg cells during infection. Prolonged antigen exposure occurs during FIV infection, which has been shown to induce Treg cells in vivo (61, 62, 68, 69).

It has been proposed that Treg cells host more integrated provirus or are more efficient producers of virions than conventional CD4⁺ T cells. We observed no evidence of decreased viral load in the CD25 depleted group within the first two weeks of FIV infection before Treg cells returned to baseline levels. Our study contradicts the findings of Su and colleagues who found that Treg cell depletion prior to HIV-1 infection in humanized mice

reduces viral load up to day 10 p.i. (28). The majority of Treg cells that were depleted in cats that received anti-CD25 mAb were most likely naïve. It is possible that Treg cells activated after FIV infection produce more FIV mRNA than conventional T cells as reported by the Tompkins group, but we did not evaluate this in our study (19). Our data indicate that Treg cells present at the time of FIV infection are not major reservoirs of virus during the first few days after infection.

Depletion of CD25⁺ Treg cells using anti-CD25 mAbs in acute infection models has raised concerns that there may be concomitant depletion of activated effector cells (70). To avoid this, it was important to administer anti-CD25 mAb in a timeframe that allowed the majority of anti-CD25 mAb to bind to Treg cells or be cleared before effector cells were generated. We infected cats with FIV-C36 12 days after administration of anti-CD25 mAb, which allowed depletion of the majority of Treg cells before infection. We found that cell mediated and humoral anti-FIV responses were not significantly different between the CD25 depleted group and the control groups, excepting anti-p24 humoral responses on days 7 and 14 p.i. This indicates that effector T and B cells were not depleted in the anti-CD25 mAb treated group. We also observed no differences in CD1a⁺ DC levels between the groups, indicating that CD25⁺ DCs were not depleted by anti-CD25 mAb treatment.

The administration of murine mAb causes immune activation as demonstrated by increased MHC II on DCs. However, we did not observe a difference in Ki67⁺ cell levels in mAb treated groups as compared to the vehicle control group. It is probable that FIV infection was the major cause of immune activation in the mAb treated groups during this

study, as FIV-C36 pathogenesis involves very high peak viremia. Any additional immune activation caused by naïve Treg cell depletion was likely overshadowed by much greater immune activation in response to FIV. We did observe that viral load was slightly but not significantly higher in the mAb treated groups, with peak viremia and PBMC proviral load highest in the CD25 depleted group. Our data confirm the observation that immune activation during lentiviral infection predisposes hosts to higher levels of viral replication (71, 72).

We conclude that Treg cells existing prior to FIV infection in cats do not play a major role in modulating acute FIV pathogenesis. We were not able to determine whether Treg cells activated and expanded or induced during acute FIV infection are beneficial or detrimental. This would be difficult to determine using anti-CD25 mAbs to target Treg cells immediately after FIV infection, as effector cells transiently upregulate CD25 and might also be targeted for depletion. The recent identification of GARP, a molecule associated with surface expression of TGF- β , as a marker of activated Treg cells may be useful in this respect (73). In support of our data, Estes et al. (26) show that Treg cell induction correlates with immune activation and suggest that Treg cells may not be able to limit robust immune activation during acute SIV infection (26). Data reviewed by Walker indicate that effector responses to pathogens may be refractory to Treg cell suppression or that Treg cell activity is inhibited until pathogen number is reduced (74). Our data support this theory, as Treg cell depletion did not have a significant effect on FIV pathogenesis.

Treg cell depletion has been proposed as a strategy to boost vaccine efficacy. Our data suggest that Treg cell depletion may be more effective when antigen is low as is the case with most vaccines. Under conditions of low antigen the balance between effector responses versus Treg suppression is more likely to be tipped toward Treg suppression. It is also important to consider timing of Treg cell depletion relative to vaccine administration. Litzinger et al. (75) found that Ontak administration one day prior to administration of recombinant vaccinia viral vaccine in mice boosted immunity significantly as compared to Ontak administration three or seven days prior to vaccine.

We report here that Treg cells do not play a major regulatory role during very early FIV pathogenesis. We found that Treg cell depletion prior to FIV infection does not reduce viral load, indicating that Treg cells are not major sites of viral replication during the first two weeks of FIV infection. Finally, Treg cells were found to expand in the periphery during acute infection, supporting data that suggests that Treg cells may play a greater regulatory role during chronic lentiviral infection.

Acknowledgements

We thank Erin Galemore, Alora LaVoy, and Rita de Cassia Simoes for their helpful assistance in this study. This work is supported by the Public Health Service grant AI065223 from the National Institute of Allergy and Infectious Diseases. Rochelle Mikkelsen is supported by the Ruth L. Kirschstein National Research Service Awards grant GM008776 as a part of North Carolina State University's Biotechnology Training Program.

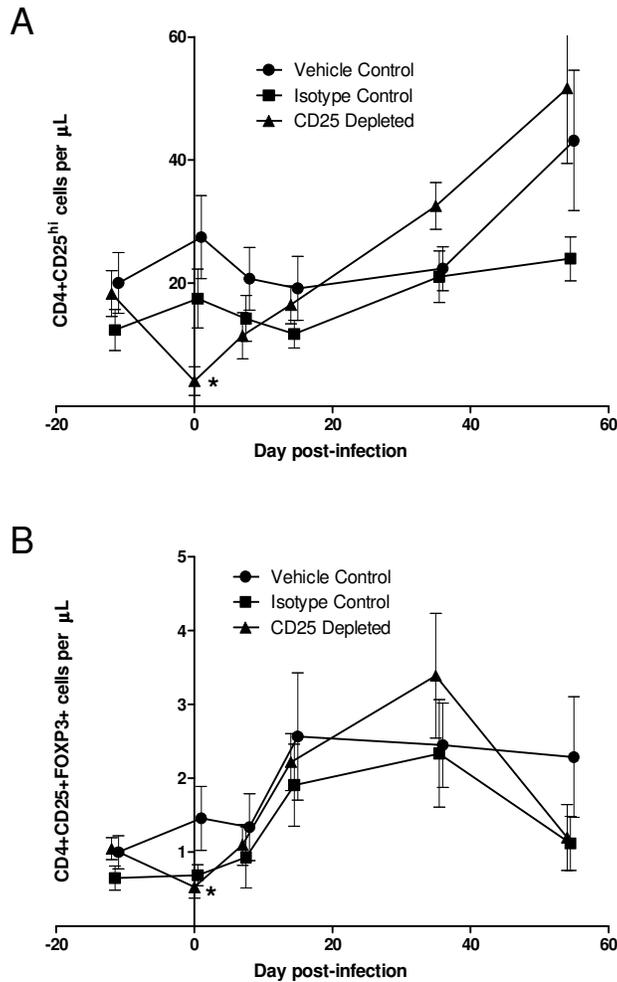


Figure 1. Treg cells are partially depleted by anti-CD25 mAb treatment. Cats received 9 mg/kg anti-CD25 mAb (9F23) (n=8) or 9 mg/kg isotype control mAb (CRL-1689) (n=8) i.p. on day -12. A third group of cats received 3 ml/kg saline on day -12 (n=8). (A) Lymphocytes were labeled with antibodies for CD4 and CD25 and analyzed by flow cytometry for CD4⁺CD25^{hi} percentages within the lymphocyte population on days -12, 0, 7, 14, 35, and 54 post-FIV infection. Percentage values were multiplied by lymphocyte absolute number to quantify absolute numbers of CD4⁺CD25^{hi} cells per ml blood. (B) FOXP3 antibody in addition to CD4 and CD25 antibodies was used to determine CD4⁺CD25⁺FOXP3⁺ cell percentages by flow cytometry. Percentage values were multiplied by lymphocyte absolute number to quantify absolute numbers of CD4⁺CD25⁺FOXP3⁺ cells per ml blood. Statistical significance was calculated compared to day 0 values. Means ± SEM are shown. * indicates $p < 0.05$.

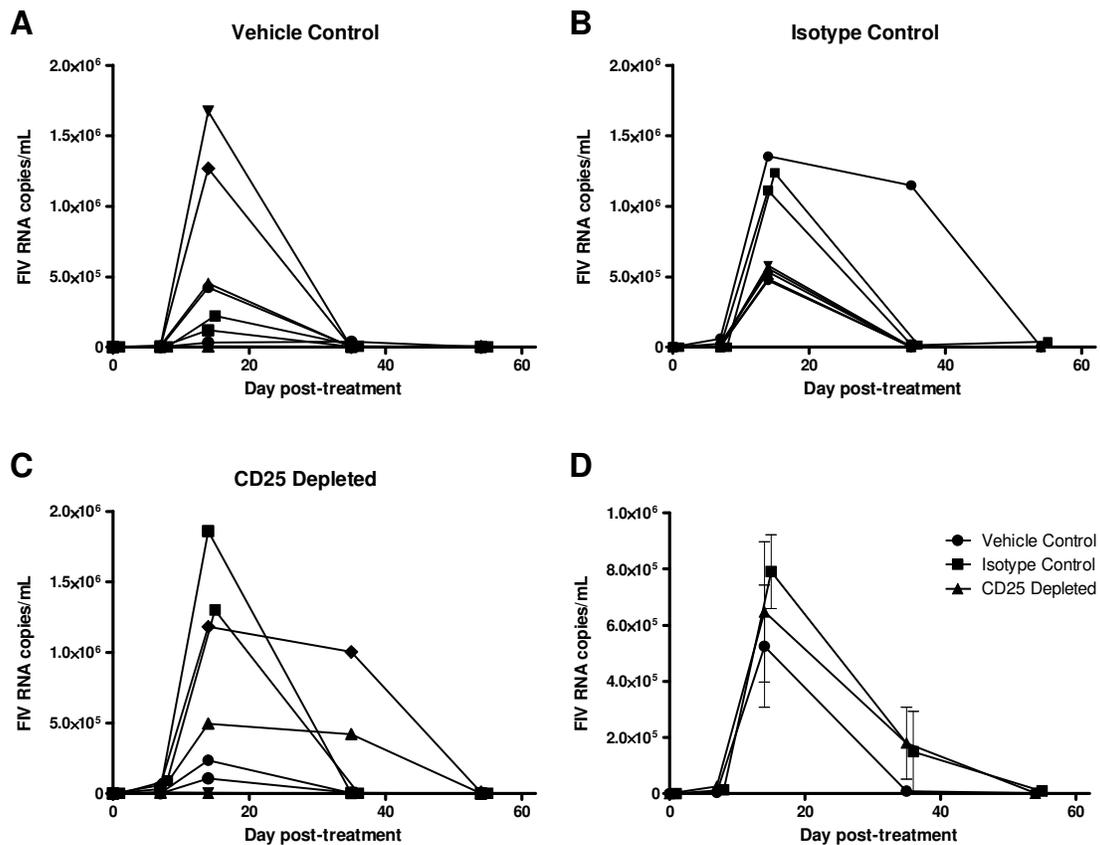


Figure 2. Anti-CD25 mAb treatment does not significantly alter plasma viremia. Cats were infected with $2 \times 10^{5.2}$ TCID₅₀ units molecular clone FIV-C36 by intravaginal and intravenous routes on day 0. To detect viremia in plasma samples, one-step quantitative real-time PCR was performed on extracted RNA samples. FIV copy number was quantified relative to an FIV C *gag* RNA standard curve. Viremia is shown in (A) vehicle control treated, (B) isotype mAb control treated, and (C) CD25 depleted cats. (D) Viremia group means (n=8/group) are shown \pm SEM.

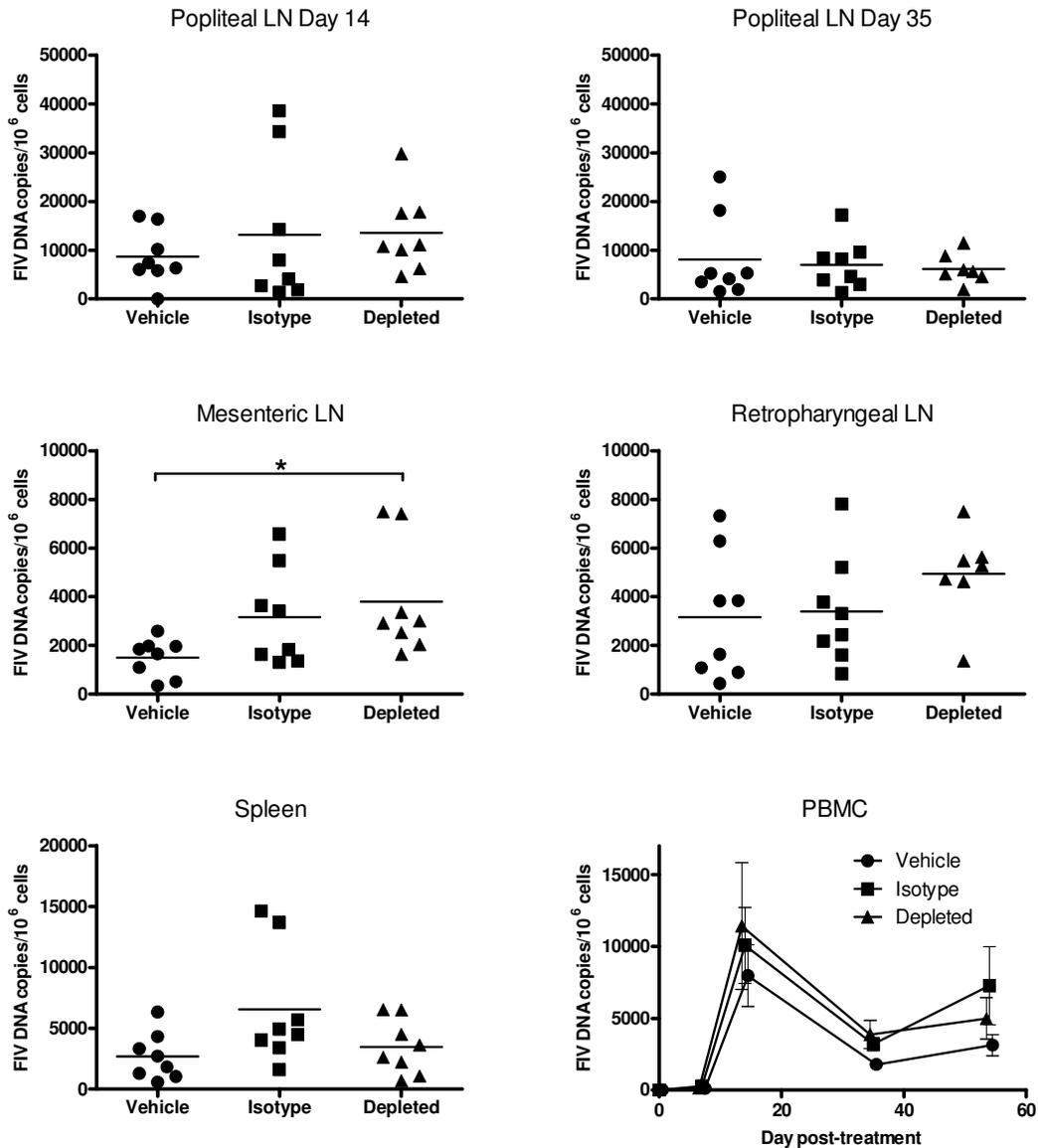


Figure 3. Proviral load is unaffected by anti-CD25 mAb treatment. Cats were infected with $2 \times 10^{5.2}$ TCID₅₀ units FIV-C36 by intravaginal and intravenous routes on day 0. To detect provirus in PBMCs and tissue samples, quantitative real-time PCR was performed on extracted DNA samples. FIV copy number was quantified relative to an FIV C *gag* DNA standard curve, and then normalized relative to CCR5 copy number. Provirus in popliteal lymph nodes was quantified on days 14 and 35 p.i. Provirus in mesenteric and retropharyngeal lymph nodes and spleen was quantified on day 54 p.i. Provirus in PBMCs was calculated on days 0, 7, 14, 35, and 54 p.i. Means \pm SEM are shown (n=8/group).

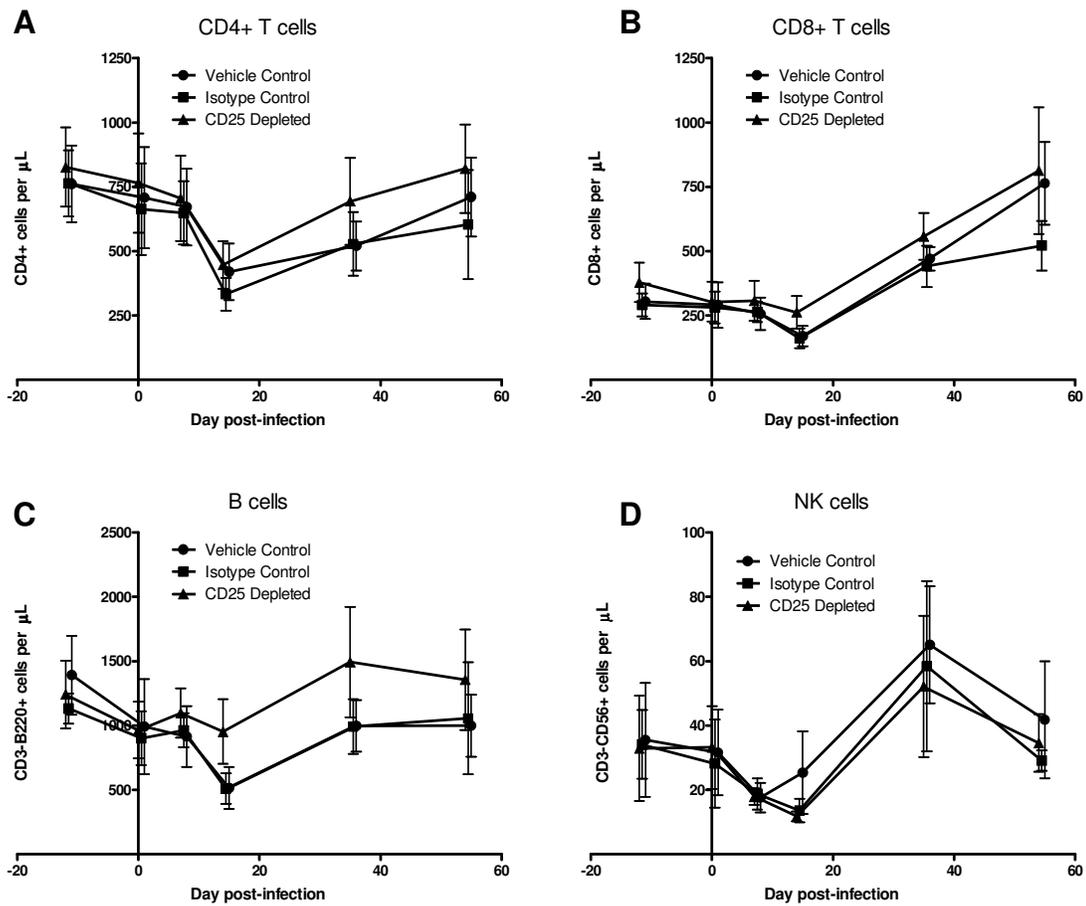


Figure 4. Peripheral lymphocyte dynamics during acute FIV-C36 infection are not altered by anti-CD25 mAb administration. Absolute numbers of (A) $\text{CD3}^+\text{CD4}^+$, (B) $\text{CD3}^+\text{CD8}^+$, (C) $\text{CD3}^-\text{B220}^+$, and (D) $\text{CD3}^-\text{CD56}^+$ cells in the periphery were quantified on days -12, 0, 7, 14, 35, and 54 p.i. based on percentages obtained by flow cytometry and absolute number of peripheral lymphocytes. Means \pm SEM are shown (n=8/group).

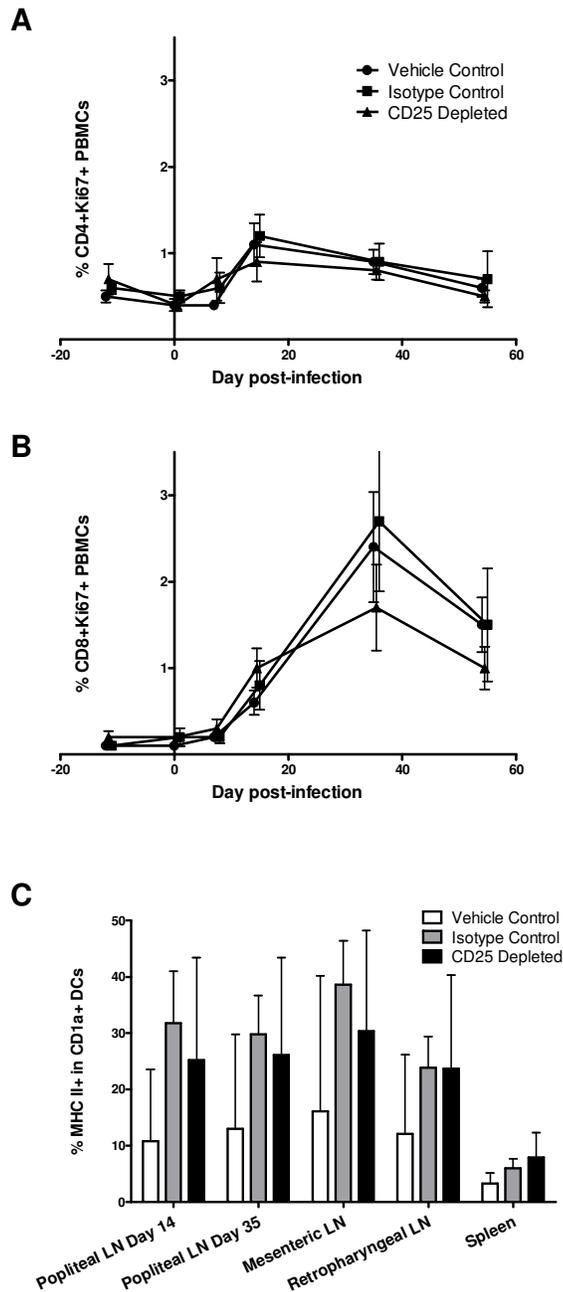


Figure 5. Dendritic cell activation but not T cell proliferation is enhanced by murine mAb treatment. Percent Ki67⁺ (A) CD4⁺ T cells and (B) CD8⁺ T cells in circulation were quantified by flow cytometry on days -12, 0, 7, 14, 35, and 54 p.i. (n=8/group). (C) Percent CD11a⁺MHC II⁺ dendritic cells were quantified in lymphoid tissues on days 14, 35, or 54 p.i. by flow cytometry (n=8/group). Means ± SEM are shown.

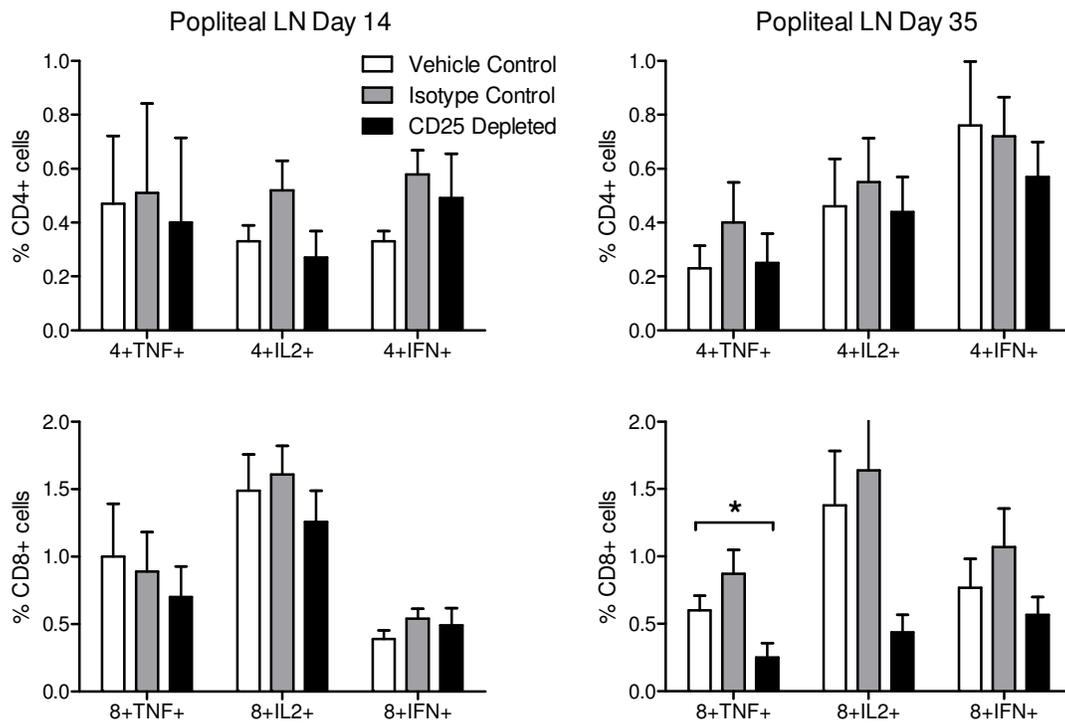


Figure 6. Cytokine-expressing CD8⁺ T cell levels are lower after CD25⁺ mAb treatment at day 35 post-FIV infection. Popliteal lymph node cells were incubated with monensin for 6 hours prior to quantification of cytokine expressing cells. Percent TNF- α -, IL-2-, and IFN- γ -expressing cells in CD4⁺ and CD8⁺ T cell populations were quantified by flow cytometry (n=8/group). Means \pm SEM are shown. * indicates $p < 0.05$.

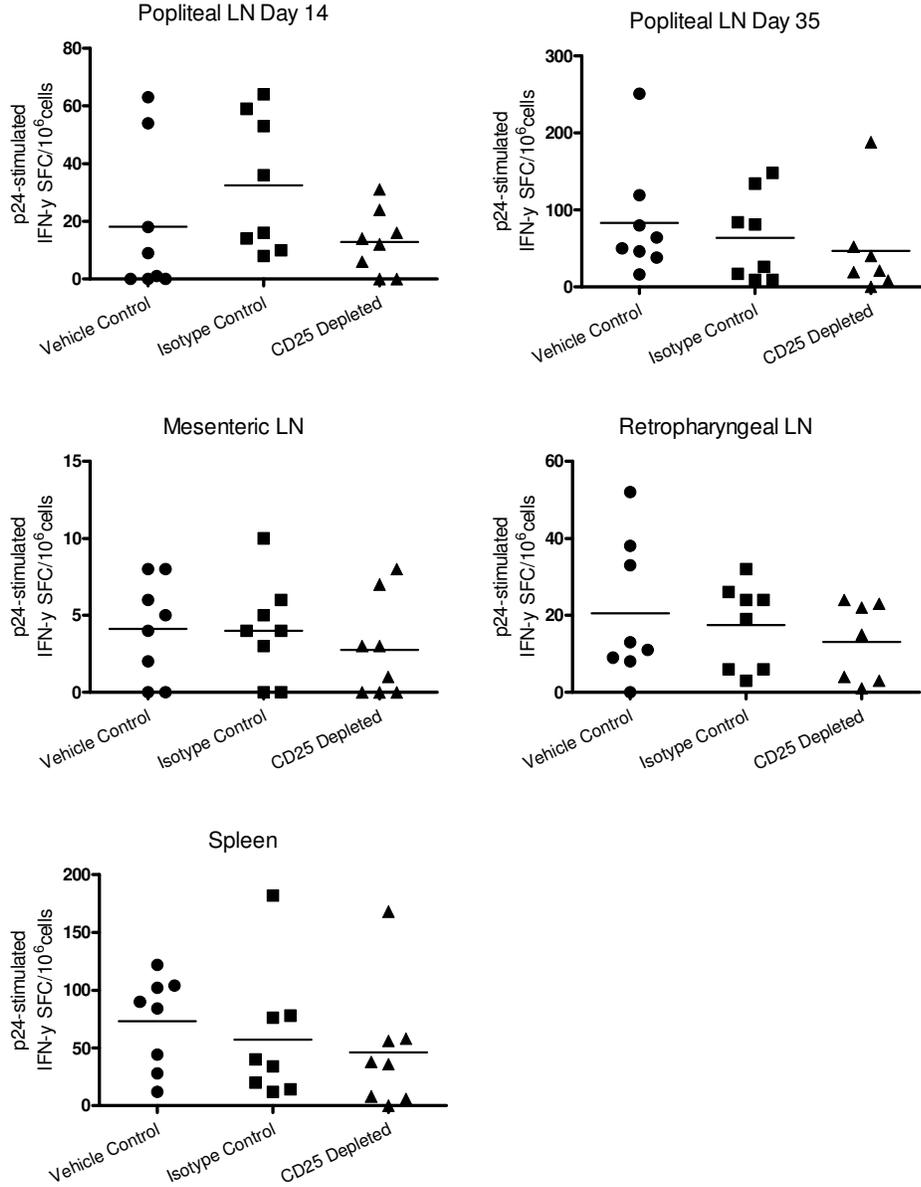


Figure 7. FIV-specific IFN- γ responses during acute infection are not altered by anti-CD25 mAb treatment. Popliteal lymph node cells from day 14 and 35 p.i. and mesenteric and retropharyngeal lymph node cells and spleen cells from day 54 p.i. were incubated for 48 hours with 100 μ g/ml FIV p24 peptides or 1% DMSO in media as a background control. IFN- γ spot forming cells (SFCs) per million cells from CD25 depleted, isotype control, and vehicle control treated groups in response to FIV p24 minus background were determined by ELISPOT (n=8/group).

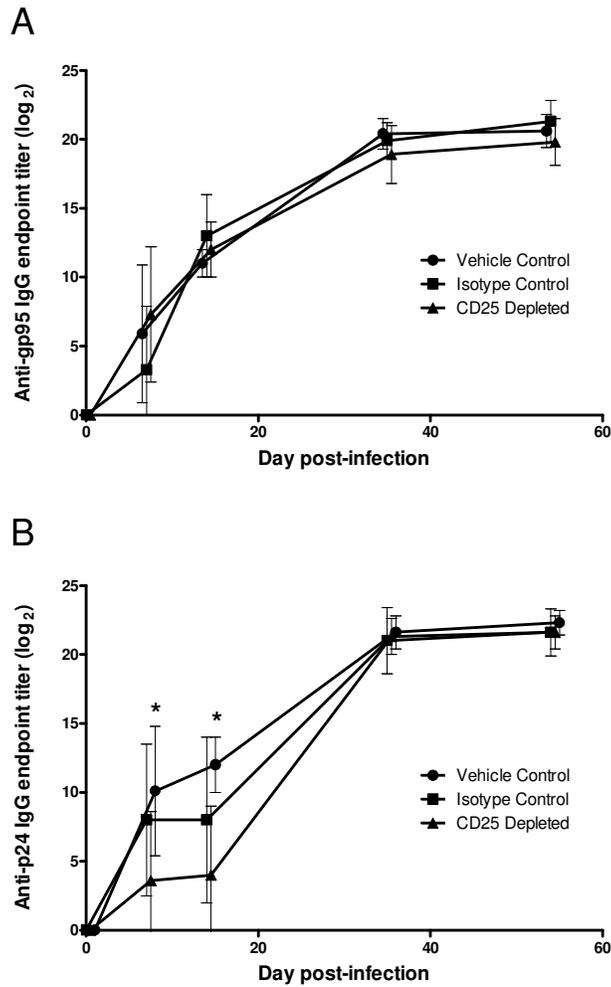


Figure 8. Anti-FIV p24, but not anti-FIV gp95, IgG titers are significantly lower after anti-CD25 mAb treatment during acute FIV infection. (A) Anti-FIV gp95 and (B) anti-FIV p24 IgG titers were quantified in serum samples on days 0, 7, 14, 35, and 54 post-FIV infection. Positive antibody titers were calculated based on a 3-fold higher optical density than day 0 pre-inoculation controls. Means \pm SEM are shown. Statistical significance was determined between treatment groups for each time point (n=8/group). * indicates $p < 0.05$.

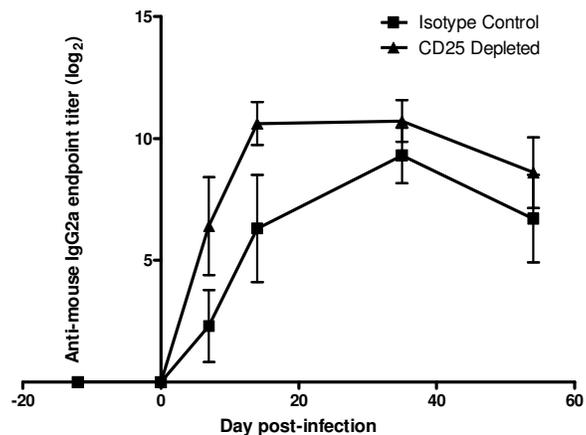


Figure 9. A trend of higher anti-mouse IgG titers are found in cats treated with anti-CD25 mAb during acute FIV infection. Serum was harvested on days -12, 0, 7, 14, 35, and 54 p.i. from cats treated with anti-CD25 mAb or isotype control mAb. ELISA plates were coated with polyclonal mouse IgG2a κ isotype antibody and then incubated with 2-fold serial dilutions of serum samples (n=8/group). Positive antibody titers were calculated based on a 3-fold higher optical density than negative controls. Means \pm SEM are shown.

References

1. Mandl, J. N., A. P. Barry, T. H. Vanderford, N. Kozyr, R. Chavan, S. Klucking, F. J. Barrat, R. L. Coffman, S. I. Staprans, and M. B. Feinberg. 2008. Divergent TLR7 and TLR9 signaling and type I interferon production distinguish pathogenic and nonpathogenic AIDS virus infections. *Nat Med* 14:1077-1087.
2. Schmitz, J. E., M. J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-Racz, M. Dalesandro, B. J. Scallon, J. Ghayeb, M. A. Forman, D. C. Montefiori, E. P. Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. *Science* 283:857-860.
3. Reimann, K. A., K. Tenner-Racz, P. Racz, D. C. Montefiori, Y. Yasutomi, W. Lin, B. J. Ransil, and N. L. Letvin. 1994. Immunopathogenic events in acute infection of rhesus monkeys with simian immunodeficiency virus of macaques. *J Virol* 68:2362-2370.
4. Song, W., E. W. Collisson, P. M. Billingsley, and W. C. Brown. 1992. Induction of feline immunodeficiency virus-specific cytolytic T-cell responses from experimentally infected cats. *J Virol* 66:5409-5417.
5. Tompkins, M. B., and W. A. Tompkins. 2008. Lentivirus-induced immune dysregulation. *Vet Immunol Immunopathol* 123:45-55.
6. Munier, M. L., and A. D. Kelleher. 2007. Acutely dysregulated, chronically disabled by the enemy within: T-cell responses to HIV-1 infection. *Immunol Cell Biol* 85:6-15.
7. Pereira, L. E., R. P. Johnson, and A. A. Ansari. 2008. Sooty mangabeys and rhesus macaques exhibit significant divergent natural killer cell responses during both acute and chronic phases of SIV infection. *Cell Immunol* 254:10-19.
8. Meythaler, M., A. Martinot, Z. Wang, S. Pryputniewicz, M. Kasheta, B. Ling, P. A. Marx, S. O'Neil, and A. Kaur. 2009. Differential CD4+ T-lymphocyte apoptosis and bystander T-cell activation in rhesus macaques and sooty mangabeys during acute simian immunodeficiency virus infection. *J Virol* 83:572-583.
9. Baker, C. A., R. Clark, F. Ventura, N. G. Jones, D. Guzman, D. R. Bangsberg, and H. Cao. 2007. Peripheral CD4 loss of regulatory T cells is associated with persistent viraemia in chronic HIV infection. *Clin Exp Immunol* 147:533-539.

10. Dunham, R. M., B. Cervasi, J. M. Brenchley, H. Albrecht, A. Weintrob, B. Sumpter, J. Engram, S. Gordon, N. R. Klatt, I. Frank, D. L. Sodora, D. C. Douek, M. Paiardini, and G. Silvestri. 2008. CD127 and CD25 expression defines CD4+ T cell subsets that are differentially depleted during HIV infection. *J Immunol* 180:5582-5592.
11. Ansari, A. A., L. E. Pereira, A. E. Mayne, N. Onlamoon, K. Pattanapanyasat, K. Mori, and F. Villinger. 2007. The role of disease stage, plasma viral load and regulatory T cells (Tregs) on autoantibody production in SIV-infected non-human primates. *J Autoimmun* 28:152-159.
12. Nilsson, J., A. Boasso, P. A. Velilla, R. Zhang, M. Vaccari, G. Franchini, G. M. Shearer, J. Andersson, and C. Chougnet. 2006. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 108:3808-3817.
13. Joshi, A., T. W. Vahlenkamp, H. Garg, W. A. Tompkins, and M. B. Tompkins. 2004. Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. *Virology* 321:307-322.
14. Ji, J., and M. W. Cloyd. 2009. HIV-1 binding to CD4 on CD4+CD25+ regulatory T cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: an additional mechanism of immunosuppression. *Int Immunol* 21:283-294.
15. Cao, W., B. D. Jamieson, L. E. Hultin, P. M. Hultin, and R. Detels. 2009. Regulatory T cell expansion and immune activation during untreated HIV type 1 infection are associated with disease progression. *AIDS Res Hum Retroviruses* 25:183-191.
16. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* 174:3143-3147.
17. Boasso, A., M. Vaccari, A. Hryniewicz, D. Fuchs, J. Nacsa, V. Cecchinato, J. Andersson, G. Franchini, G. M. Shearer, and C. Chougnet. 2007. Regulatory T-cell markers, indoleamine 2,3-dioxygenase, and virus levels in spleen and gut during progressive simian immunodeficiency virus infection. *J Virol* 81:11593-11603.
18. Epple, H. J., C. Loddenkemper, D. Kunkel, H. Troger, J. Maul, V. Moos, E. Berg, R. Ullrich, J. D. Schulzke, H. Stein, R. Duchmann, M. Zeitz, and T. Schneider. 2006. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in

- untreated HIV infection and normalize after suppressive HAART. *Blood* 108:3072-3078.
19. Mexas, A. M., J. E. Fogle, W. A. Tompkins, and M. B. Tompkins. 2008. CD4(+)CD25(+) regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol*.
 20. Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins. 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol* 172:4752-4761.
 21. Aandahl, E. M., J. Michaelsson, W. J. Moretto, F. M. Hecht, and D. F. Nixon. 2004. Human CD4+ CD25+ regulatory T cells control T-cell responses to human immunodeficiency virus and cytomegalovirus antigens. *J Virol* 78:2454-2459.
 22. Pereira, L. E., F. Villinger, N. Onlamoon, P. Bryan, A. Cardona, K. Pattanapanyasat, K. Mori, S. Hagen, L. Picker, and A. A. Ansari. 2007. Simian immunodeficiency virus (SIV) infection influences the level and function of regulatory T cells in SIV-infected rhesus macaques but not SIV-infected sooty mangabeys. *J Virol* 81:4445-4456.
 23. Kinter, A. L., R. Horak, M. Sion, L. Riggin, J. McNally, Y. Lin, R. Jackson, A. O'Shea, G. Roby, C. Kovacs, M. Connors, S. A. Migueles, and A. S. Fauci. 2007. CD25+ regulatory T cells isolated from HIV-infected individuals suppress the cytolytic and nonlytic antiviral activity of HIV-specific CD8+ T cells in vitro. *AIDS Res Hum Retroviruses* 23:438-450.
 24. Weiss, L., V. Donkova-Petrini, L. Caccavelli, M. Balbo, C. Carbonneil, and Y. Levy. 2004. Human immunodeficiency virus-driven expansion of CD4+CD25+ regulatory T cells, which suppress HIV-specific CD4 T-cell responses in HIV-infected patients. *Blood* 104:3249-3256.
 25. Eggena, M. P., B. Barugahare, N. Jones, M. Okello, S. Mutalya, C. Kityo, P. Mugenyi, and H. Cao. 2005. Depletion of regulatory T cells in HIV infection is associated with immune activation. *J Immunol* 174:4407-4414.
 26. Estes, J. D., Q. Li, M. R. Reynolds, S. Wietgreffe, L. Duan, T. Schacker, L. J. Picker, D. I. Watkins, J. D. Lifson, C. Reilly, J. Carlis, and A. T. Haase. 2006. Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection. *J Infect Dis* 193:703-712.

27. Tenorio, A. R., J. Martinson, D. Pollard, L. Baum, and A. Landay. 2008. The relationship of T-regulatory cell subsets to disease stage, immune activation, and pathogen-specific immunity in HIV infection. *J Acquir Immune Defic Syndr* 48:577-580.
28. Jiang, Q., L. Zhang, R. Wang, J. Jeffrey, M. L. Washburn, D. Brouwer, S. Barbour, G. I. Kovalev, D. Unutmaz, and L. Su. 2008. FoxP3+CD4+ regulatory T cells play an important role in acute HIV-1 infection in humanized Rag2^{-/-}gammaC^{-/-} mice in vivo. *Blood* 112:2858-2868.
29. Joshi, A., H. Garg, M. B. Tompkins, and W. A. Tompkins. 2005. Preferential feline immunodeficiency virus (FIV) infection of CD4+ CD25+ T-regulatory cells correlates both with surface expression of CXCR4 and activation of FIV long terminal repeat binding cellular transcriptional factors. *J Virol* 79:4965-4976.
30. Oswald-Richter, K., S. M. Grill, N. Shariat, M. Leelawong, M. S. Sundrud, D. W. Haas, and D. Unutmaz. 2004. HIV infection of naturally occurring and genetically reprogrammed human regulatory T-cells. *PLoS Biol* 2:E198.
31. Holmes, D., G. Knudsen, S. Mackey-Cushman, and L. Su. 2007. FoxP3 enhances HIV-1 gene expression by modulating NFkappaB occupancy at the long terminal repeat in human T cells. *J Biol Chem* 282:15973-15980.
32. Chase, A. J., H. C. Yang, H. Zhang, J. N. Blankson, and R. F. Siliciano. 2008. Preservation of FoxP3+ regulatory T cells in the peripheral blood of human immunodeficiency virus type 1-infected elite suppressors correlates with low CD4+ T-cell activation. *J Virol* 82:8307-8315.
33. Kinter, A. L., M. Hennessey, A. Bell, S. Kern, Y. Lin, M. Daucher, M. Planta, M. McGlaughlin, R. Jackson, S. F. Ziegler, and A. S. Fauci. 2004. CD25(+)CD4(+) regulatory T cells from the peripheral blood of asymptomatic HIV-infected individuals regulate CD4(+) and CD8(+) HIV-specific T cell immune responses in vitro and are associated with favorable clinical markers of disease status. *J Exp Med* 200:331-343.
34. Giorgi, J. V., Z. Liu, L. E. Hultin, W. G. Cumberland, K. Hennessey, and R. Detels. 1993. Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. *J Acquir Immune Defic Syndr* 6:904-912.
35. Deeks, S. G., C. M. Kitchen, L. Liu, H. Guo, R. Gascon, A. B. Narvaez, P. Hunt, J. N. Martin, J. O. Kahn, J. Levy, M. S. McGrath, and F. M. Hecht. 2004. Immune

- activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. *Blood* 104:942-947.
36. Liu, Z., W. G. Cumberland, L. E. Hultin, H. E. Prince, R. Detels, and J. V. Giorgi. 1997. Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression. *J Acquir Immune Defic Syndr Hum Retrovirol* 16:83-92.
 37. Levacher, M., F. Hulstaert, S. Tallet, S. Ullery, J. J. Pocidallo, and B. A. Bach. 1992. The significance of activation markers on CD8 lymphocytes in human immunodeficiency syndrome: staging and prognostic value. *Clin Exp Immunol* 90:376-382.
 38. Smithberg, S. R., J. E. Fogle, A. M. Mexas, S. K. Reckling, S. M. Lankford, M. B. Tompkins, and G. A. Dean. 2008. In vivo depletion of CD4(+)CD25(+) regulatory T cells in cats. *J Immunol Methods* 329:81-91.
 39. Ohno, K., R. Goitsuka, K. Kitamura, A. Hasegawa, T. Tokunaga, and M. Honda. 1992. Production of a monoclonal antibody that defines the alpha-subunit of the feline IL-2 receptor. *Hybridoma* 11:595-605.
 40. de Rozieres, S., C. K. Mathiason, M. R. Rolston, U. Chatterji, E. A. Hoover, and J. H. Elder. 2004. Characterization of a highly pathogenic molecular clone of feline immunodeficiency virus clade C. *J Virol* 78:8971-8982.
 41. Stevens, R., K. E. Howard, S. Nordone, M. Burkhard, and G. A. Dean. 2004. Oral immunization with recombinant listeria monocytogenes controls virus load after vaginal challenge with feline immunodeficiency virus. *J Virol* 78:8210-8218.
 42. Burkhard, M. J., C. K. Mathiason, T. Bowdre, and E. A. Hoover. 2001. Feline immunodeficiency virus Gag- and Env-specific immune responses after vaginal versus intravenous infection. *AIDS Res Hum Retroviruses* 17:1767-1778.
 43. Dean, G. A., and N. C. Pedersen. 1998. Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. *J Virol* 72:9436-9440.
 44. Nishimura, Y., M. Shimojima, E. Sato, Y. Izumiya, Y. Tohya, T. Mikami, and T. Miyazawa. 2004. Downmodulation of CD3epsilon expression in CD8alpha+beta- T cells of feline immunodeficiency virus-infected cats. *J Gen Virol* 85:2585-2589.

45. Tompkins, M. B., D. H. Gebhard, H. R. Bingham, M. J. Hamilton, W. C. Davis, and W. A. Tompkins. 1990. Characterization of monoclonal antibodies to feline T lymphocytes and their use in the analysis of lymphocyte tissue distribution in the cat. *Vet Immunol Immunopathol* 26:305-317.
46. Dean, G. A., A. LaVoy, and M. J. Burkhard. 2004. Peptide mapping of feline immunodeficiency virus by IFN-gamma ELISPOT. *Vet Immunol Immunopathol* 100:49-59.
47. Tompkins, M. B., M. E. Bull, J. L. Dow, J. M. Ball, E. W. Collisson, B. J. Winslow, A. P. Phadke, T. W. Vahlenkamp, and W. A. Tompkins. 2002. Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis. *J Infect Dis* 185:1077-1093.
48. Woo, J. C., and P. F. Moore. 1997. A feline homologue of CD1 is defined using a feline-specific monoclonal antibody. *Tissue Antigens* 49:244-251.
49. Pedersen, N. C., C. M. Leutenegger, J. Woo, and J. Higgins. 2001. Virulence differences between two field isolates of feline immunodeficiency virus (FIV-APetaluma and FIV-CPGammar) in young adult specific pathogen free cats. *Vet Immunol Immunopathol* 79:53-67.
50. Stevens, R., A. Lavoy, S. Nordone, M. Burkhard, and G. A. Dean. 2005. Pre-existing immunity to pathogenic *Listeria monocytogenes* does not prevent induction of immune responses to feline immunodeficiency virus by a novel recombinant *Listeria monocytogenes* vaccine. *Vaccine* 23:1479-1490.
51. Staats, H. F., W. G. Nichols, and T. J. Palker. 1996. Mucosal immunity to HIV-1: systemic and vaginal antibody responses after intranasal immunization with the HIV-1 C4/V3 peptide T1SP10 MN(A). *J Immunol* 157:462-472.
52. de Parseval, A., and J. H. Elder. 2001. Binding of recombinant feline immunodeficiency virus surface glycoprotein to feline cells: role of CXCR4, cell-surface heparans, and an unidentified non-CXCR4 receptor. *J Virol* 75:4528-4539.
53. de Rozieres, S., C. H. Swan, D. A. Sheeter, K. J. Clingerman, Y. C. Lin, S. Huitron-Resendiz, S. Henriksen, B. E. Torbett, and J. H. Elder. 2004. Assessment of FIV-C infection of cats as a function of treatment with the protease inhibitor, TL-3. *Retrovirology* 1:38.
54. Baecher-Allan, C., V. Viglietta, and D. A. Hafler. 2004. Human CD4+CD25+ regulatory T cells. *Semin Immunol* 16:89-98.

55. Ziegler, S. F. 2007. FOXP3: not just for regulatory T cells anymore. *Eur J Immunol* 37:21-23.
56. Lankford, S., C. Petty, A. Lavoy, S. Reckling, W. Tompkins, and G. A. Dean. 2007. Cloning of feline FOXP3 and detection of expression in CD4+CD25+ regulatory T cells. *Vet Immunol Immunopathol*.
57. Gavin, M. A., J. P. Rasmussen, J. D. Fontenot, V. Vasta, V. C. Manganiello, J. A. Beavo, and A. Y. Rudensky. 2007. Foxp3-dependent programme of regulatory T-cell differentiation. *Nature* 445:771-775.
58. Gavin, M. A., T. R. Torgerson, E. Houston, P. DeRoos, W. Y. Ho, A. Stray-Pedersen, E. L. Ocheltree, P. D. Greenberg, H. D. Ochs, and A. Y. Rudensky. 2006. Single-cell analysis of normal and FOXP3-mutant human T cells: FOXP3 expression without regulatory T cell development. *Proc Natl Acad Sci U S A* 103:6659-6664.
59. Allan, S. E., S. Q. Crome, N. K. Crellin, L. Passerini, T. S. Steiner, R. Bacchetta, M. G. Roncarolo, and M. K. Levings. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int Immunol* 19:345-354.
60. Lund, J. M., L. Hsing, T. T. Pham, and A. Y. Rudensky. 2008. Coordination of early protective immunity to viral infection by regulatory T cells. *Science* 320:1220-1224.
61. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol* 6:1219-1227.
62. Apostolou, I., and H. von Boehmer. 2004. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med* 199:1401-1408.
63. Petty, C. S., M. B. Tompkins, and W. A. Tompkins. 2008. Transforming growth factor-beta/transforming growth factor-betaRII signaling may regulate CD4+CD25+ T-regulatory cell homeostasis and suppressor function in feline AIDS lentivirus infection. *J Acquir Immune Defic Syndr* 47:148-160.
64. Bendinelli, M., M. Pistello, S. Lombardi, A. Poli, C. Garzelli, D. Matteucci, L. Ceccherini-Nelli, G. Malvaldi, and F. Tozzini. 1995. Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin Microbiol Rev* 8:87-112.

65. Antons, A. K., R. Wang, K. Oswald-Richter, M. Tseng, C. W. Arendt, S. A. Kalam, and D. Unutmaz. 2008. Naive precursors of human regulatory T cells require FoxP3 for suppression and are susceptible to HIV infection. *J Immunol* 180:764-773.
66. Klein, L., K. Khazaie, and H. von Boehmer. 2003. In vivo dynamics of antigen-specific regulatory T cells not predicted from behavior in vitro. *Proc Natl Acad Sci U S A* 100:8886-8891.
67. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional CD25+ CD4+ regulatory T cells by antigen-processing dendritic cells. *J Exp Med* 198:235-247.
68. Liang, S., P. Alard, Y. Zhao, S. Parnell, S. L. Clark, and M. M. Kosiewicz. 2005. Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus. *J Exp Med* 201:127-137.
69. Knoechel, B., J. Lohr, E. Kahn, J. A. Bluestone, and A. K. Abbas. 2005. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med* 202:1375-1386.
70. Couper, K. N., P. A. Lanthier, G. Perona-Wright, L. W. Kummer, W. Chen, S. T. Smiley, M. Mohrs, and L. L. Johnson. 2009. Anti-CD25 antibody-mediated depletion of effector T cell populations enhances susceptibility of mice to acute but not chronic *Toxoplasma gondii* infection. *J Immunol* 182:3985-3994.
71. Cecchinato, V., E. Trynieszewska, Z. M. Ma, M. Vaccari, A. Boasso, W. P. Tsai, C. Petrovas, D. Fuchs, J. M. Heraud, D. Venzon, G. M. Shearer, R. A. Koup, I. Lowy, C. J. Miller, and G. Franchini. 2008. Immune activation driven by CTLA-4 blockade augments viral replication at mucosal sites in simian immunodeficiency virus infection. *J Immunol* 180:5439-5447.
72. Pandrea, I., C. Coleman, T. Gaufin, R. Gautam, M. Pattison, C. Monjure, A. A. Lackner, and C. Apetrei. 2008. Experimentally-induced immune activation in natural hosts of SIVs results in significant increases in viral replication. In *Keystone Symposia HIV Pathogenesis*, Banff, Canada.
73. Wang, R., L. Kozhaya, F. Mercer, A. Khaitan, H. Fujii, and D. Unutmaz. 2009. Expression of GARP selectively identifies activated human FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A* 106:13439-13444.
74. Walker, L. S. 2009. Regulatory T cells overturned: the effectors fight back. *Immunology* 126:466-474.

75. Litzinger, M. T., R. Fernando, T. J. Curiel, D. W. Grosenbach, J. Schlom, and C. Palena. 2007. IL-2 immunotoxin denileukin diftitox reduces regulatory T cells and enhances vaccine-mediated T-cell immunity. *Blood* 110:3192-3201.

DISSERTATION SUMMARY

The studies described in this dissertation were designed to fill knowledge gaps regarding the role of regulatory T (Treg) cells during lentiviral pathogenesis in a natural host. Studies exploring Treg cell activity in HIV-1 infected patients are limited to in vivo observational correlative data or in vitro cell assays, and are not capable of determining consequences of Treg cell activity in vivo. Studies designed to explore the role of Treg cells during experimental SIV infection have been limited by the utilization of methods that insufficiently disrupt Treg cell activity in vivo, including blocking only one mechanism of Treg cell immunosuppression, or using diphtheria toxin conjugated human IL-2 (Ontak), which did not deplete Treg cells in SIV-infected African green monkeys (1-3). We sought to better define the dimensions of Treg cell influence during the acute and chronic phases of lentiviral infection by using anti-CD25 monoclonal antibody (mAb) therapy and the feline immunodeficiency virus (FIV)/cat model, a natural host model for HIV-1 infection.

Before depleting Treg cells in FIV-infected cats, we first sought to validate our model of Treg cell depletion in uninfected cats using a mouse anti-feline CD25 mAb. We found that a single high dose of anti-CD25 mAb, given intraperitoneally, was sufficient to deplete the majority of CD4⁺CD25^{hi} Treg cells from feline lymphoid tissues and blood by day 11 post-depletion. CD4⁺ T cell absolute number was decreased in anti-CD25 mAb treated cats, indicating that Treg cells were depleted as opposed to CD25 receptor being shed from Treg cells after treatment. Additionally, as mouse anti-CD25 mAb-coated Treg cells were cleared in the blood, murine-specific IgG titers increased in cat serum. FOXP3 mRNA levels in

lymphoid tissues remained low until day 35 post-depletion, indicating that Treg cells were depleted for at least 5 weeks following anti-CD25 mAb administration. However, we recognized that Treg cell depletion was transient, and though the majority of Treg cells were depleted, some CD4⁺CD25^{hi}FOXP3⁺ cells remained after CD25 depletion. Some cats were immunized with FIV p24-GST prior to Treg cell depletion. Humoral and cell-mediated anti-FIV p24-GST immune responses were retained during Treg cell depletion, indicating that anti-CD25 mAb treatment did not deplete effector T and B cells. It was noted that anti-CD25 mAb administration should not be administered during an active immune response, when effector cells upregulate CD25.

Next, we treated cats chronically infected with FIV-NCSU₁ with saline, isotype control mAb, or anti-CD25 mAb. We found that anti-FIV cell-mediated immune responses were significantly increased while Treg cells were depleted. However, the effect was transient, as FIV-specific responses were again repressed as Treg cells returned after depletion. The anti-FIV cellular responses that were revealed were pre-existing, and not expanded, during Treg cell depletion. Transient depletion of Treg cells during asymptomatic lentiviral infection when viral set-point was low did not adversely affect viral burden or induce damaging proinflammatory cytokines. Because FOXP3 was upregulated in Treg cells of isotype control FIV⁺ cats as they responded to foreign mouse protein, but not in Treg cells of FIV naïve cats, we conclude that FIV infection results in elevated Treg cell activity under steady-state conditions and may prime for heightened Treg cell responses to new antigens. Importantly, Treg cell depletion in FIV-infected cats allowed the generation of 3 to 4 log higher antibody titers during a primary immune response to mouse protein, possibly allowing

a window of opportunity to introduce a novel vaccine or therapy. The more robust primary humoral immune response observed in Treg cell-depleted FIV-infected cats was likely due to removal of Treg cells that were excessively activated over the course of FIV infection.

We found that Treg cells do not harbor more integrated provirus than conventional CD4⁺ T cells during chronic FIV infection. Thus, depletion of Treg cells does not represent a useful strategy to eliminate a latent viral reservoir. The most clinically significant conclusion from this study is that eliminating Treg cells for a short period of time during chronic lentiviral infection would relieve suppression of the adaptive antiviral immune response and allow an opportunity to administer a therapeutic vaccine. The FIV/cat model would be ideal to test this therapeutic strategy.

In our next study, cats were treated with saline, isotype control mAb, or anti-CD25 mAb before infection with the acutely pathogenic molecular clone FIV-C36. We found that the feline immune system is able to mount effector T and B cell responses to FIV in the presence of Treg cells in control treated cats. Our methodology of CD25⁺ cell depletion did not eliminate effector T or B cells or dendritic cells in CD25 depleted cats. Treg cell depletion prior to infection did not significantly alter acute FIV pathogenesis in regard to viremia or provirus levels or CD4⁺ T cell counts. We conclude that naïve Treg cells do not play a major role during acute infection of a highly pathogenic FIV strain, when massive amounts of immune activation induced by FIV infection may override any amount of immunosuppression exerted by Treg cells. It is possible that Treg cell depletion prior to infection with a less pathogenic FIV strain could modulate FIV pathogenesis, when activation status of the immune system is lower. We found that the Treg cell:T effector cell

ratio increases in the periphery during acute FIV infection. Others have reported that the Treg cell:T effector cell ratio increases in the periphery and in lymphoid tissues during lentiviral infection (4-7), and that Treg cells are more suppressive during lentiviral infection (8-10). We propose that Treg cell influence may be more robust during chronic lentiviral infection, when Treg cells are activated and matured, as compared to acute infection.

Additional studies are needed for further insight into the role of Treg cells during lentiviral infection. Bromodeoxyuridine (BrdU) administration in cats before and after FIV infection would delineate the *in vivo* proliferation rate of Treg cells in different tissue sites during FIV infection. Repeated isolation of Treg cells from feline lymphoid tissues following FIV infection would allow sequential functional analysis of Treg cell suppressive activity and help determine when Treg cell function is modulated during infection. A study to investigate the affect of lentiviral vaccination on Treg cell activity may shed light on recent results of the Merck STEP HIV vaccine trial in humans (11). The administration of an FIV vaccine in FIV naïve cats may induce the activation and maturation of FIV-specific Treg cells. Subsequent FIV infection of these cats may have adverse effects if induced Treg cells suppress FIV-specific immune responses. It also remains to be determined whether efficacy of an FIV vaccine, whether of preventative or therapeutic nature, could be boosted when administered in conjunction with Treg cell depletion. In addition, the affect of FIV infection on a pre-existing immune response and the corresponding population of induced Treg cells remains to be determined.

Our studies have made major contributions towards understanding the role of Treg cells during lentiviral infection. This dissertation describes, for the first time, the effective

modulation of Treg cells in an animal model of natural lentiviral infection. Furthermore, the method of Treg cell depletion that was employed to conduct the studies described here mirrors the FDA-approved technology that could be used to deplete Treg cells in humans, namely, Ontak, and the anti-CD25 mAbs, basiliximab and daclizumab. Further studies will be needed to determine the applicability of Treg cell depletion in patients chronically infected with HIV-1, but the work described here indicates that anti-CD25 mAb therapy in conjunction with a therapeutic vaccine may be beneficial in certain HIV-1 infected patients.

References

1. Cecchinato, V., E. Trynieszewska, Z. M. Ma, M. Vaccari, A. Boasso, W. P. Tsai, C. Petrovas, D. Fuchs, J. M. Heraud, D. Venzon, G. M. Shearer, R. A. Koup, I. Lowy, C. J. Miller, and G. Franchini. 2008. Immune activation driven by CTLA-4 blockade augments viral replication at mucosal sites in simian immunodeficiency virus infection. *J Immunol* 180:5439-5447.
2. Hryniewicz, A., A. Boasso, Y. Edghill-Smith, M. Vaccari, D. Fuchs, D. Venzon, J. Nacsa, M. R. Betts, W. P. Tsai, J. M. Heraud, B. Beer, D. Blanset, C. Chougnet, I. Lowy, G. M. Shearer, and G. Franchini. 2006. CTLA-4 blockade decreases TGF-beta,IDO, and viral RNA expression in tissues of SIVmac251-infected macaques. *Blood* 108:3834-3842.
3. Pandrea, I., C. Coleman, T. Gaufin, R. Gautam, M. Pattison, C. Monjure, A. A. Lackner, and C. Apetrei. 2008. Experimentally-induced immune activation in natural hosts of SIVs results in significant increases in viral replication. In *Keystone Symposia HIV Pathogenesis*, Banff, Canada.
4. Andersson, J., A. Boasso, J. Nilsson, R. Zhang, N. J. Shire, S. Lindback, G. M. Shearer, and C. A. Chougnet. 2005. The prevalence of regulatory T cells in lymphoid tissue is correlated with viral load in HIV-infected patients. *J Immunol* 174:3143-3147.
5. Boasso, A., M. Vaccari, A. Hryniewicz, D. Fuchs, J. Nacsa, V. Cecchinato, J. Andersson, G. Franchini, G. M. Shearer, and C. Chougnet. 2007. Regulatory T-cell markers, indoleamine 2,3-dioxygenase, and virus levels in spleen and gut during progressive simian immunodeficiency virus infection. *J Virol* 81:11593-11603.
6. Epple, H. J., C. Loddenkemper, D. Kunkel, H. Troger, J. Maul, V. Moos, E. Berg, R. Ullrich, J. D. Schulzke, H. Stein, R. Duchmann, M. Zeitz, and T. Schneider. 2006. Mucosal but not peripheral FOXP3+ regulatory T cells are highly increased in untreated HIV infection and normalize after suppressive HAART. *Blood* 108:3072-3078.
7. Nilsson, J., A. Boasso, P. A. Velilla, R. Zhang, M. Vaccari, G. Franchini, G. M. Shearer, J. Andersson, and C. Chougnet. 2006. HIV-1-driven regulatory T-cell accumulation in lymphoid tissues is associated with disease progression in HIV/AIDS. *Blood* 108:3808-3817.
8. Vahlenkamp, T. W., M. B. Tompkins, and W. A. Tompkins. 2004. Feline immunodeficiency virus infection phenotypically and functionally activates immunosuppressive CD4+CD25+ T regulatory cells. *J Immunol* 172:4752-4761.

9. Mexas, A. M., J. E. Fogle, W. A. Tompkins, and M. B. Tompkins. 2008. CD4(+)CD25(+) regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol*.
10. Ji, J., and M. W. Cloyd. 2009. HIV-1 binding to CD4 on CD4+CD25+ regulatory T cells enhances their suppressive function and induces them to home to, and accumulate in, peripheral and mucosal lymphoid tissues: an additional mechanism of immunosuppression. *Int Immunol* 21:283-294.
11. Sekaly, R. P. 2008. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J Exp Med* 205:7-12.