ABSTRACT

MILLER, MICHELLE MARIE. The Role of Feline Glycoprotein A Repetitions Predominant (GARP) in Regulatory T Cell Mediated Immune Dysfunction During FIV Lentiviral Infection (Under the direction of Dr Mary Tompkins)

Feline Immunodeficiency Virus (FIV) is an important pathogen for companion animal medicine which is homologous in structure and pathogenesis to the human lentivirus, HIV, and therefore serves as an appropriate model for the study of HIV-AIDS in a naturally infected host. Our lab has described the contributions of T regulatory (Treg) cells to the disease progression and immunologic dysfunction associated with FIV. We previously reported that membrane-associated TGFβ (mTGFβ) on Treg cells mediates suppressor function against $CD4^+$ T helper (Th) cells, and that FIV infection of cats results in activation of these $mTGF\beta^+CD4^+CD25^+$ Treg cells in vivo. This dissertation focuses on the role of a novel protein, Glycoprotein A Repetitions Predominant (GARP), during FIV infection and provides the first description of GARP in the feline system, demonstrating that it serves as a membrane anchor for TGFβ on the surface of feline Treg cells. We examine co-expression of TGFβ and GARP on $CD4^+CD25^+$ Treg cells from chronically FIV-infected cats and demonstrate that $GARP^+$ Treg cells are activated during infection and function as highly effective suppressors of $CD4^+$ Th cells. Having demonstrated that $GARP^+$ Treg cells are activated during infection, we next investigated the mechanism of activation. To determine if FIV infection was sufficient to directly activate $GARP^+$ Treg cells, purified $CD4^+CD25^+$ cells from FIV negative cats were infected in vitro and phenotypic and functional changes analyzed. Between 3 and 6 days post in vitro infection, GARP and TGFβ expression were increased on $CD4^+CD25^+$ cells. These $CD4^+CD25^+GARP^+$ cells also expressed the Treg specific transcription factor, FoxP3, demonstrating that FIV infection alone induces an

activated regulatory cell phenotype. To determine if these cells were functionally activated, 6 day in vitro FIV-infected $CD4^+CD25^+$ cells were co-cultured with ConA activated $CD4^+CD25^-$ Th cells for 24 hours and IL2 levels I the culture supernatant measured. In vitro infection-activated $CD4^+CD25^+$ cells were capable of suppressing IL2 production from activated Th cells. From these data, we hypothesize that the in vivo activation of GARP:TGFβ expressing Treg cells by direct FIV infection contributes to the $CD4^+$ T cell anergy via ligation of TGFβ-RII on the surface of $CD4^+CD25^-$ Th cells. Progression through the chronic stage of infection is characterized by a generalized $CD4^+$ T cell depletion but despite this, the percentage of Treg cells in the $CD4^+$ population remains constant. Having previously demonstrated that soluble TGFβ is sufficient to convert activated $CD4^+CD25^-$ Th cells into suppressor cells, we asked whether the expanded $CD4^+CD25^+GARP^+mTGF\beta^+$ population identified during FIV infection could engage in TGFβ-mediated signaling with $TGF\beta\text{-}RII^+CD4^+CD25^-$ Th and convert these cells into regulatory cells, thereby maintaining Treg numbers by recruitment of new Treg cells from the Th pool during FIV infection. Here, we demonstrate that $GARP^+$ Treg cells activated during FIV-infection in vivo are capable of converting activated Th cells into phenotypic and functional Treg cells in vitro. This process is mediated by mTGFβ on the Treg cells and TGFβ-RII on the target Th cells as the addition of TGFβ/ TGFβ-RII blocking antibodies prior to co-culture abrogated conversion. Taken together, these data suggest that FIV infection is characterized by the expansion and activation of infected $GARP^+$ Treg cells and continued immune dysfunction results from conversion of FIV-activated Th cells into regulatory cells. Death of FIV-infected T cells coupled to the conversion of Th cells leads to a continual cycle of Th depletion consistent with CD4 decline culminating in AIDS pathogenesis. In addition to providing a mechanistic

perspective for disease progression, this discovery suggests a novel point of therapeutic intervention. Further research aimed at providing more detailed longitudinal data for conversion dynamics over the course of infection and the specificities of the TGFβ-mediated conversion signaling pathways will provide important information for future Treg-based immunotherapy development.

The Role of Feline Glycoprotein A Repetitions Predominant (GARP) in Regulatory T Cell Mediated Immune Dysfunction During FIV Lentiviral Infection

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

Immunology

Raleigh, North Carolina

2013

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DEDICATION

To my husband for his support and patience.

To my parents for being proud of me no matter what I've accomplished or how long it took me to get there.

To my sisters for being the best friends I could ever ask for.

BIOGRAPHY

Michelle Marie Miller was born Michelle Marie Dollar in a rural Oklahoma town and after living in Seattle, Washington for several years her family moved to North Carolina where Michelle attended middle and high school. She was active in sports as well as fine and performing arts throughout school and was exceptionally mercurial when deciding her academic future. She initially joined North Carolina State University's College of Design to pursue a degree in Architecture after graduating high school but quickly determined that her interests lay in veterinary medicine. She transferred to a Zoology major and worked for several years in small animal medicine before developing a strong inclination for laboratory work. She spent the last few years of her undergraduate career fulfilling the requirements for a second degree in Chemistry, completing two internships in RTP and meeting her future husband. Graduation, a wedding and a full-time job later, Michelle finally found her passion: Research. She applied to the NCSU Immunology doctoral program in 2008 while working as a research associate at Pfizer Animal Health in RTP, NC. Upon acceptance, she began the program full-time, taking a position in the Tompkins lab studying FIV as a model for HIV-AIDS. She has accepted a post-doctoral position at NCSU College of Veterinary Medicine following her graduation and looks forward to a career in scientific research.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor and mentor, Mary, for providing me with a stable, supportive environment in which to mature as a research scientist over the last four years. You have always kept me on target but allowed me the freedom to make a few mistakes along the way so that I could learn and grow. I would also like to thank Wayne for his tireless editing of my manuscripts and his constant supply of ideas for my research. I hope that I haven't kept you too busy during your first few years of retirement. Thank you to Jonathan, for being a sounding board and a second mentor to me. Your encouragement has meant a lot. To Linda, thank you for listening and for the occasional wine-therapy. To Deb, thank you for your endless helpfulness (and superior organization!). To everyone in the Tompkins lab over the years, thank you for your assistance and camaraderie. Thank you to the Hess lab for teaching me the art/headache of sequencing. To the Hudson lab, thank you for the many, many samples you have provided for my research. To everyone else in the Immunology program, both faculty and students, thank you for making the last four years an exceptional experience.

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Infection with Feline Immunodeficiency Virus Directly Activates $CD4^+CD25^+$ T Regulatory Cells in vitro.

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1. INTRODUCTION

Feline Immunodeficiency Virus or FIV is a common lentiviral pathogen of domestic and exotic cat species which has proven a useful scientific model for advancements in both human and veterinary medicine. Disease estimates for FIV infection in domestic cats differ based on global location with reported values as high as 24% in some cohorts in Japan (1) but as low as 2.5% in non-feral domestic populations in the United States (2). Although a safe and effective vaccine is commercially available to prevent FIV infection in household pets, there is currently no treatment for this disease and infection, once established, is lifelong (3-5). Development of a suitable therapeutic for infection has broad reaching application from companion animal medicine to wildlife conservation. The FIV lentivirus bears striking similarities to the human lentiviral pathogen HIV in both virus structure and disease pathogenesis. While murine models are commonly utilized for HIV research, mice must be genetically modified to be susceptible to HIV infection (6) while FIV infection in the *felis catus* host provides a natural model of infection. The feline animal model is also more affordable than the related simian lentivirus model (SIV) and involves less stringent ethical considerations than human or simian based research. FIV thus provides a valuable veterinary research model with important translational applications to a human pathogen of global importance.

This lab has previously identified a significant role for the unique subset of T cells denoted as $CD4^+CD25^+$ T regulatory (Treg) cells during FIV infection (7-9). These regulatory cells are able to suppress $CD4^+$ T helper (Th) cells and $CD8^+$ cytotoxic T cells (CTLs) during both

acute and chronic FIV, contributing to viral persistence and promoting the immune dysfunction characteristic of chronic lentiviral infection. The Treg cell-specific expression of membrane bound TGFβ (mTGFβ) has been documented in human, murine and feline systems (7, 10, 11) and we have previously demonstrated that Treg cells activated during FIV infection are able to suppress cytokine production and proliferation of Th cells in a TGFβ-dependent manner (7, 8, 12). These data lead us to conclude that the unique expression of mTGFβ enables Treg cells to promote Th cell aznergy through TGFβ-RII ligation and subsequent SMAD signaling in the Th target. What remains uncertain, however, are the specific mechanism(s) by which mTGFβ is expressed and the manner in which these $mTGF\beta^{+}$ Treg cells become activated during infection.

This dissertation directly addresses both of these uncertainties by first identifying a novel protein GARP which is essential for mTGFβ expression in Treg cells. We demonstrate the similarities between feline and human GARP and provide data to support the suppressive nature of GARP-expressing Treg cells in chronically FIV-infected cats when compared to noninfected cats. We identify the expansion of TGFβ receptor (TGFβ-RII) expressing Th cells during chronic FIV infection in vivo, supporting a role for TGFβ signaling in Treg-mediated Th suppression. In vitro FIV infection of purified Treg cells reveals that the virus is not only capable of productively infecting Treg cells as we have previously demonstrated (13-15) but that infection itself is sufficient for Treg cell activation.

In addition to the well established populations of natural Treg cells (nTreg cells), there is evidence to suggest a population of Treg cells can be induced during viral infection by conversion of non-suppressive Th cells (16-18). In this lab, we have demonstrated that in vitro treatment of activated $CD4^+CD25^-$ Th cells with soluble recombinant TGFβ (rTGFβ) and the plant lectin Concanavalin A (ConA) converts a significant proportion of these Th cells into phenotypic and functional suppressor cells (19). Using our feline GARP model, we demonstrate that conversion of Th cells into iTregs by treatment with rTGFβ/ConA is characterized by the induction of Treg specific markers including GARP and the Treg specific transcription factor, FoxP3. Having demonstrated that Treg cells are directly activated by FIV infection, upregulate immunoactive GARP:TGFβ complexes on their surface, and are thereby able to suppress activated Th cells through TGFβ-RII engagement on the Th surface, we ask whether the TGFβ-mediated signaling of Treg cells activated during in vivo infection is sufficient to convert these activated Th cells into induced suppressor cells. Here we provide evidence for Treg-mediated conversion of Th cells following FIV infection and demonstrate that converted cells display phenotypic and functional characteristics consistent with Treg suppressor cells. This discovery provides a key explanation for the maintenance of Treg cell numbers despite a decline in T cell counts during chronic FIV and further supports a model in which Treg conversion of Th cells contributes to lentivirus-associated immune dysfunction. These data suggest that modulation of Treg activity may be important for therapeutic treatment and lentivirus elimination.

2. BACKGROUND AND LITERATURE REVIEW

A. Feline Immunodeficiency Virus

Feline Immunodeficiency Virus or FIV was first isolated from a domestic cat (*felis catus*) by Pederson et al. in 1987 and described as a T-lymphotropic virus causing immune deficiency in the infected feline host (20). Species-specific strains of FIV have since been identified in cheetahs and African lions as well although pathogenesis appears restricted to the domestic cat (21). FIV exhibits homology to the human pathogen, Human Immunodeficiency Virus or HIV, and the physical as well as physiologic similarities have been extensively reviewed (22). HIV itself is the causative agent for Acquired Immune Deficiency Syndrome (AIDS) which is of global importance. World Health Organization statistics estimate that 34 million people are currently infected with HIV with annual deaths around 1.7 million (WHO 2012). Preliminary drug development and therapeutic research for the HIV pandemic is largely limited to translational research using animal models of lentiviral disease. This can be problematic as the only natural host of HIV is humans. Animal models include mice that are genetically altered to induce HIV susceptibility or simians infected with the related lentivirus, SIV (6, 23). The latter has some limitations as SIV pathogenesis varies widely between simian species and does not typically cause an AIDS-like disease in the natural host such as that observed following human HIV infection (24). Therefore, FIV represents a unique and appropriate model of natural infection for the study of HIV-AIDS and offers many benefits over the use of other lentivirus models including cost and accessibility of test subjects. Importantly, FIV infection in domestic cats results in AIDS immunopathogenesis

and can thereby be used as a model for long-term studies of disease progression as well as short-term vaccination studies.

I. Natural History of Infection

FIV infection can occur through horizontal transmission by sexual contact and bite wounds or through vertical transmission from queen to offspring (20, 25, 26). Disease progression then closely follows that established for HIV with three or four stages described (27, 28). The first stage, acute infection, is characterized by an initial $CD4^+$ T cell lymphopenia with the lymphocyte count nadir loosely associated with a peak in plasma- and cell-associated viremia (29). Experimental infections have demonstrated an early virus specific humoral immune response with FIV-specific antibodies detected as soon as two weeks post infection (PI) (30, 31). Conflicting reports make it unclear whether robust humoral responses are beneficial or detrimental as initial antibody detection has been correlated with a decline in viral load in some studies but with enhancement of disease progression in others (32, 33). Nevertheless, B cell dynamics and antibody production during FIV infection remain important subjects for successful vaccine development (34). Acute infection is also characterized by the expansion of virus-specific CTLs, leading to an increase in overall $CD8^+$ T cell numbers and an inverted CD4:CD8 ratio which typically persists for the duration of infection (35-38). Other clinical signs of acute infection include febrile episodes and lymph node enlargement which resolve by six months PI at which time the infection has progressed to the asymptomatic or chronic stage. This stage is often referred to as clinical latency as infected cats do not display overt indicators of disease despite maintaining a low, nearly

undetectable viremia (39). The precise span of time for asymptomatic infection widely differs between cats but is generally measured in years before culminating in the onset of an AIDS-like disease (38). An additional stage termed ARC (AIDS Related Complex) is frequently described as an intermediate stage between asymptomatic infection and AIDS onset that is characterized by the development of persistent respiratory infections, gingivitis and other nonfatal but recurrent infections by pathogens normally controlled by T cell responses in the immune competent host (40, 41). Most FIV related deaths occur during the feline AIDS (FAIDS) stage and are attributed to development of wasting disease, sepsis, and other opportunistic infections (41). Similar to HIV, some deaths have been documented prior to FAIDS as a result of B cell lymphoma (42).

II. Molecular Mechanisms of Infection

FIV-$NCSU_1$, the strain of FIV utilized in this lab, was isolated from a naturally infected cat brought into the NCSU veterinary hospital and has been characterized as broadly tropic for $CD4^+$ T cells, $CD8^+$ T cells and B cells in vivo (39). Unlike HIV, which recognizes the CD4 protein on T cell membranes as a primary receptor, $NCSU_1$ and other FIV strains utilize CD134 (OX40), a costimulatory molecule expressed on $CD4^+$ Th cells and $CD8^+$ CTLs following TCR ligation and on either quiescent or activated Treg cells (39, 43-45). Following the ligation of the FIV virion to CD134, a change in conformation of the FIV surface proteins exposes an important residue for recognition of the secondary receptor, CXCR4, which is necessary for initiation of membrane fusion events (46-49). As with CXCR4-tropic HIV strains, FIV-$NCSU_1$ produces syncytia of infected cells and is thus easily

visualized during in vitro assays (50). Our lab has previously demonstrated the productive infection of purified cell populations in vitro and shown that both $CD4^+$ Th and Treg cells support FIV infection (14). We have further demonstrated that $CD4^+CD25^+$ Treg cells are preferentially infected, support productive infection as measured by the release of infectious virions, and that this heightened susceptibility correlates with increased CXCR4 expression (13-15).

After CXCR4-mediated entry into the host cell, the FIV capsid uncoats in preparation for reverse transcription. Each FIV virion contains two positive-strand RNA copies of virus which are reverse transcribed into double stranded DNA by an RNA-dependent DNA polymerase that is also packaged within the virion. Other proteins important for this process that are of viral origin include the reverse transcriptase (RT) with intrinsic RNaseH activity, the tRNA primer and deoxyuridine triphosphatase (dUTPase). The newly transcribed double-stranded DNA is transported into the nucleus and incorporated into the host cell genome by the action of Integrase (IN), another protein of viral origin. The location of provirus insertion was initially assumed to be random in distribution but more recent reports have suggested that preferential integration occurs at specific sites within the host genome (51). One study in particular by Rohdewohld et al suggests that retroviral incorporation may occur in areas containing cistrons which are important regions for transcription factor recruitment and cis-acting gene regulation (52-54). Additional reports suggest that aspects such as degree of transcriptional activity (55), association with nucleosomes (56), or CpG methylation status (57) can influence integration efficiency. In addition, selection of specific

integration sites has been partially attributed to the intrinsic properties of the Integrase protein itself, making comparisons across retroviruses difficult as FIV Integrase may be unique to other retroviruses including HIV (58).

Following successful integration, viral genome transcription is carried out by normal cellular processes as the FIV LTR regions contain multiple TATA boxes and binding sites recognized by transcription factors such as AP-1, NFκB or C/EBP and by the cellular polymerase transcriptional complex (59). The FIV provirus genome consists of three main open reading frames (ORFs) encoding the major FIV components *gag, pol* and *env* which are present in the HIV proviral genome as well. Numerous accessory proteins are encoded within these ORFs and are reviewed in Elder et al 2008 (27). Major differences between HIV and FIV genes include the Vpr, Vpu and Nef accessory proteins that are present in HIV but not FIV. A complex system of splicing and transport of FIV gene products leads to the eventual accumulation of all necessary proteins for the formation of infectious virions in the cytoplasm of the host cell. Assembly occurs in association with the cell membrane and virion budding releases enveloped particles from the cell surface which begin the cycle again upon reaching a CXCR4-bearing target (60).

III. Immune Dysfunction in the FIV Host

Acute FIV infection provokes robust cell-mediated immune responses but despite this, FIV persists in the infected host and disease progression into the chronic phase is characterized by a wide-spread immune dysfunction (61). CD4 T cells that are activated during the initial

immune response become anergic as demonstrated by their diminished IL2 production and impaired proliferation upon antigenic restimulation in vitro. This progressive immune dysfunction can also be observed when FIV-specific CTLs that were effective early during infection begin to lose the ability to respond to viral antigens and later a general dysfunction in the total CTL population is observed by memory cell loss of response to other, non-FIV recall antigens (62, 63). Cytokine profiles of infected subjects have revealed that progression into chronic FIV and the development of immune dysfunction is marked by altered cytokine levels. These have been consistently characterized as an increase in IFN-γ, TNF-α and IL10 but a decrease in IL2 and IL12 (64-66). The gradual decline in CD4 T cell numbers over the course of FIV-infection has been well documented and correlates with the decline in IL2 (67, 68) but evidence suggests that it is the decline in T cell function rather than number that is a major factor in FIV/HIV pathogenesis (69-72). In support of this, our lab has previously demonstrated that FIV-infected cats lack the functional T cell responses necessary to control a nonpathogenic strain of *Toxoplasma gondii* during FIV co-infection as compared to noninfected controls and that this dysfunction occurs as soon as 16 weeks following infection, consistent with a model for FIV-mediated T cell anergy (71). A similar deficiency in T cell function was demonstrated by Dean et al. following co-infection with *Listeria monocytogenes* (73). Increases in cell anergy and apoptosis during chronic FIV infection have also been documented and further contribute to the state of dysfunction. We have demonstrated expansion of CTLA-4^{+}B7.1^{+} T cells during chronic FIV infection and hypothesized that signaling though this pathway by T-T cell interactions induces anergy (74). Taken together, these results suggest that the induction of anergy in T cell populations is

central to immune dysfunction and the chronic nature of FIV infection. While these observations describe the results of immune subversion, the question of how this occurs remains unanswered. A paradigm develops during FIV infection with the well characterized immune suppression occurring simultaneous to an immune hyperactivation within specific lymphocyte subsets. This activation has been documented by the development of polyclonal B cell responses as well as the replacement of the naïve T cell phenotype with activation phenotypes (75-77). Our lab and others have provided strong evidence that during the course of FIV or HIV infection, it is the activation of regulatory cell populations which contributes to the suppression of T cell populations and thereby leads to altered cytokine levels in the immunocompromised host. Central to this theory was the identification of activated Treg cells during FIV/HIV infection (9, 78, 79). While Treg activation is antigen-specific, the ensuing suppression occurs in a non-antigen-specific fashion. The activation of Treg cells during FIV infection therefore provides a mechanistic explanation for the global immune suppression observed during chronic stages of infection despite the emergence of activation phenotypes in T and B cell subsets and has been the focus of this lab for many years.

B. T Regulatory Cell Function

CD4 Treg cells are currently defined by constitutive expression of the high affinity IL2 receptor, CD25, and the transcription factor FoxP3 (80, 81). Additional markers have been proposed including the surface proteins CTLA-4 and GITR or the intranuclear transcription factor Helios (82, 83). Activated Treg cells function to suppress the activity of other immune cell subsets including $CD4^+$ Th cells and $CD8^+$ CTLs. Treg cells are traditionally described

as circulating cells of thymic origin with TCR receptors weakly responsive to self-antigen which function in immune surveillance to suppress autoreactive T and B cells (84). Since the initial discovery and characterization of Treg cells, researchers have described additional subpopulations of regulatory cells which arise during infection and are pathogen specific (16, 85-87). These pathogen-induced regulatory cells or adaptive Treg cells have been identified in Hepatitis B and C viruses, Herpes virus and HIV infections and evidence suggests that they are able to shape disease pathogenesis (16, 17, 78, 88, 89). While FIV-specificity within the Treg pool has yet to be determined, Vahlenkamp et al (12) first described Treg cells from chronic FIV-infected cats as being chronically activated in vivo and capable of suppressing activated $CD4^{+}CD25$- Th cells in vitro. These results suggest that FIV infection leads to the activation of a regulatory cell subset which then contributes to T cell dysfunction by induction of anergy in Th cells. The specific mode of suppression utilized for Th cell suppression, the mechanism by which these regulatory cells become activated and the potential for adaptive Treg cell development during FIV infection are the focus of this dissertation.

I. Mechanisms of Treg mediated suppression

While it is now widely accepted that a subset of CD4 T cells function to induce peripheral tolerance, the specific mechanisms of Treg-mediated suppression remain controversial. Mediation of dendritic cell presentation by CTLA-4 signaling, Granzyme/perforin-dependent killing of target cells, direct Th cell inhibition through IL10 and TGFβ cytokine signaling, direct Th inhibition through B7.1:CTLA-4 signaling, and IL2 deprivation of Th target cells

have all been proposed as methods of Treg-mediated suppression (74, 90-94). It is suggested that Treg cells can be divided into subpopulations with unique mechanisms of suppression but whether these subgroups represent terminally differentiated lymphocyte subsets or whether some plasticity is maintained in the Treg pool is yet to be determined. The most commonly accepted theory is that the method and strength of Treg cell activation may determine the subsequent mechanism of Treg-mediated suppression and that these suppression mechanisms are intrinsically redundant (95).

Despite the suggestion that multiple mechanisms of Treg function exist, there is substantial evidence that the majority of Treg cells function in a similar manner following activation in vivo. Early studies on immune tolerance in graft rejection have demonstrated that $CD4^{+}CD25^{+}$ Treg cells promote transplant tolerance as depletion of these cells leads to accelerated T cell mediated graft rejection (96). The Treg-specific modulation of these alloresponses are attributed to signaling via IL10, CTLA-4 and TGFβ (90, 97). Studies using a mouse model for autoimmune diabetes have demonstrated that activated Treg cells provide protection against disease development in Non-obese Diabetic (NOD) mice, consistent with the known role for Treg cell in maintaining tolerance (98, 99). Gregg et al (100) demonstrated that expanding Treg cells in young NOD mice had a protective effect against autoimmune diabetes development as measured by a significant delay in disease onset when compared to age-matched control mice. Conversely, expansion of Treg cells in older, insulitis-positive NOD mice did not result in protection when compared to age-matched controls. Further investigation into the difference between Treg cells induced in young

versus old NOD mice revealed that only the Treg cells expanded in the young NOD mice displayed significant levels of membrane-bound TGFβ (mTGFβ) and that blocking TGFβ subsequently abrogated Treg-specific suppression (100). TGFβ is a pleiotropic cytokine with roles in angiogenesis, cell growth, inflammation and apoptosis and although TGFβ is secreted in a latent, soluble form from various cell populations including $CD4^+$ Treg cells, Treg cells are unique in the ability to express a membrane bound form of TGFβ (100, 101). Distinguishing between the effects of soluble and membrane bound protein on target cells is potentially problematic but Nakamura et al. published definitive evidence that Treg cells expressing mTGFβ suppressed Th targets in a contact dependent manner by utilizing transwell assays and further demonstrated abrogation of suppression by blocking TGFβ (11, 102). These data support the hypothesis that mTGFβ is a primary mechanism for Treg-mediated suppression of Th target cells.

Having previously demonstrated that Treg cells are phenotypically and functionally activated in chronic and acute FIV infection (8, 9), our lab proposed that the Treg-mediated immune suppression during infection was dependent upon a TGFβ mechanism. After providing evidence of TGFβ-RII upregulation on Th and CTL target populations during infection, we demonstrated that freshly isolated $CD4^+CD25^+$ cells from FIV infected cats are able to suppress Th cells as measured by a reduction in activated Th cell IL2 production and proliferation following co-culture and that this suppression was abrogated by the addition of blocking antibodies to TGFβ (9, 19, 103). We also provided evidence that $mTGFβ^+$ Treg cells from FIV-infected cats suppress $CD8^+$ CTLs and that SMAD phosphorylation

associated with signaling through the TGFβ-RII was present in the target cell population, (7). These data strongly suggest that during both acute and chronic FIV infection, a population of $CD4^{+}CD25^{+}TGF\beta^{+}$ Treg cells are actively engaged in suppression of immune cells via a mTGFβ-dependent mechanism.

II. mTGFβ, GARP, and a Model for Suppression

TGFβ is a pleiotropic cytokine with roles ranging from cell migration and tumor metastasis to angiogenesis and embryogenesis (104-107). It is produced by many different cell types from lymphocytes to epithelial cells but while three isoforms have been described (TGFβ1, 2 and 3), the specific form important in T cell signaling is TGFβ1 and will be referred to simply as TGFβ for the remainder of this dissertation (91, 108). The production and secretion of TGFβ from T cells is a fairly complex process which begins with the synthesis of a 75 kDa homodimeric pro-TGFβ molecule. This molecule is proteolytically cleaved into two distinct but closely associated protein dimers in the golgi and the N-terminus becomes a 25 kDa mature TGFβ dimer while the C-terminus becomes the Latency Associated Peptide or LAP (109). Although distinct protein components, mature TGFβ and LAP are non-covalently associated into a single complex which then becomes disulfide linked to an additional protein component termed Latent TGFβ Binding Protein or LTBP and this entire complex is referred to as the Large Latent Complex or LLC (110, 111). At this stage, the LLC is directed from the golgi to the cell surface to be secreted into the extracellular milieu. Specific residues of the LTBP interact with components of the extracellular matrix to anchor the LLC outside of the cell (110). Cleavage or denaturation of LAP occurs in order to release

the active TGFβ dimer and this has been attributed to changes in pH or interactions with other proteins such as integrin αvβ6, thrombospondin (TSP-1) or protease (112-114). Although this secretory pathway has been ascribed to CD4 T cell production of TGFβ, the mTGFβ identified on the surface of Treg cells has been shown to be in complex with LAP but not LTBP, suggesting a mechanism by which the latent TGFβ complex is either picked up by the Treg cell from the extracellular matrix following its cleavage from LTBP or is diverted to a different pathway specific for membrane expression prior to LTBP association in the golgi (10, 112, 115). These hypotheses have been purely speculative and no evidence in support of either was available until the recent discovery of a protein called Glycoprotein A Repetitions Predominant or GARP.

GARP message was initially isolated and described in the early 1990's from the human chromosomal region 11q13-11q14 which was over expressed in a breast carcinoma cell line (116). Initial studies found that the encoded protein was prevalent during embryogenesis, particularly in the endothelium and during later stages of development in megakaryocytes of both humans and mice (117). It was not until 2008 that GARP expression was identified in mature Treg cells by the lab of D. Unatmaz (118). In this study, T cells were isolated from human donors, purified into Th or Treg subsets, and differential gene expression was determined by microarray analysis. This analysis revealed that the GARP gene product (LRRC32) was preferentially expressed in Treg cells and these findings were confirmed by PCR analysis as well (118). This group further demonstrated that GARP mRNA was upregulated after TCR activation of Treg cells. Retroviral transduction of GARP into non-

regulatory Th cell clones resulted in the induction of FoxP3 expression and the acquisition of suppressor function (118). As FoxP3 has been shown to be a key mediator of Treg cell phenotype and function, these studies suggest that GARP could function upstream of FoxP3 during induction of gene expression important for the Treg lineage (119). However, later studies demonstrated through siRNA targeting that silencing GARP expression in Treg cells only moderately decreased FoxP3 expression or suppressor capacity, suggesting that GARP may only be important for induction of FoxP3 and not for sustaining expression during later stages of activation (120).

The protein encoded by LRRC32 was then identified as being expressed on the surface of Treg cells and mutational analysis revealed that deletion of the cytoplasmic portion of the protein did not affect Treg phenotype or function but the transmembrane region and the extracellular region were crucial in this regard as depletion of either region abrogated Treg suppressor function (118). These data support an important role for this cell surface protein in Treg-mediated suppression beyond regulation of FoxP3 at the mRNA level. These findings were confirmed when GARP was shown to physically bind to the LAP portion of inactive TGFβ and co-immunoprecipitation techniques demonstrated all three of these molecules complexed on the surface of Treg cells (121). It is proposed that GARP plays a role in the expression of mTGFβ by shuttling the molecule to the cell surface prior to formation of the LLC and serves as an anchor, a suggestion which is supported by the fact that these molecules become complexed in a golgi dependent manner prior to surface expression and elimination of GARP by siRNA strategies completely prevents the surface

expression of TGFβ (121, 122). A proposed scheme for TGFβ, LAP and GARP association follows below (Figure 1). Taken together, these studies suggest GARP is a functional Treg surface marker which plays a direct role in the mechanism of Treg-mediated suppression through mTGFβ but also functions as a high order modulator of the Treg lineage through the induction of FoxP3.

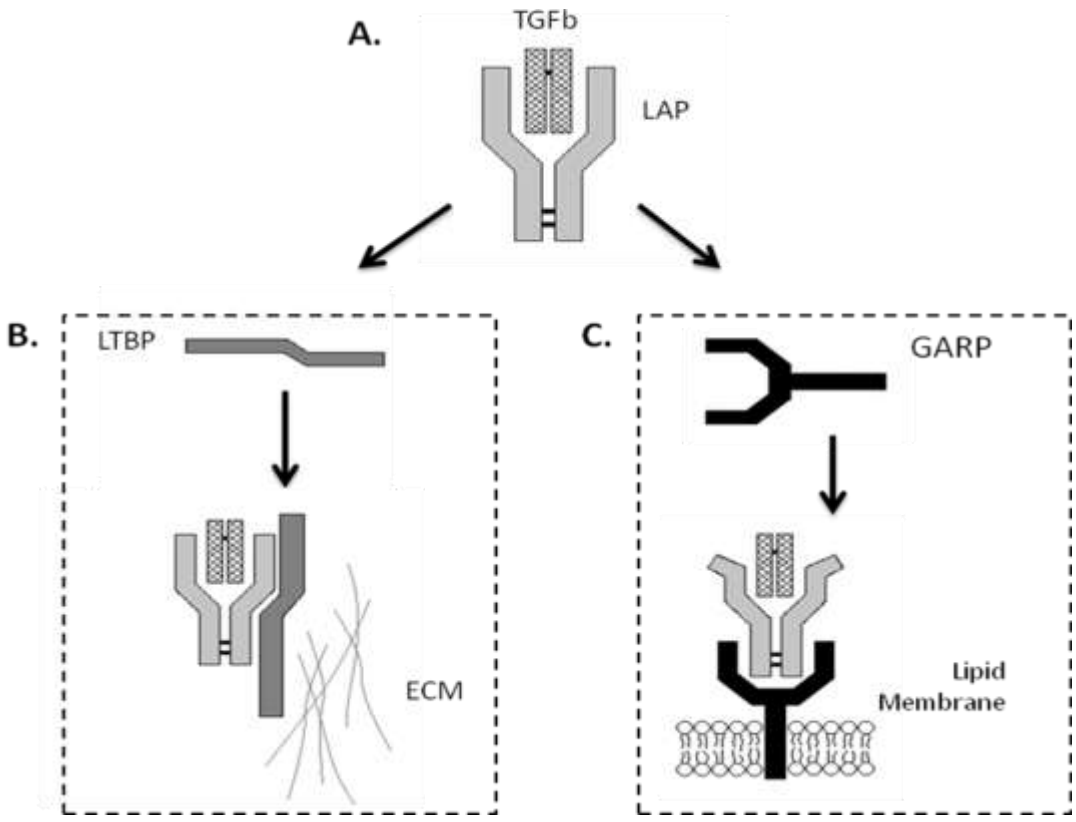

FIGURE 1. **Proposed Mechanism for GARP expression in Treg cells.** **A**. After the pro-TGFβ molecule is processed in the golgi, the mature TGFβ homodimer is found associated with Latency Associated Peptide (LAP). **B**. In most cell types, the TGFβ:LAP complex becomes associated with Latent TGFβ Binding Protein (LTBP) and is secreted into the extracellular matrix (ECM). **C**. In Treg cells, the GARP protein associates with the TGFβ:LAP complex and shuttles it to the surface of the cell where the complex becomes anchored to the outside of the cell membrane.

The activation status of GARP bound TGFβ remains uncertain. Activation of latent TGFβ in the extracellular milieu reportedly requires the physical constraints imposed by LTBP during the association of LAP with activating molecules such as αV integrins (112). As GARP replaces this interaction for surface bound TGFβ, GARP may serve a similar role to LTBP in facilitating LAP cleavage while ensuring that TGFβ signaling occurs proximal to the Treg cell surface by its dual role as a membrane anchor. In support of this, Stockis et al. (121) first demonstrated that GARP would only associate with LAP-bound TGFβ and not mature TGFβ alone. They further demonstrated that GARP transfected T cell clones did not engage in TGFβ signaling in vitro even following TCR stimulation (121). While these studies convincingly show that GARP bound TGFβ is in a latent form immediately following surface expression, they do not preclude activation following association with other cell types as these studies utilized homogenous populations of $GARP^+$ cells. From these data we can only reliably conclude that GARP itself is insufficient to activate LAP:TGFβ upon association. More recent reports have demonstrated that αvβ6 integrins expressed on the surface of transfected 293 cells are efficient activators of GARP bound TGFβ, providing the first direct evidence of Treg expressed mTGFβ activation during cell to cell contact (123). From this we can speculate that other proteins expressed on either Th target cells or APCs are capable of GARP-bound TGFβ activation as well. Such a phenomenon suggests that antigen specific contact between Treg cells and APCs bearing the activating protein results in proximal signaling via active TGFβ on the Treg cell and suppression of other antigen-specific cells in close proximity. A more detailed analysis of proteins capable of activating mTGFβ in this

manner and the subsequent evaluation of cell expression patterns of these activating molecules will be necessary to address these hypotheses.

III. Induced Treg Cells and GARP

The heterogenous nature of the regulatory T cell population has become widely accepted and it is known that at least two major subpopulations exist (87, 99, 124). The first are the natural Treg (nTreg) cells that are specific for self antigen and originate in the thymus. The second are referred to as either adaptive Treg (aTreg) cells when derived in vivo or induced Treg (iTreg) cells when derived in vitro. Whether aTreg cells represent a population of Treg lineage cells which require further differentiation after exit from the thymus or whether they originate from the conversion of antigen-specific Th cells remains uncertain (87). In vitro stimulation of activated Th cells with soluble TGFβ (sTGFβ) has been shown to facilitate the conversion of these cells into phenotypic and functional iTreg cells (125-129). The concentration of TGFβ required for this conversion process is artificial and some researchers would argue that these conditions are not physiologically relevant. However, researchers do agree that nTreg, a Treg and iTreg are alike in both phenotype and function.

Wang et al. (120) reported that TGFβ-induced Treg cells do not express surface GARP and that during HIV infection, there was no detectable expansion in $GARP^+$ cells, implying that activation and expansion of Treg cells during lentiviral infection does not take place within the natural Treg compartment. However, we have found that FIV infection is characterized by activation and expansion of Treg cells which express GARP:TGFβ (Appendix I). This

raises the question, do feline adaptive Treg cells express GARP? Here we identify that GARP is expressed on the surface of feline Th cells that have been converted into iTreg cells after ConA and sTGFβ treatment following our previously reported conversion protocol and we hypothesize that activation of $GARP^+$ Treg cells during FIV infection may facilitate TGFβ-induced Th conversion via mTGFβ:TGFβ-RII signaling. While Treg cell produced TGFβ-mediated conversion of Th cells has been previously reported, these studies have focused on secreted TGFβ from in vitro activated Treg cells. Here we have developed a novel system for evaluating Th conversion by in vivo activated Treg cells and demonstrate the potential for in vivo conversion during FIV-lentiviral infection for the first time. Conversion of activated Th cells into iTreg cells by Treg-mediated signaling provides a plausible explanation for the maintenance of Treg cell numbers during FIV infection (13). The continued maintenance of the Treg cell pool at the expense of Th depletion would exacerbate T cell decline and contribute to AIDS-pathogenesis.

C. Significance

Treg activation contributes to the chronic state of immune dysfunction in the FIV-infected host. We have previously demonstrated that these activated Treg cells are able to suppress $CD4^+$ Th and $CD8^+$ CTL responses in a mTGFβ-dependent manner (7, 19). These results are supported by findings in murine and human models for autoimmune disease which demonstrate the critical role TGFβ plays in Treg cell function (100, 130, 131). The characterization of GARP provides a mechanistic explanation for Treg specific expression of membrane bound TGFβ and subsequent immunosuppression of TGFβ-RII-bearing target

cells. Treg cell therapies could be developed by either administering immunosuppressive $GARP^+$ cells or, conversely, by interfering with GARP-dependent suppression on endogenous Treg cells. The ability to modulate Treg function is not the only use for this protein, however, as tracking the expression dynamics of GARP during various infection states allows for a more detailed understanding of Treg activation and tissue distribution during pathogenesis. Here, GARP has provided a means for understanding the activation of Treg cells during FIV and for effectively isolating suppressor cells without risk of including activated Th cells. $GARP^+$ Treg cells during FIV infection have been shown to contribute to $CD4^+$ T helper anergy and the results of these studies have implicated $GARP^+$ Treg cells as important immune modulators during disease pathogenesis. These results suggest that manipulation of this Treg population could provide the key to effective disease management and treatment in the future.

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3. Feline Glycoprotein A Repetitions Predominant (GARP) Anchors TGFβ on the Surface of Activated $CD4^+CD25^+$ Regulatory T Cells and Mediates AIDS Lentivirus-Induced T Cell Immunodeficiency

This chapter has been accepted for publication in AIDS Research and Human Retroviruses (1).

A. Abstract

Using the FIV model for AIDS-lentivirus infection, our laboratory has previously demonstrated that T regulatory (Treg) cell-mediated immune T and B cell dysfunction contributes to lentivirus persistence and chronic disease through membrane bound TGFβ (mTGFβ). Studying Treg cells in the context of infection has been problematic as no inducible marker for activated Treg cells had been identified. However, recent reports in human Treg studies have described a novel protein, Glycoprotein A Repetitions Predominant (GARP), as a unique marker of activated human Treg cells that anchors mTGFβ. Herein we extend these studies to the feline Treg system, identifying feline GARP and demonstrating that human and feline GARP proteins are homologous in structure, expression pattern and ability to form a complex with TGFβ. We further demonstrate that GARP and TGFβ form a complex on the surface of activated Treg cells and that these $GARP^+TGF\beta^+$ Treg cells are highly efficient suppressor cells. Analysis of expression of this Treg activation marker in the FIV-AIDS model reveals an up-regulation of GARP expressing Treg cells during chronic FIV infection. We demonstrate that the $GARP^+$ Treg cells from FIV-infected cats suppress T helper cells in vivo and that blocking GARP or TGFβ eliminates this suppression. These

data suggest that GARP is expressed in complex with TGFβ on the surface of activated Treg cells and plays an important role in TGFβ^{+} Treg-mediated T cell immune suppression during lentivirus infection.

B. Introduction

CD4^{+} regulatory T cells (Treg cells), currently defined by constitutive expression of the high affinity IL2 receptor CD25 and the transcription factor FoxP3, play an important role in controlling autoimmune disease (2, 3). Treg cells also shape the pathogenesis of viral infections by controlling inflammation from excessive activation of T and B effector subsets (4-8). The study of Treg population dynamics and function has thus become important for an array of diseases. However, their purification has been problematic as there has been no marker unique to Treg cells. Historically, the transcription factor FoxP3 has been used as a ubiquitous marker for CD4^{+}CD25^{+} Treg populations, but the transient expression of FoxP3 has since been demonstrated in non-regulatory, activated T helper cells in both the human and feline immune systems, limiting the use of this marker in identifying pure populations of Treg cells (9, 10) (Tompkins unpublished). Additionally, FoxP3 and CD25 are considered constitutive markers of Treg cells and cannot be used to evaluate activation status. The novel protein GARP or Glycoprotein A Repetitions Predominant (LRRC32), has only recently been described as a unique activation marker of human Treg cells and has been shown to correlate with suppressor function (11-13). This surface marker thus presents a method for isolating pure Treg populations and for evaluating activation status.

Importantly, human GARP has been shown to bind TGFβ within the Treg cell before being targeted for membrane expression (12, 14). The GARP:TGFβ complex is then displayed on the Treg cell surface with GARP anchoring the complex via its transmembrane region, leaving the majority of the protein exposed on the extracellular surface with TGFβ (11, 12, 14). Human GARP (hGARP) has a short cytoplasmic tail devoid of overt signaling residues, indicating that the dominant role for this protein is to display surface TGFβ (11, 15). While numerous mechanisms for Treg cell-mediated suppression have been proposed, studies on murine, human and feline Treg cells have identified TGFβ signaling to be important (6, 16-19). In the case of autoimmune disease, it has been reported that membrane bound TGFβ (mTGFβ) mediates T cell suppression by ligation of the TGFβ receptor (TGFβ-RII) expressed on the surface of activated target Th cells (17-20). We have demonstrated that engagement of the TGFβ-RII on target cells activates the SMAD pathway (6), which may in turn induce the expression of FoxP3, a transcription repressor of IL2. Using the well established feline immunodeficiency virus (FIV) model for HIV infection, we have demonstrated an important role for mTGFβ in Treg cell-mediated suppression of $CD4^+CD25^-$ Th cells in a contact-dependent manner (6, 19). This suppression can be abrogated by the addition of blocking antibodies to TGFβ on the Treg cell or TGFβ-RII on the target cell (19), providing evidence that AIDS lentiviruses may induce T cell immunodeficiency by activating $mTGF\beta^+$ immunosuppressive Treg cells. When taken together, these studies suggest that GARP is not only a marker of activated Treg cells but, by anchoring TGFβ on the cell surface, represents an important component of Treg cell-mediated immune suppression.

Here, we are the first to identify GARP in the *Felis catus* genome and evaluate expression of this protein on feline Treg cells. We isolate and sequence feline GARP (fGARP) mRNA and identify expression of two fGARP protein isoforms in Treg cells. We extend our findings for mTGFβ expression on the surfaces of feline $CD4^+CD25^+$ T cells in association with fGARP by surface phenotyping of this novel protein and show that feline Treg but not Th cells display fGARP-bound TGFβ complexes on their surfaces. We also demonstrate that the fGARP-TGFβ complex on Treg cells is capable of mediating suppression of Th cells and report a significantly higher level of fGARP:TGFβ complex expression on the Treg cell surface from chronic FIV-infected cats when compared to noninfected cats. We further demonstrate that $GARP^+$ Treg cells isolated from FIV-infected cats are functionally suppressors of Th cells in vitro and that this suppression is abrogated by the addition of GARP or TGFβ blocking antibodies. These data suggest that the GARP-TGFβ complex plays a role in FIV-induced immunodeficiency. We propose that $GARP^+TGF\beta^+$ Treg cells represent a dedicated regulatory population activated in response to lentivirus infection, mediating suppression and contributing to the immune dysfunction in AIDS lentivirus infections.

C. Materials and Methods

Cats and in vivo FIV infection

Specific pathogen-free (SPF) cats were obtained from Liberty Research, Inc. (Waverly, NY) at 6 months of age, and housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Protocols were approved by the North

Carolina State University Institutional Animal Care and Use Committee. FIV^+ cats had been previously inoculated with the $NCSU_1$ isolate of FIV (21) and FIV-infected cats were housed separately from FIV negative cats. All chronic cats had been infected in excess of two years at the time of this study.

Blood and lymph node cell collection

Whole blood was collected by jugular venipuncture into EDTA container tubes. Where indicated, peripheral blood mononuclear cells (PBMC) were isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient as previously described (22). Peripheral lymph node (PLN) cells were obtained by lymph node biopsy and single cell suspensions were prepared by repeatedly injecting sterile PBS into the tissue with an 18 gauge needle. After isolation, cell counts and viability were obtained using Trypan Blue dye exclusion. Cell viability was >90% in all experiments.

Real time PCR

FACS purified populations of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells from PLN and peripheral blood were used for quantification of GARP, FoxP3 and GAPDH mRNA by reverse transcription and real-time PCR. Total RNA extraction was carried out using Qiagen's RNeasy plus mini kits (Qiagen, Valencia, CA). RNA was used in reverse transcription reactions using Oligo(dT) primers and the Promega Reverse transcription system according to the manufacturer's instructions (Promega, Madison, WI). Products were assessed in triplicate for the specific mRNA of interest. Development and use of FoxP3 and GAPDH

primers has been described elsewhere (19). fGARP primers were designed to amplify a 155 base pair product within the 1431 base pair (bp) open reading frame (ORF) sequence (f 5'-GCCGGAACTCCAGCACCCA-3', r 5'- CCGCACCTCAAAGGCTCGCA-3'). fGARP RT-PCR amplification product was separated on a 1% agarose gel and analyzed with the VersaDoc Imaging System (BioRad, Hercules, CA) to confirm uniform amplification of a single product. The SYBR Green Taqman PCR master mix (Applied Biosystems, Life Technologies, New York) was used for quantification according to manufacturer's guidelines. All three primer sets were optimized to an annealing temperature of 61°C and reactions carried out in an ICycler PCR machine. Quantification was performed with the delta delta CT method for fold change in expression or the delta CT method for relative expression with GAPDH as the housekeeping gene.

Amplification and cloning of GARP ORFs

For sequencing analysis of fGARP, primers were designed to span the predicted 1431 (f 5'-CCACAGCAACGTGCTCATGG-3', r 5'- GGCCGGGCCGTTCTACAGTC-3') or 1851 bp (f 5'-GTCCCCTCAATGCTCCCGCT-3', r 5'- GGCCGGGCCGTTCTACAGTC -3') ORF. cDNA was synthesized from feline $CD4^+CD25^+$ T cells using the Promega Reverse Transcription System with an Oligo(dT) Primer (Promega, Madison, WI). PCR amplification of the 1431 or 1851 bp ORF product was performed using the HotStar HiFidelity PCR Kit (Qiagen, Valencia, CA) on a Mastercycler Pro thermocycler (Eppendorf, Hauppauge, NY) programmed with the following cycling conditions: initial denaturation/hot start at 95°C for 15 min; 40 cycles with denaturation at 95°C for 15s, annealing at 60.4°C for

45s and extension at 72°C for 2 min; final elongation step at 72°C for 10 min. The 1431 bp product was analyzed by electrophoresis on a 1.0% Agarose gel; the appropriate weight band was visualized with GelRed (Biotium, Hayward CA), then excised and purified using the QIAquick Gel Extraction Kit following the supplied manufacturer's protocol. TA Cloning and ligation was performed using pGEM T Easy Vector system (Promega, Maison WI) and chemically competent *E.coli* (GC10; Genesee Scientific, San Diego, CA) were transformed with recombinant plasmids. Samples were sent to Eurofins MWG Operon for sequencing. Results were analyzed using Geneious Pro software (23).

Flow cytometry analysis

At least $5x10^5$ PBMC or PLN cells were stained for surface expression of various markers using specific antibodies. Mouse anti-feline CD25 (mAb 9F23) was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan) and conjugated to fluorescein isothiocyanate (FITC). Mouse anti-feline CD4 (mAb 30A) and CD8 (mAb 3.357) were developed in our laboratory (24). Anti-TGFβ (MAB240) was purchased from R&D systems and conjugated to allophycocyanin (APC). Phycoerythrin (PE)-conjugated anti-TGFβ-RII (FAB241P) was purchased from R&D. Unconjugated and FITC-conjugated anti-GARP IgG2b monoclonal antibodies (LRRC32, Plato-1) were purchased from Enzo life Sciences (Ann Arbor, MI). Isotype specific, PE-conjugated anti-murine IgG2b was purchased from Jackson ImmunoResearch Laboratories. Three or four color flow cytometry was performed on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA). Each antibody combination was analyzed in duplicate and lymphocytes were gated based on forward versus

side scatter. Gated events were analyzed using CellQuest software and gates determined by isotype controls.

Western Blot and Co-Immunoprecipitation

PLN cells were collected from FIV negative cats, and $CD4^+CD25^+$ cells were FACS sorted using anti-CD4 and anti-CD25 antibodies. $10\text{-}15x10^6$ sorted cells were used for preparation of sample lysate. Gels were transferred to PVDF membranes and analyzed using either anti-GARP or anti-TGFβ followed by HRP-conjugated goat anti-rabbit IgG and detected by chemiluminescence. Where indicated, an IgG1 specific HRP-conjugated rabbit anti-mouse IgG was used to detect TGFβ as anti-GARP was IgG2b. The Pierce Co-Immunoprecipitation (Co-IP) Kit was used for isolation of GARP from $CD4^+CD25^+$ cell lysate following the supplied protocol. Negative controls consisted of a column with inactive resin or with activated resin bound to anti-GARP but PBS in place of sample lysate.

Cell culture and stimulation

Unfractionated PBMCs, PLN cells or various purified T cell subsets were cultured in RPMI medium containing 10% heat inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine and 1 mM HEPES buffer. For suppression assays, cells were maintained in AIM V Serum-Free Medium (Invitrogen Life Technologies, New York) to eliminate potential TGFβ contamination from FBS. All cells were cultured at $2x10^6$ total cells per mL of culture media. For ConA stimulation, 5ug/mL ConA was added to the media for the indicated length of time. For LPS/IL2 stimulation,

cells were cultured with 10 ug/mL LPS for two days, washed and cultured for two more days in fresh media supplemented with 100 U/mL recombinant human IL2 (rhIL2) (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD).

T regulatory cell suppression assay

Purified $CD4^+CD25^+$ cells from FIV negative cat PLNs were stimulated with LPS/rhIL2 for four days then FACS sorted into $CD4^+CD25^+$ or $CD4^+CD25^+GARP^-$ effector populations. In the case of FIV-infected cats, freshly isolated $CD4^+CD25^+$ and $CD4^+CD25^+GARP^-$ cells, in the absence of in vitro stimulation, were used as effectors. For initial suppression assays utilizing FIV negative $CD4^+CD25^+$ cells stimulated for four days as effector cells (Figure 4), PBMCs were collected from the same cats on day four and FACS sorted into Antigen Presenting Cells (APCs) by depletion of CD4 and CD8 expressing cells or $CD4^+CD25^-$ target cells. For all studies utilizing $CD4^+CD25^+$ cells from chronically FIV-infected cats (Figures 6 and 7), PLNs were used as a source of $CD4^+CD25^-$ target cells and APCs as well. The sorted cells were combined at a 1:1 APC to target cell ratio and stimulated with 5ug/mL of ConA for one hour, washed then cultured in fresh media. Activated $CD4^+CD25^+$ or $CD4^+CD25^+GARP^-$ effector cells were then added to the cultures to give a final effector:target:APC ratio of 1:2:2. Controls consisted of ConA stimulated $CD4^+CD25^-$ cells plus APCs without effector cells or unstimulated $CD4^+CD25^-$ cells as effector cells. After 24 hours, 100uL of the supernatant from each well was analyzed in triplicate by IL2 ELISA using the Feline IL2 DuoSet DY1890 (R&D Systems, Minneapolis, MN) as per manufacturer's protocol. In blocking studies, unconjugated anti-GARP (Plato-1, Enzo Life

Science, Ann Arbor, MI), neutralizing anti-TGFβ-RII (AF-241-NA, R&D Systems, Minneapolis, MN) and/or anti-TGFβ (MAB240 R&D Systems, Minneapolis, MN) were added at 100 ug/mL to the effector cells 30 minutes prior to addition of APCs and target cells to the co-culture. For analysis of proliferation, $CD4^+CD25^-$ target cells were labeled with the CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Invitrogen Life Technologies, New York) according to the manufacturer's instructions and then co-cultured as described for 72 hours prior to analysis. Proliferation Index (PI) was determined using ModFit LT Software (Verity Software House) and the percent suppression was calculated as follows: [[(PI stimulated $CD4^+CD25^-$ alone) – (PI with effector cells)] / (PI stimulated $CD4^+CD25^-$ alone)] x 100.

Statistical Analysis

The Mann-Whitney *U* test (*t* test for nonparametric data) was used for pair wise comparison of parameters (e.g. surface molecule expression). Differences were considered to be significant at $p<0.05$.

D. Results

Sequence and predicted structure of fGARP in $CD4^+CD25^+$ lymphocytes.

The human GARP (hGARP) gene resides at chromosome locus 11q14 and encodes a transmembrane protein (25). Full length mRNA sequence for hGARP (NCBI Reference Sequence: NM_005512.2) was obtained and a blast against the whole genome shotgun

(WGS) sequence for *Felis catus* revealed a region with 87% nucleotide (nt) and 85% amino acid (aa) sequence homology (Accession number ACBE01342003.1). Using the GARFIELD Genome Browser (26) this region was mapped to feline chromosome D1. The human genes adjacent to hGARP (LRRC32) on chromosome 11 are displayed in Figure 1A. Homologous regions in the feline genome also mapped to feline chromosome D1, supporting the identification of fGARP at this location (Figure 1A).

The identified fGARP region encodes two potential open reading frames (ORFs) of 1431 and 1851 bp for which primers were designed. RT-PCR was performed on RNA isolated from FACS sorted $CD4^+CD25^+$ or $CD4^+CD25^-$ lymphocytes from FIV negative cats. Both ORFs were successfully amplified from $CD4^+CD25^+$ cells, but neither product was amplified from $CD4^+CD25^-$ lymphocytes, consistent with the known restriction of hGARP gene expression to Treg cells (Figure 1B). The 1431 bp product was then cloned and sequenced (GenBank Accession number JX297379). In Figure 1C, the corresponding amino acid translation is aligned with the hGARP protein sequence and identical residues highlighted. As predicted, the two proteins are homologous with fGARP representing a truncated version of hGARP. The transmembrane domain resides at the C-terminus and is underlined in Figure 1C. The differences between fGARP and hGARP are at the N-terminus.

To create an *in silico* model for the fGARP protein, the aa translation of the 1431 bp ORF was submitted to the Swiss-Model server (27) using the solved crystal structure of human TLR-3 ectodomain, 2AOZ chain A, as has been done previously for hGARP (11). The

protein models differ in overall length and density. Specifically, two looped regions, indicated by asterisks in Figure 1D, are more disordered in the fGARP model than in the hGARP model and may highlight functional differences between these two proteins.

TGFβ is found in complex with GARP on feline lymphocytes

hGARP:TGFβ complexes localize to the surface of Treg cells after intracellular association (14, 28). To confirm surface expression of fGARP:TGFβ, PBMCs were isolated from FIV negative cats and analyzed by flow cytometry for GARP and TGFβ expression. We observed however, that dual staining for fGARP and TGFβ resulted in a decrease in percentage of positive cells for each protein compared to single color analysis, suggesting the close association of these molecules results in steric hindrance (data not shown). Therefore, percentages of gated $CD4^+CD25^+$ cells stained for fGARP or TGFβ were compared. As shown in Figure 2A, the $CD4^+CD25^+$ population expressed comparable numbers of $fGARP^+$ and $mTGF\beta^+$ cells while $CD4^+CD25^-$ cells were negative. Dual staining with fGARP and FoxP3 antibodies confirmed that $fGARP^+$ cells also expressed FoxP3, supporting the classification of these cells as Treg cells (Fig. 2B).

As hGARP has been found in close association with and thought to provide an anchor for TGFβ on the surface of human Treg cells (12, 14) and demonstration of dual expression by flow cytometry was problematic, the association of these two proteins in feline cells was further analyzed by immunoprecipitation (IP). Western blot analysis of whole cell lysates from purified feline $CD4^+CD25^+$ cells confirmed the presence of fGARP and TGFβ proteins

(Fig. 2C). Interestingly, two bands were identified for fGARP at approximately 70 kDa and 55 kDa. Previous hGARP publications have described this protein as 80 kDa in size (25) but two hGARP isoforms of 552 aa and 662 aa are available through GenBank (accession numbers AAH52210.2 and AAH70079.1), consistent with our discovery of two molecular weights for fGARP. TGFβ was detected at 25kDa, consistent with the mature form of this protein (29). Anti-GARP was used to IP lysate from purified $CD4^+$ T cells for analysis of TGFβ. As shown in Figure 2D, the fGARP protein immunoprecipitated from $CD4^+$ cells was complexed with mature TGFβ, confirming the association of these two proteins in vivo.

Surface staining identifies upregulation of fGARP:TGFβ on activated $CD4^+CD25^+FoxP3^+$ Treg cells

hGARP:TGFβ is reported to be up-regulated on the surface of Treg cells following activation (14, 28). As we and others have demonstrated phenotypic and functional activation of naïve Treg cells by LPS/IL2 stimulation (30, 31) , FACS purified $CD4^+CD25^+$ cells from FIV negative cats and activated by LPS/rhIL2 were analyzed by flow cytometry for expression of fGARP, FoxP3 and TGFβ. A significant increase in the percentage of $fGARP^+$ cells, as well as mean fluorescence intensity (MFI) was observed in the stimulated group (Fig. 3A and 3B). The percentage of $FoxP3^+$ cells was not significantly altered following $CD4^+CD25^+$ activation, but expression level of this protein as measured by MFI was increased following stimulation in cells that were constitutively $FoxP3^+$ (Fig. 3C and 3D). mTGFβ was also expressed on a significantly larger proportion of stimulated $CD4^+CD25^+$ cells over control cells (Fig. 3E). fGARP and FoxP3 mRNA levels were also up-regulated after stimulation

(Fig. 3F). These data demonstrate that activation of feline $CD4^+CD25^+$ Treg cells results in up-regulation of surface fGARP and TGFβ, as well as intracellular FoxP3 message and protein.

fGARP$^+$CD4$^+$CD25$^+$ Treg cells are potent suppressors of CD4$^+$CD25$^-$ Th cells

As we have previously demonstrated an important role for mTGFβ in Treg cell-mediated suppression (6) , demonstration of fGARP-anchored mTGFβ on activated Treg cells by Co-IP (Fig. 2D) is consistent with a TGFβ-mediated suppressor function. To demonstrate the suppressor activity of these cells, IL2 production from ConA-stimulated $CD4^+CD25^-$ Th cells was measured by ELISA following co-culture with LPS/rhIL2-activated $CD4^+CD25^+$ Treg cells or activated $CD4^+CD25^+$ Treg cells depleted of fGARP expressing cells. As shown in Figure 4, ConA-stimulated $CD4^+CD25^-$ cells produced high levels of IL2, whereas non-stimulated $CD4^+CD25^-$ cells produced little IL2. In contrast, co-culture with LPS/rhIL2 stimulated Treg cells decreased IL2 production, confirming the suppressor function of these Treg cells. Depletion of $fGARP^+$ cells from the activated Treg cell population resulted in an increase in IL2 production (Fig. 4), demonstrating that $fGARP^+$ cells function as suppressor cells. These data provide evidence that the fGARP:TGFβ complex is a contributing factor to Treg-mediated suppression of $CD4^+CD25^-$ T helper cells and further indicate that $fGARP^+$ Treg cells represent a highly efficient Treg suppressor subset.

fGARP mRNA levels and surface fGARP:TGFβ expression on Treg cells is elevated during chronic FIV infection

We have reported that immunosuppressive Treg cells are chronically activated during long-term FIV infection (31). As shown in Figure 3, in vitro activation of feline $CD4^+CD25^+$ T cells by LPS/rhIL2 resulted in the up-regulation of surface fGARP and TGFβ. To determine whether in vivo activation of immunosuppressive Treg cells by FIV infection correlated with the up-regulation of fGARP:mTGFβ expression, mRNA was extracted from freshly isolated $CD4^+CD25^+$ or $CD4^+CD25^-$ lymphocytes from either FIV negative or chronically FIV-infected cats and mRNA levels analyzed by real time RT-PCR. As expected, the $CD4^+CD25^+$ population from chronically FIV infected cats expressed increased fGARP mRNA as compared to FIV negative cats (Fig. 5A). PBMCs from chronically infected cats were then analyzed for surface fGARP and TGFβ expression by flow cytometry. As shown in Figure 5B, $CD4^+CD25^+$ cells from FIV-infected cats had significantly higher fGARP expression as compared to FIV negative PBMCs. fGARP was not detected on the surface of $CD4^+CD25^-$ cells regardless of infection status, further supporting data that fGARP is a Treg cell specific protein. Similar to fGARP expression, TGFβ expression was higher on the $CD4^+CD25^+$ cells than on $CD4^+CD25^-$ cells. As the contact-dependent suppression by $fGARP^+$ Treg cells is mediated by ligation to TGFβ receptor (TGFβ-RII) on the surface of target cells, TGFβ-RII expression on freshly isolated $CD4^+CD25^-$ targets was evaluated. The percentage of $CD4^+CD25^-$ cells expressing TGFβ-RII is significantly greater in FIV-infected cats than FIV negative cats (Fig. 5C).

$CD4^+CD25^+GARP^+$ Treg cells from chronic FIV-infected cats are capable of suppressing $CD4^+CD25^-$ Th cells

To confirm the activation of $CD4^+CD25^+$ Treg cells during chronic FIV infection and to further evaluate the contribution of $fGARP^+$ Treg cells to $CD4^+CD25^-$ Th suppression, a suppression assay was performed utilizing PLN cells from chronically FIV-infected cats. Purified $CD4^+CD25^-$ target cells and APCs were stimulated for one hour with ConA, washed and freshly isolated, purified $CD4^+CD25^+$ cells were added to the culture. After 24 hours of co-culture, supernatant was collected and assayed for IL2 by ELISA. As shown in Figure 6, $CD4^+CD25^+$ Treg cells from chronically FIV-infected cats suppressed IL2 production by Th cells. Additionally, depletion of $fGARP^+$ cells from the effector pool resulted in an increase in Th cell IL2 production (Fig. 6).

To further confirm that the presence of GARP:TGFβ complexes on these effector cells was responsible for the suppression, PLN cells from chronically FIV-infected cats were used in a blocking assay. Briefly, FACS purified $CD4^+CD25^+$ cells were cultured for 30 minutes with blocking antibodies for GARP, blocking antibodies for TGFβ and TGFβ-RII, or with a combination of all three antibodies. APCs and CFSE labeled $CD4^+CD25^-$ target cells were then added to these cultures. After 24 hours, supernatants were analyzed by IL2 ELISA and, as shown in figure 7A, the addition of anti-GARP antibodies to these cultures abrogated the suppressor capacity of the $CD4^+CD25^+$ cells as measured by Th cell IL2 production. Addition of TGFβ/TGFβ-RII blocking antibodies reduced the suppressor capacity to a similar degree (Fig. 7A), and treatment with all three blocking antibodies also had this effect,

consistent with our model for the GARP:TGFβ complex mediating suppression of Th cells. After 72 hours in co-culture, the CFSE labeled target cells were analyzed for proliferation and percent suppression was determined (Fig. 7B.) As shown in figure 7B, the IL2 suppression measured in figure 7A was correlated with a decrease in proliferation when $CD4^+CD25^-$ target cells were co-cultured with activated $CD4^+CD25^+$ effectors but not when they were co-cultured with nonsuppressive $CD4^+CD25^-$ Th cells. Importantly, the addition of GARP or TGFβ blocking antibodies to the $CD4^+CD25^+$ effector cells prior to co-culture resulted in a decrease in percent suppression (Fig. 7B). These data support the hypothesis that chronic lentivirus infection induces $GARP^+mTGF\beta^+$ activated Treg cells that suppress Th cells and mediate lentivirus-induced immune dysfunction in a TGFβ-dependent manner.

E. Discussion

The Glycoprotein A Repetitions Predominant (GARP) gene product, first described in human breast carcinoma cells (25), was subsequently shown to be expressed in lymphocytes but restricted to CD4+CD25+ Treg cells activated through the TCR (32). As membrane associated TGFβ (mTGFβ) has been implicated in Treg-mediated immune suppression (6, 17, 19), Stockis et al. (12) examined the possible relationship between GARP and mTGFβ and reported that latent TGFβ was bound to GARP on the surface of Treg cell clones. While a number of studies have documented the role of activated $FoxP3^+$ Treg cells in mediating T cell suppression and immune deficiency in AIDS lentivirus infections (7, 33, 34) , the role of mTGFβ, and in particular the GARP:TGFβ complex, is largely unknown. We have reported

that mTGFβ may be an important mediator of Treg-induced T cell immune suppression in AIDS lentivirus infections (6, 19) . Here, we are the first to identify GARP in the feline species and its role in Treg cell-mediated suppression in FIV infection. Moreover, we present evidence that fGARP complexes with mTGFβ on the surface of Treg cells and may play a major role in TGFβ-mediated T cell immune suppression.

Two fGARP ORFs were identified and mRNA corresponding to the two different length fGARP transcripts was amplified from feline $CD4^+CD25^+$ T cells but not from $CD4^+CD25^-$ Th cells. The 1651 bp fGARP mRNA product differs from the 1431 bp fGARP product only at the three prime end, as these ORFs are within the same frame and differ only in the location of the start codon. This additional region of the mRNA when translated represents the N terminus or extracellular tail of the fGARP protein. Translating the 1431 bp region and modeling this amino acid sequence to human TLR-3 reveals that fGARP may retain the ability to fold into the same horseshoe-shaped tertiary structure proposed for hGARP (11) . Two isoforms of fGARP were identified by Western blot analysis, consistent with the two hGARP proteins previously identified and submitted to GenBank (accession numbers AAH52210.2 and AAH70079.1). Whatever differences between hGARP and fGARP may exist, the data reported herein suggest that they are structurally similar and that they perform the same function as accessory proteins in mediating the suppressor activity of Treg cells.

In support of the association of fGARP and TGFβ on feline Treg cells, flow cytometric analysis revealed that these surface proteins were expressed on similar numbers of

$CD4^+CD25^+$ lymphocytes but not on $CD4^+CD25^-$ Th. These $TGF\beta^+fGARP^+CD4^+CD25^+$ were shown to express FoxP3, consistent with the established phenotype of Treg cells. While dual staining for GARP and TGFβ on activated Treg cells proved to be problematic, likely due to steric hindrance, supporting the close association of the two proteins, analysis of $CD4^+CD25^+$ cells by Co-IP revealed that the fGARP protein was complexed with mature TGFβ in $CD4^+CD25^+$ lymphocytes. These data support previous findings by Stockis, et al. (12) who reported that hGARP formed a complex with TGFβ. However, Stockis et al. focused on soluble TGFβ rather than membrane-bound TGFβ in suppressor signaling and they did not investigate the contact-dependent suppressor capacity of these cells. Other studies have found that Th cells transduced with GARP are capable of suppressing CD4 T cell proliferation in a contact-dependent manner and that silencing the expression of GARP in $CD4^+CD25^+$ Treg cells reduces suppressor function (11, 14). The role of TGFβ was not evaluated in these later studies. These results suggest that activation and suppressor function of GARP-bound TGFβ should be evaluated within the context of the Treg cell membrane.

Studies have demonstrated that the hGARP surface protein is only expressed on activated Treg cells (11, 32) while FoxP3 is constitutively expressed on resting Treg cells (35) but is up-regulated after activation (8, 36). In agreement with these findings, LPS/rhIL2 stimulation of feline $CD4^+CD25^+$ lymphocytes resulted in the up-regulation of fGARP and FoxP3 message and protein as seen by real-time RT-PCR and flow cytometry. Flow cytometric analysis also revealed up-regulation of mTGFβ protein following LPS/rhIL2 activation of $CD4^+CD25^+$ Treg cells. These results support our conclusion that

fGARP:TGFβ complexes are expressed on the surface of feline Treg cells and that these complexes are up-regulated following activation.

Assay of IL2 production by stimulated $CD4^+CD25^-$ target cells co-cultured with in vitro activated $CD4^+CD25^+$ Treg cells demonstrated reduced suppressor capability in fGARP-depleted $CD4^+CD25^+$ Treg cells, indicating that $fGARP^+$ Treg cells are potent immune suppressors. Similar results were obtained from hGARP studies in which $GARP^+$ Treg cells were found to be more efficient suppressors than $GARP^-$ Treg cells (32). Studies by Wang et al. (32) on hGARP and the studies here show that while GARP-expressing Treg cells do not mediate total Th cell suppression, they are major mediators of T cell immune suppression. The redundancy and overlap of Treg suppressor mechanisms has been widely accepted (37-39) and the results reported herein do not provide a definitive model for Treg cell-mediated suppression but do identify the GARP:TGFβ complex as an important cell surface regulatory mechanism utilized by Treg cells in the suppression of Th cells. Collectively, these studies support the proposal that GARP-bound TGFβ on the surface of activated Treg cells mediates Treg suppression of Th cells.

To further pursue this question, with the understanding that AIDS lentivirus infections, including FIV, activate the Treg cells in vivo (5, 31, 33) , we evaluated Treg expression of GARP and TGFβ in FIV-infected cats. A significant increase in fGARP and TGFβ expression was observed on freshly isolated $CD4^+CD25^+$ T cells from cats with chronic FIV infection. While Wang et al. (15) did not examine freshly isolated Treg cells from HIV^+

donors, they did report GARP expression on stimulated Treg cells from HIV patients and found no significant difference in Treg cell expression between HIV^+ and HIV^- donors post-stimulation. However, there was a significantly larger population of $CD4^+CD25^+FoxP3^+$ cells in the HIV^+ patients, suggesting that HIV infection may not significantly alter $GARP^+$ Treg cell numbers but may expand nonregulatory T cells expressing FoxP3. It is unclear whether the HIV^+ donors in this study were receiving chemotherapeutic treatments that could further complicate comparisons to HIV^- donors as antiretroviral therapy has been shown to alter numbers and phenotypes of Treg cells (40) . Our findings suggest that lentiviral infection is capable of activating $GARP^+$ Treg cells in vivo.

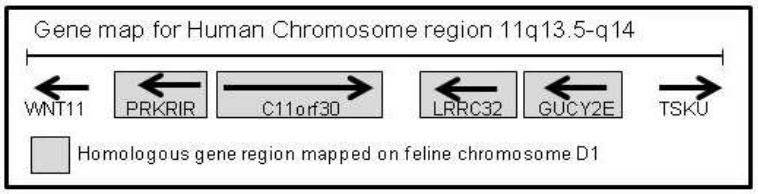
We have previously demonstrated that FIV preferentially and productively infects Treg cells (8, 41, 42) and that freshly isolated Treg cells from FIV infected cats are not only productively infected, but are activated and capable of suppressing Th cells (31, 42) . We have also demonstrated that the Th cells express higher levels of TGFβ-RII during chronic FIV infection (6) . As we propose that $TGF\beta^+$ Treg cells engage TGFβ-RII on Th target cells, expression of TGFβ-RII on the target cell would promote Treg cell-mediated suppression. In this study, we extend these findings, demonstrating that elimination of $fGARP^+$ cells from the $CD4^+CD25^+$ Treg population from chronically FIV-infected cats reduced Treg-mediated suppressor activity. Blocking assays further demonstrated that pretreatment of the in vivo activated $CD4^+CD25^+$ Treg cells from FIV-infected cats with neutralizing antibodies to GARP, TGFβ and/or TGFβ-RII eliminated this suppressor capacity as measured by both Th target cell IL2 production and target cell proliferation. These data

suggest FIV infection activates Treg cells, up-regulating fGARP:TGFβ complexes on their surface, inducing their immunosuppressive function against TGFβ-RII^{+} Th cells via TGFβ signaling. The up-regulation of GARP and TGFβ on Treg cells may not be unique to FIV infection but may occur with other chronic virus infections known to activate immunosuppressive Treg cells such as hepatitis and herpes viruses (4, 43-45) . Further research will be necessary to evaluate the existence of a GARP^{+} Treg cell population in these additional models of disease.

F. Figures

FIGURE 1. **Predicted fGARP gene locus, mRNA expression dynamics and protein composition are homologous to hGARP**. **A**. The predicted feline GARP (LRRC32) gene is located on feline chromosome D1 and is flanked by genes homologous with those adjacent to hGARP. **B**. The mRNA product corresponding to the 1431 or 1851 GARP ORFs was amplified from purified feline $CD4^{+}CD25^{+}$ or $CD4^{+}CD25^{-}$ cells, products separated on 1% agarose gel, and imaged. Both gene products were detected only in $CD4^{+}CD25^{+}$ cells. **C.** The sequenced fGARP gene product was translated into the corresponding amino acid (aa) sequence (F) and aligned to the hGARP aa sequence (H). Shaded boxes identify matching aa residues; asterisks indicate changes in which the aa are not of similar composition. Categories used for composition were acidic, basic, aliphatic, aromatic, hydroxylic, sulfur-containing and amidic. The C-terminal transmembrane domain is underlined. **D**. Ribbon diagram of the predicted fGARP protein structure modeled by comparison to human TLR-3. Asterisks highlight disordered loops. **E**. The corresponding space-filling model. Arrows identify the C-terminus where the transmembrane domain has been deleted for this image.

A



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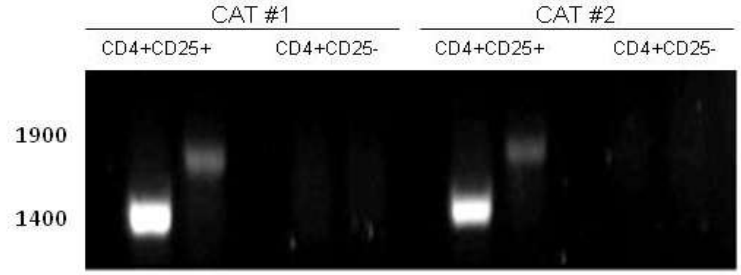

C

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F  ----------------------------------------------------

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F  ----------------------MDIEDGAFEALPHLAHLNLSRNSLTCI  27

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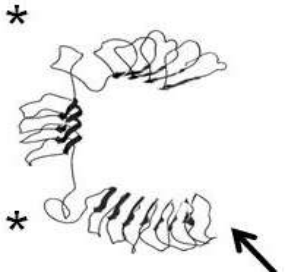

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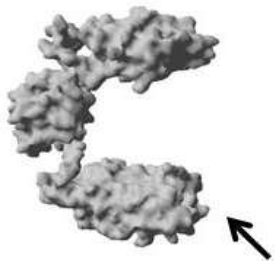
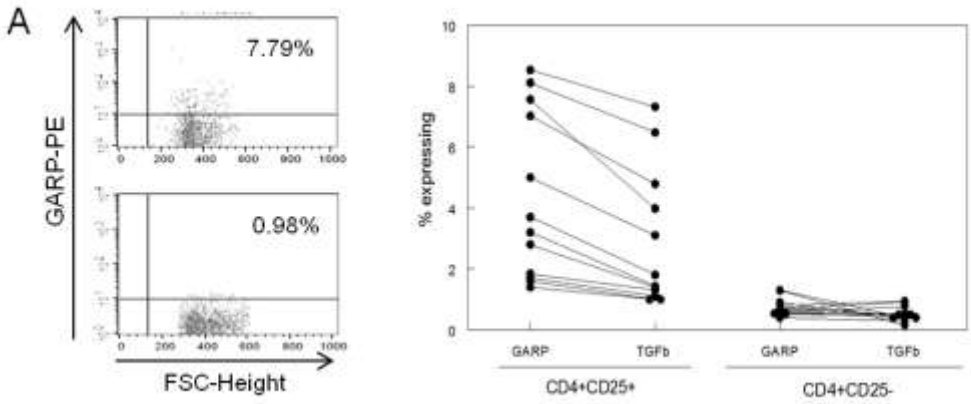


FIGURE 2. **fGARP and TGFβ form a complex that is expressed on the surface of $CD4^+CD25^+FoxP3^+$ cells, consistent with a regulatory phenotype. A**. Lymphocytes were analyzed for GARP and TGFβ expression by flow cytometry. Representative gating for GARP expression within the $CD4^+CD25^+$ (top) and $CD4^+CD25^-$ (bottom) populations is shown on the left. The total percentage of cells expressing GARP or TGFβ in each population is shown on the right with lines connecting each cat (N = 10). Elevated levels of GARP and TGFβ are seen only on $CD4^+CD25^+$ cells. **B.** Lymphocytes were analyzed for GARP surface expression and intracellular FoxP3 expression. Population shown is gated on $CD4^+CD25^+$ cells. GARP expressing $CD4^+CD25^+$ cells were found to co-express FoxP3. **C.** Whole cell lysate from purified feline $CD4^+CD25^+$ cells was analyzed by Western blot for GARP and TGFβ. **D.** Purified $CD4^+$ cells from two different cats were analyzed by Co-IP. Lysates were immunoprecipitated using anti-GARP and then blotted with anti-TGFβ. Negative Control 1 corresponds to sample immunoprecipitated with anti GARP and blotted with only secondary HRP conjugated antibody. Negative control 2 corresponds to sample run through a nonactivated resin and blotted with anti-TGFβ.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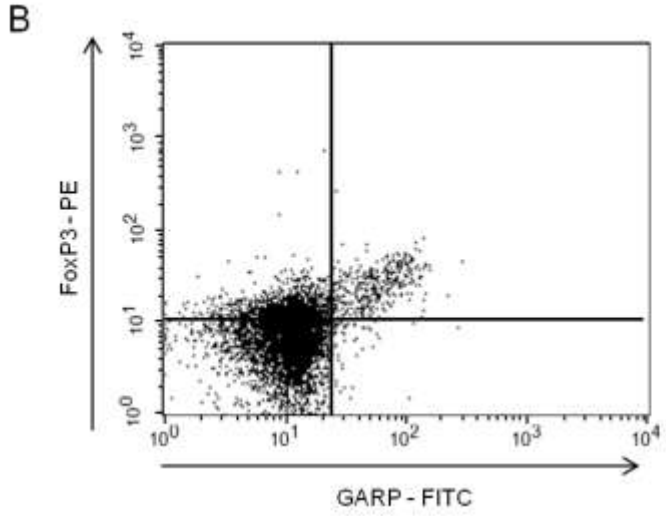
FSC-Height
% expressing
GARP
TGFb
GARP
TGFb
CD4+CD25+
CD4+CD25-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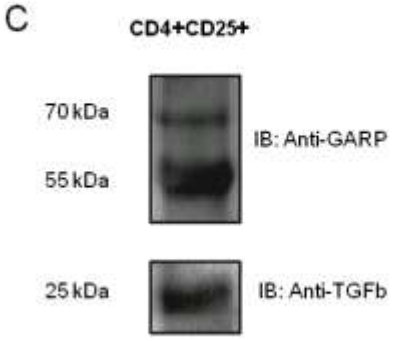
FoxP3 - PE
GARP - FITC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55 kDa
IB: Anti-GARP
25 kDa
IB: Anti-TGFb

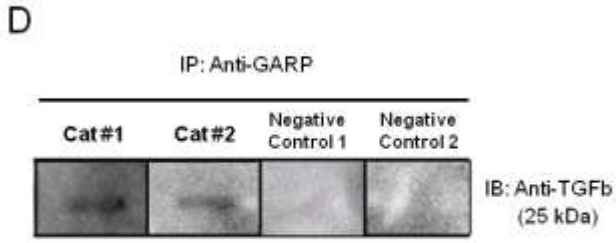

D
IP: Anti-GARP
Cat #1
Cat #2
Negative Control 1
Negative Control 2
IB: Anti-TGFb (25 kDa)

FIGURE 3. **GARP, FoxP3 and TGFβ protein and message are up-regulated in $CD4^+CD25^+$ lymphocytes following in vitro activation.** $CD4^+CD25^+$ lymphocytes were sorted from PLN of FIV negative cats and activated in vitro by LPS/rhIL2 stimulation and analyzed for surface expression of GARP or TGFβ, or for intracellular expression of FoxP3 by flow cytometry. **A**. Percent of cells expressing GARP, **B**. Mean fluorescence intensity (MFI) of GARP expression, **C**. Percent of cells expressing FoxP3, **D**. MFI of FoxP3 expression, **E**. Percent of cells expressing TGFβ. **F**. Sorted $CD4^+CD25^+$ lymphocytes were analyzed for GARP and FoxP3 message before and after stimulation by Real-Time RT-PCR and results are shown as fold change over GAPDH (-ΔΔCT). (error bars represent standard error of the mean of 4 independent experiments, *$p < .05$ Mann-Whitney)

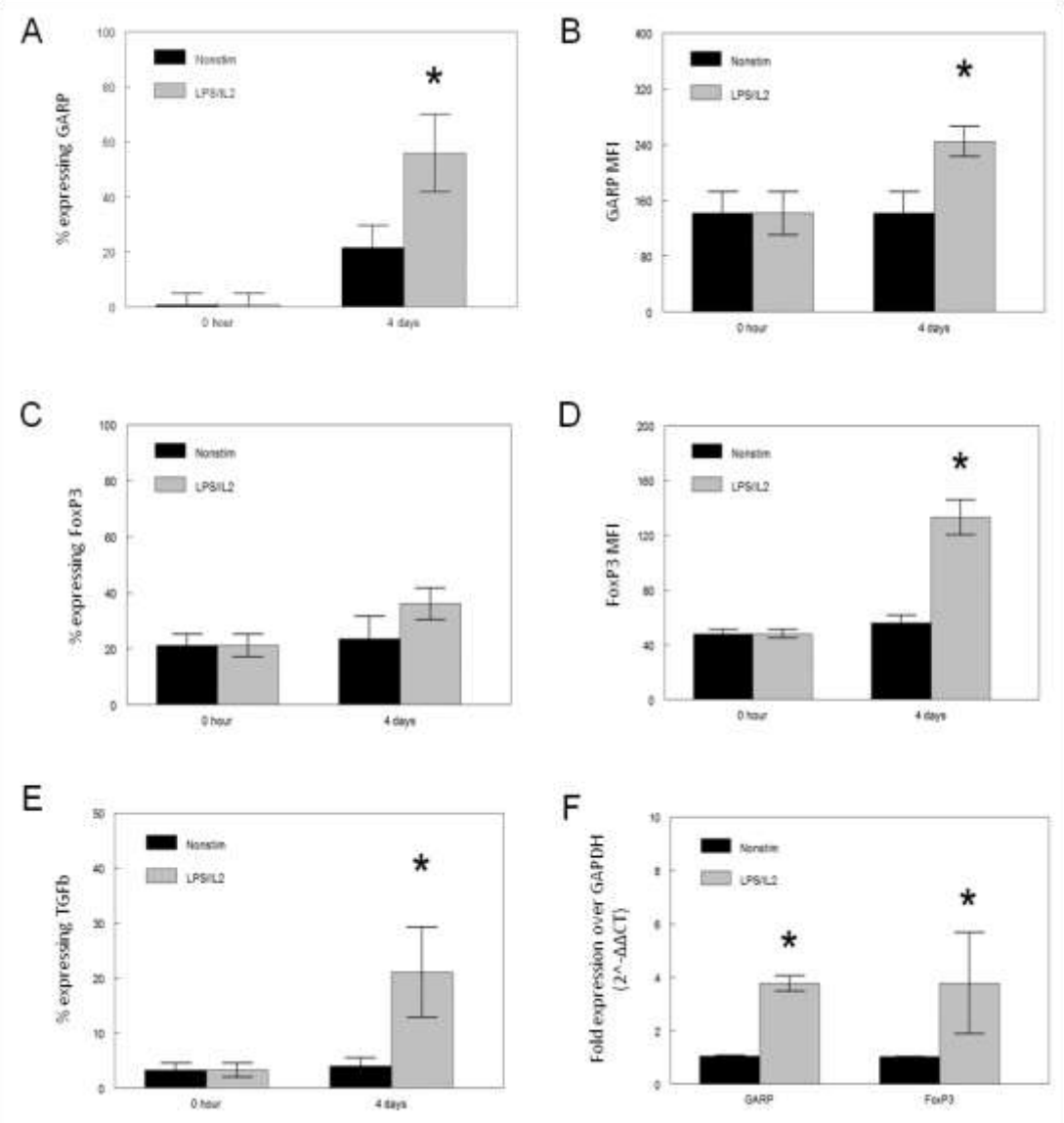
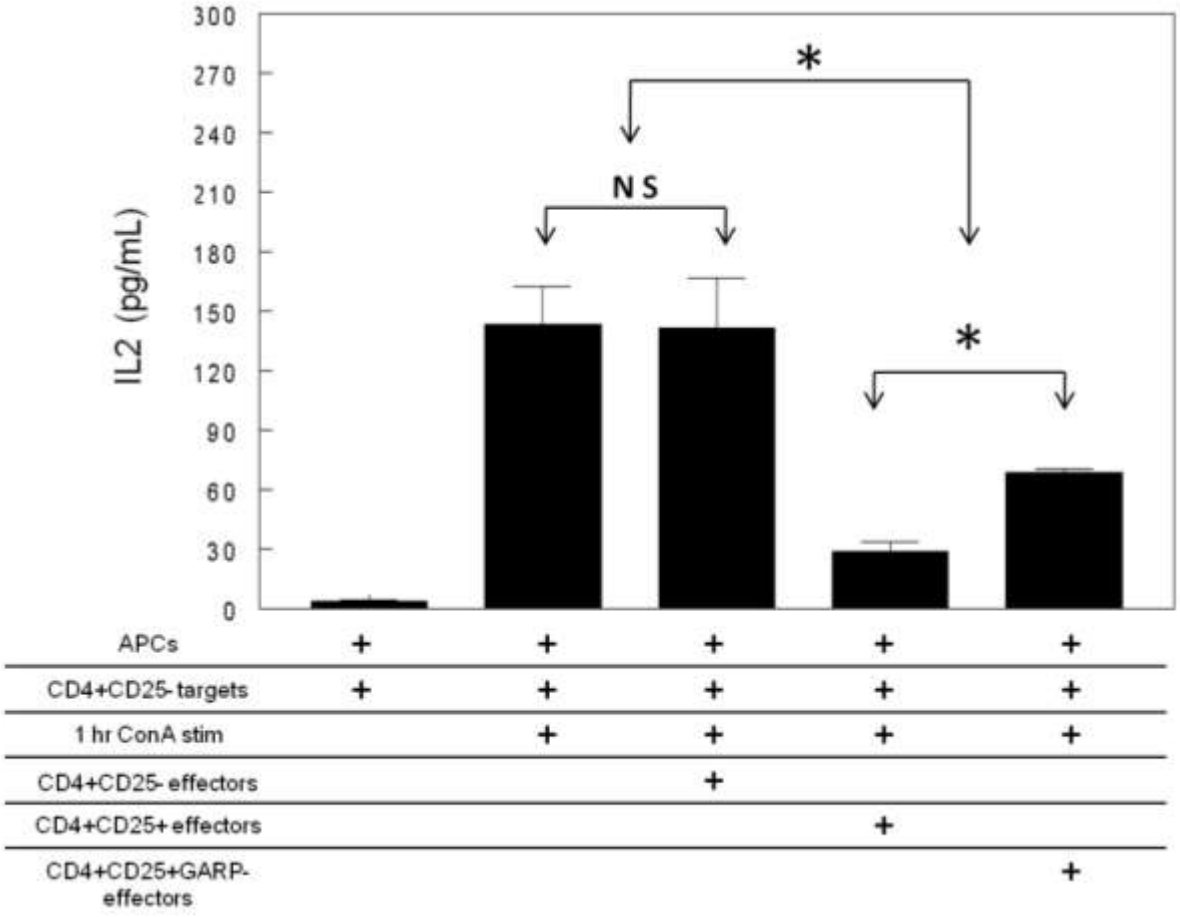

A
% expressing GARP
Nonstim
LPS/IL2
*
0 hour
4 days
B
GARP MFI
Nonstim
LPS/IL2
*
0 hour
4 days
C
% expressing FoxP3
Nonstim
LPS/IL2
0 hour
4 days
D
FoxP3 MFI
Nonstim
LPS/IL2
*
0 hour
4 days
E
% expressing TGFb
Nonstim
LPS/IL2
*
0 hour
4 days
F
Fold expression over GAPDH
(2^-ΔΔCT)
Nonstim
LPS/IL2
*
*
GARP
FoxP3

FIGURE 4. **Depletion of GARP$^+$ Treg cells decreases Treg-mediated suppression of CD4$^+$CD25$^-$ Th cells.** CD4$^+$CD25$^+$ effector cells were obtained by FACS purification from SPF cat PLNs and activated with LPS/IL2 treatment for four days as described. CD4$^+$CD25$^-$ Th cells were used as targets and were obtained from freshly isolated PBMCs on the day of co-culture addition. APCs were prepared by FACS depletion of CD4$^+$ and CD8$^+$ PBMCs at this time as well and added at a 1:1 ratio with Th cells. Where indicated, these cells were stimulated for 1 hour with 5 ug/uL of ConA, washed and then plated. CD4$^+$CD25$^-$, LPS/rhIL2 activated CD4$^+$CD25$^+$, or activated CD4$^+$CD25$^+$GARP$^-$ populations were used as effector cells at an 1:2 effector:target ratio. Supernatant was collected after 24 hours of co-culture and IL2 analyzed by ELISA in triplicate. (data shown is representative of two separate experiments, error bars represent standard error of the mean, *$p < .05$ Mann-Whitney)


300
270
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IL2 (pg/mL)
*
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APCs + + + + +
CD4+CD25- targets + + + + +
1 hr ConA stim + + + +
CD4+CD25- effectors +
CD4+CD25+ effectors +
CD4+CD25+GARP- effectors +

FIGURE 5. **GARP expression on $CD4^+CD25^+$ and TGFβ-RII expression on $CD4^+CD25^-$ cells are up-regulated during chronic FIV infection.** **A**. Lymphocyte populations from chronic FIV infected cat or FIV negative cat PLNs were FACS purified into $CD4^+CD25^+$ or $CD4^+CD25^-$ populations. RNA was extracted from each population and analyzed for GARP mRNA levels by real time RT-PCR (n = 3). Data shown as fold expression over GAPDH (error bars represent the standard error of the mean). **B.** Freshly isolated PBMCs from chronically FIV infected cats (n = 11) or FIV- cats (n = 10) were analyzed by flow cytometry for the expression of GARP and TGFβ by gating on $CD4^+CD25^+$ or $CD4^+CD25^-$ populations. Data expressed as percentage of gated cells, gating determined by isotype controls. **C**. FIV^- and chronic FIV^+ PBMCs (n = 5) were analyzed by flow cytometry for the expression of TGFβ receptor (TGFβ-RII) (*$p < .05$ Mann-Whitney, mean shown for each group).

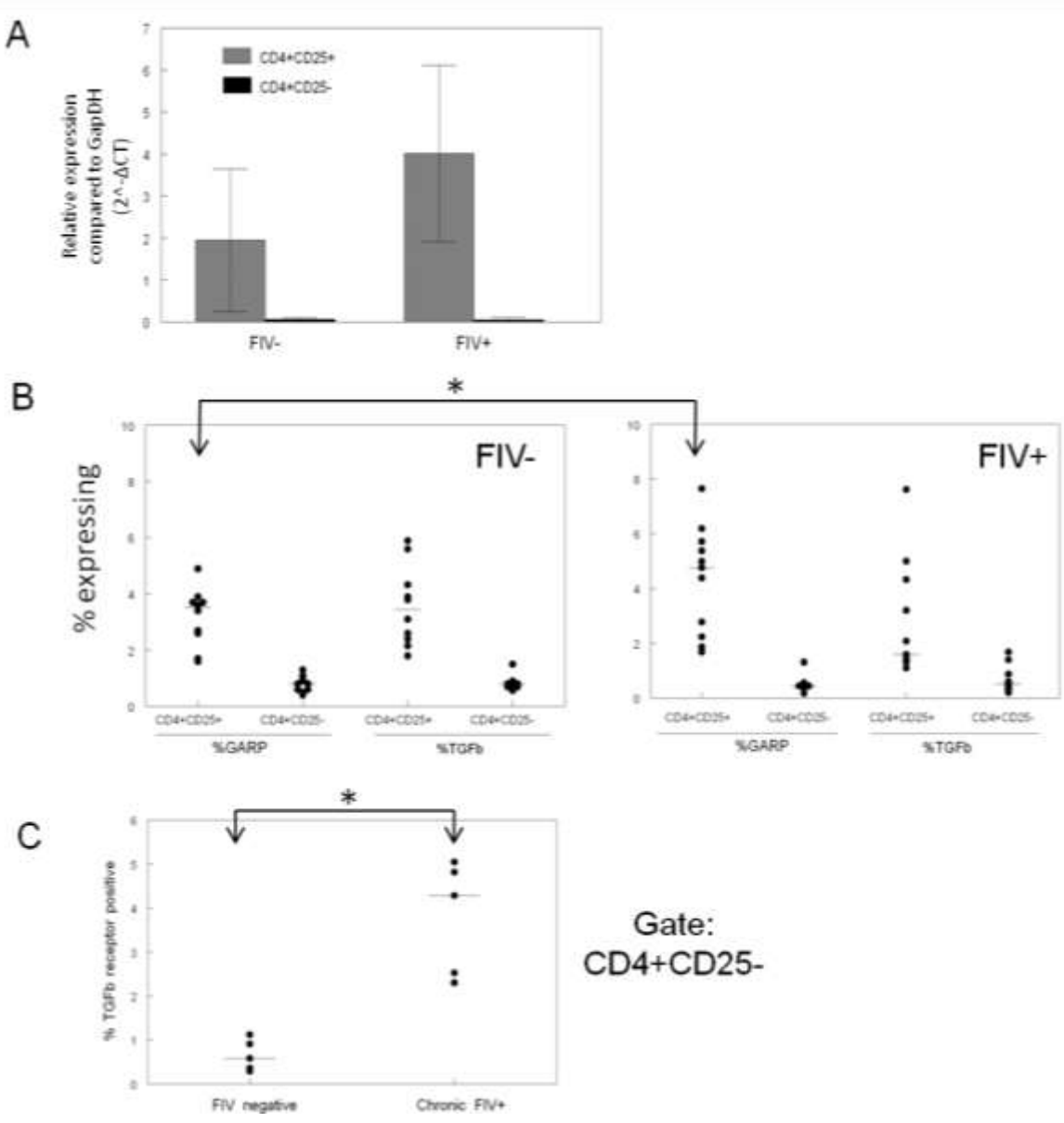
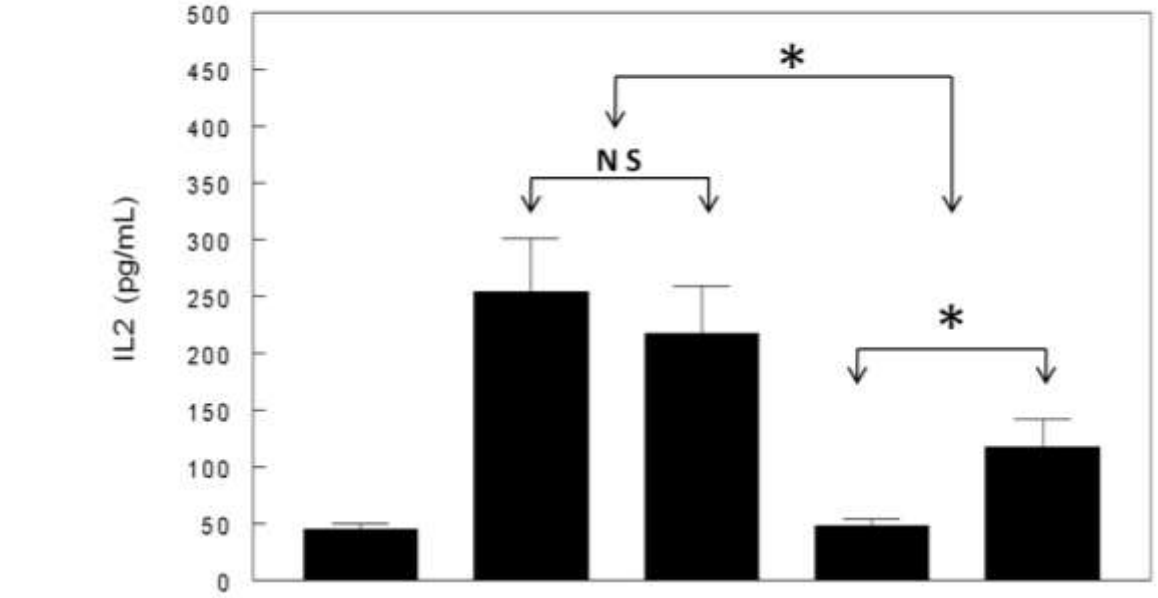
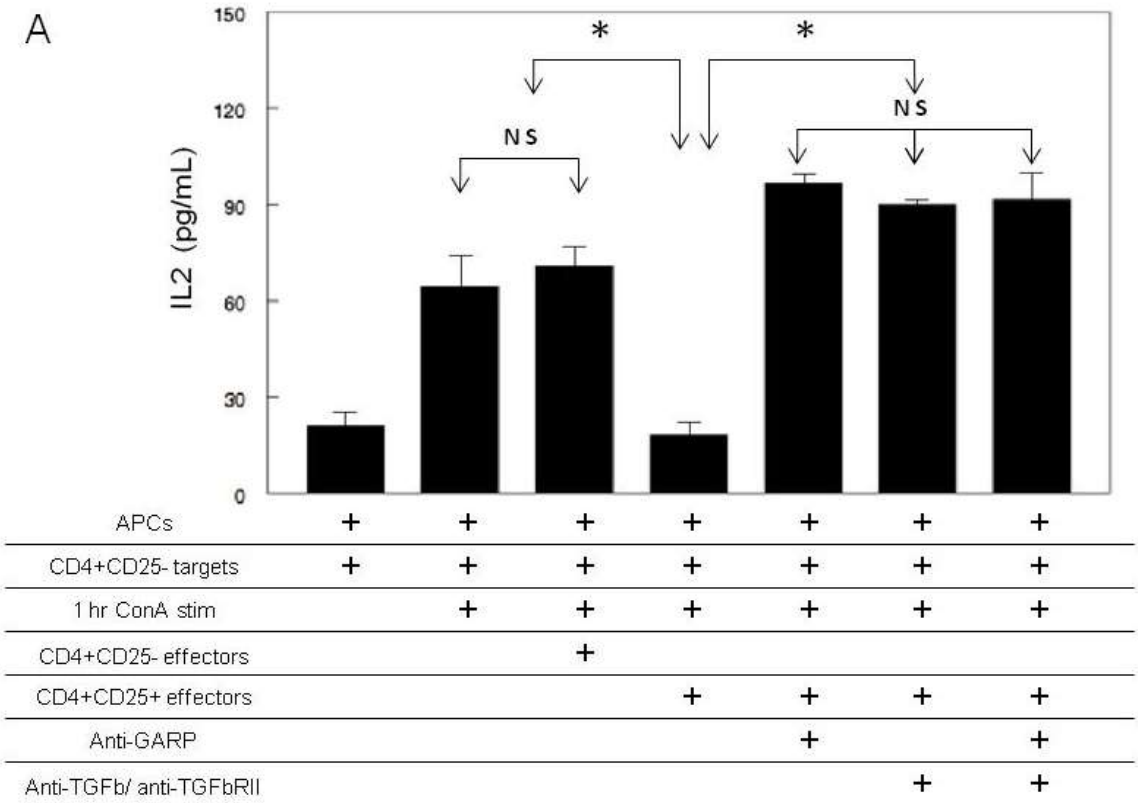

A
CD4+CD25+
CD4+CD25-
Relative expression compared to GapDH (2^-ΔCT)
FIV-
FIV+
B
*
FIV-
FIV+
% expressing
CD4+CD25+
CD4+CD25-
%GARP
%TGFb
C
*
% TGFb receptor positive
FIV negative
Chronic FIV+
Gate:
CD4+CD25-

FIGURE 6. **$CD4^+CD25^+$ Tregs from chronic FIV-infected cats suppress $CD4^+CD25^-$ Th and this is partially dependent upon GARP expression.** Lymphocyte populations from chronic FIV infected cat PLNs were FACS purified. $CD4^+CD25^-$ cells were used as target cells. APCs were prepared by FACS depletion of $CD4^+$ and $CD8^+$ lymphocytes and added at a 1:1 ratio with Th cells. Where indicated, these cells were stimulated for 1 hour with 5 ug/mL of ConA , washed and then plated. Freshly isolated $CD4^+CD25^-$, $CD4^+CD25^+$, or $CD4^+CD25^+GARP^-$ populations were used as effector cells with an effector:target ratio of 1:2. Supernatant was collected after 24 hours of co-culture and IL2 was analyzed by ELISA in triplicate. (data shown is representative of three independent experiments, error bars represent the standard error of the mean, *$p < .05$ Mann-Whitney).



APCs	+	+	+	+	+
CD4+CD25- targets	+	+	+	+	+
1 hr ConA stim		+	+	+	+
CD4+CD25- effectors			+		
CD4+CD25+ effectors				+	
CD4+CD25+GARP- effectors					+

FIGURE 7. **Blocking GARP and/or TGFβ signaling abrogates suppressor capacity of $CD4^+CD25^+$ Treg cells isolated from FIV-infected cats.** Lymphocyte populations from chronic FIV infected cat PLNs were FACS purified. Freshly isolated $CD4^+CD25^-$ or $CD4^+CD25^+$ were used as effector cells. Where indicated, $CD4^+CD25^+$ cells were plated with 100 ug/mL each anti-GARP, anti-TGFβ and/or anti-TGFβ-RII neutralizing antibodies for 30 minutes. CD4+CD25- cells were used as target cells and were labeled with CFSE immediately after purification. APCs were prepared by FACS depletion of $CD4^+$ and $CD8^+$ lymphocytes and added at a 1:1 ratio with already labeled Th cells. Where indicated, these cells were stimulated for 1 hour with 5 ug/mL of ConA , washed and then plated with the pretreated $CD4^+CD25^+$ cells at an effector:target ratio of 1:2. **A**. Supernatant was collected after 24 hours of co-culture and IL2 was analyzed by ELISA in triplicate. (data shown is pooled from two independent experiments, error bars represent the standard error of the mean, *$p < .05$ Mann-Whitney). **B**. CFSE proliferation was analyzed using ModFit LT and the percent inhibition of proliferation was calculated for each of the effector cell groups as follows: [[(PI stimulated $CD4^+CD25^-$ alone) – (PI with effector cells)] / (PI stimulated $CD4^+CD25^-$ alone)] x 100. (Data is representative of two independent experiments).


A
150
120
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IL2 (pg/mL)
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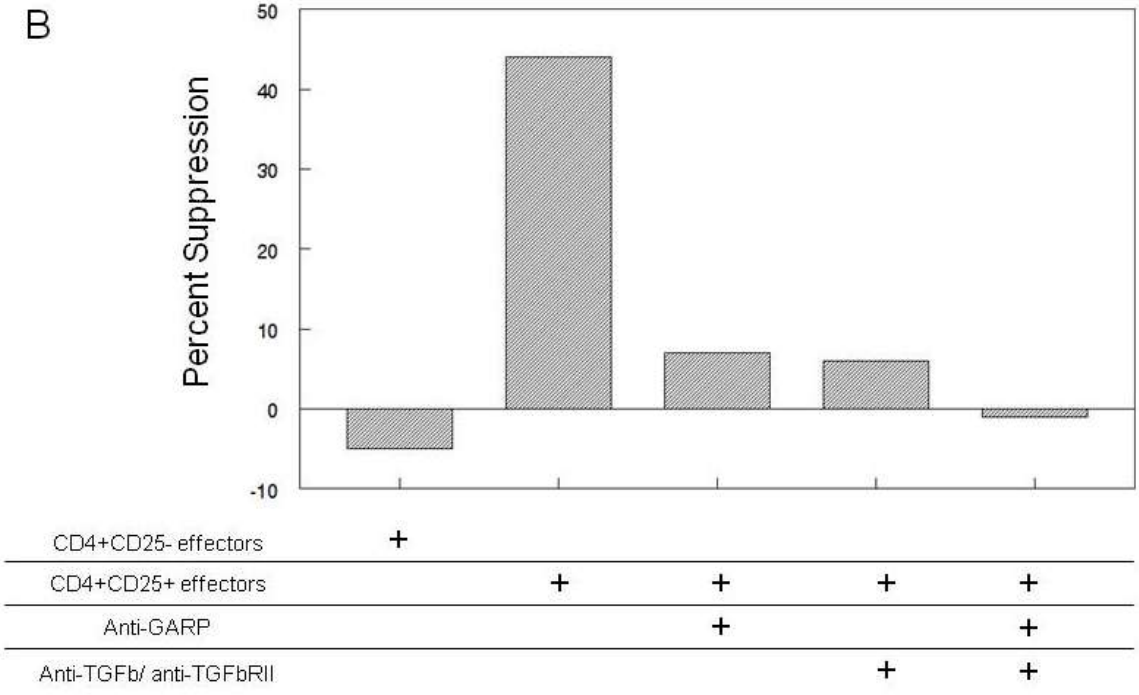
NS
NS
APCs + + + + + + +
CD4+CD25- targets + + + + + + +
1 hr ConA stim + + + + + +
CD4+CD25- effectors +
CD4+CD25+ effectors + + + +
Anti-GARP + +
Anti-TGFb/ anti-TGFbRII + +


B
50
40
30
20
10
0
-10
Percent Suppression
CD4+CD25- effectors +
CD4+CD25+ effectors + + + +
Anti-GARP + +
Anti-TGFb/ anti-TGFbRII + +

G. References

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4. Infection with Feline Immunodeficiency Virus Directly Activates $CD4^+CD25^+$ T Regulatory Cells

This chapter has been reformatted and submitted to the Journal of Virology as a Short-Form paper.

A. Abstract

AIDS-lentivirus infections are characterized by progressive activation of $CD4^+CD25^+$ T regulatory (Treg) cells. The mechanism behind this activation remains enigmatic but may be a result of direct virus infection or an indirect effect of chronic antigenic stimulation. Here, we demonstrate that in vitro treatment of purified $CD4^+CD25^+$ Treg cells with live virus, but not UV inactivated virus, results in upregulation of the Treg activation markers GARP and membrane-bound TGFβ. We further demonstrate that these FIV-infected Treg cells, in the absence of any additional stimulation, suppress activated $CD4^+CD25^-$ Th cells in vitro. To our knowledge, this is the first report demonstrating that lentiviral infection is, in itself, sufficient to activate Treg cells and may contribute to Treg-mediated immune dysfunction in the FIV host.

B. Introduction

$CD4^+$ regulatory T cells (Treg cells), currently defined by constitutive expression of the high affinity IL2 receptor, CD25, and the transcription factor FoxP3, play an important role in controlling autoimmune disease and shaping the pathogenesis of viral infections by regulating expansion of T and B effector subsets (1-7). Treg cells play an important role in

controlling inflammation associated with immune responses to pathogens, and curtailing excessive tissue damage. However, they may prematurely abort protective T and B cell immune responses as well, allowing the establishment of persistent infection and chronic immune activation leading to more severe disease pathogenesis. This has been demonstrated in herpes virus, B and C hepatitis virus, and AIDS lentivirus infections (1, 3, 8-10), supporting the speculation that Treg cells can contribute to the chronic nature of these infections by limiting the antiviral response. In HIV and FIV, immunosuppressive Treg cells are first activated during acute infection and remain phenotypically and functionally activated throughout the chronic stage (6, 11), suggesting that they may contribute to virus persistence and subsequent immunodeficiency.

While it is well established that $CD4^+$ and $CD8^+$ T cell immune dysfunction following FIV or HIV infection is associated with early and long-term activation of Treg cells, the precise mechanism(s) of activation has yet to be resolved. Although Treg cell activation is associated with virus infection of the host, it is not known whether virus antigens or direct virus infection activates these cells. In support of the latter, we have demonstrated that feline $CD4^+CD25^+$ cells sustain a productive, non-cytopathic FIV infection in vitro that correlates with over-expression of the FIV co-receptor CXCR4 and constitutive activation of transcription factors such as AP1 and C/EBP that bind to and activate the FIV promoter (12, 13). We have also reported that as early as 1 week post FIV infection, $CD4^+CD25^+$ T cells are phenotypically and functionally activated (7). In concert with our findings, Oswald-Richter et al. (14) also reported that Treg cells from HIV-infected individuals express the

HIV co-receptor CCR5 and are highly susceptible to HIV infection and replication. Thus, understanding the activation dynamics of these cells during the early stages of lentivirus infection is important to understanding disease progression. We (15) and others (16, 17) have presented evidence that membrane bound TGFβ (mTGFβ) is up-regulated on activated Treg cells. More recently, we have demonstrated that activated feline Treg cells also display a surface marker, Glycoprotein A Repetitions Predominant or GARP, and that this transmembrane protein anchors mTGFβ on the surface of Treg suppressor cells (18). As Treg cells from FIV-infected cats display increased expression of mTGFβ, GARP, and FoxP3 (18), and are highly suppressive, we asked if these characteristics could be replicated in purified Treg cells infected in vitro and thus establish a role for direct Treg cell activation by FIV infection. Here, we demonstrate that FIV infection of purified Treg cells is able to directly activate these cells and induce suppressor function against activated $CD4^+$ T helper cells. These results suggest that the activation of Treg cells during acute and chronic FIV infection is a direct consequence of viral infection and not simply a result of general immune activation.

C. Materials and Methods

Cats and Sample Collection

Specific pathogen-free (SPF) cats were obtained from Liberty Research, Inc. (Waverly, NY) at 6 months of age, and housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee. LN were biopsied

and single-cell suspensions were prepared by gently and repeatedly injecting sterile PBS using an 18G needle until the cells were released from the tissue. For suppression assay targets, whole blood (28 ml/cat) was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) and lymphocytes separated by Ficoll-Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St-Louis, MO) following the manufacturer's guidelines. Cell counts and viability were determined by trypan blue dye exclusion and viability was always >90%.

In vitro infection and cell cultures

$CD4^+CD25^+$ cells were FACS purified by positive selection with feline specific CD4 and CD25 antibodies. Mouse anti-feline CD25 (mAb 9F23) was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan) and mouse anti-feline CD4 (mAb 30A) was developed in our laboratory (19). For in vitro infections, cells were cultured in medium containing 10% heat inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine and 1 mM HEPES buffer at $2x10^6$ total cells per mL. Infection with live virus or UV inactivated virus was performed at an MOI of 2.5 using the $NCSU_1$ isolate of FIV (20). Briefly, purified cells were washed with fresh media and pelleted by centrifugation. Virus was added to the cell pellet and incubated on ice for 2 hours. Cells were washed in fresh media and plated into 12 well plates. UV inactivation of FIV consisted of exposing virus to 1 microjoule of UV light for indicated times prior to administration following the outlined procedure. In vitro infection was confirmed using Gag-specific PCR for cell-associated virus and p24 ELISA for production of viral Gag protein into the culture

media. These methods have been described in detail elsewhere (13). For LPS/IL2 activation, purified $CD4^+CD25^+$ cells were cultured with 10 ug/mL LPS for two days, washed and cultured for two more days in fresh media supplemented with 100 U/mL recombinant human IL2 (rhIL2) (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD).

Real time PCR

FACS purified $CD4^+CD25^+$ T cells from PLN were used for quantification of GARP, FoxP3 and GAPDH mRNA by reverse transcription and real-time PCR. Total RNA extraction was carried out using Qiagen's RNeasy plus mini kits (Qiagen, Valencia, CA). RNA was used in reverse transcription reactions using Oligo(dT) primers and the Promega Reverse transcription system according to the manufacturer's instructions (Promega, Madison, WI). Products were assessed in triplicate for the specific mRNA of interest. Development and use of FoxP3 or GAPDH primers (15) and of GARP primers (18) has been described elsewhere.

Flow Cytometry

At least $5x10^5$ cells were stained for surface expression of various markers using specific antibodies. Anti-TGFβ (MAB240) was purchased from R&D systems and conjugated to allophycocyanin (APC). FITC-conjugated anti-GARP (LRRC32, Plato-1) was purchased from Enzo life Sciences (Ann Arbor, MI). One or two color flow cytometry was performed on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA). PE conjugated rat anti-mouse FoxP3 (FJK-16s) intracellular staining kit was purchased from eBioscience

(San Diego CA) and intracellular staining was performed as per manufacturer's instructions. Each antibody combination was analyzed in duplicate and lymphocytes were gated based on forward versus side scatter. Gated events were analyzed using CellQuest software and gates determined by isotype controls.

IL2 Suppression Assay

$CD4^+CD25^-$ cells were FACS purified from FIV negative cat PBMCs and used as target cells. APCs were prepared by FACS depletion of CD4 and CD8 (19) expressing lymphocytes and added at a 1:1 ratio with target cells. Where indicated, these cells were stimulated for 1 hour with 5 ug/mL ConA, washed, CFSE labeled and plated. LPS activated $CD4^+CD25^+$, FIV-infected $CD4^+CD25^+$ or $CD4^+CD25^+GARP^-$ and UV-inactivated $CD4^+CD25^+$ or $CD4^+CD25^+GARP^-$ were used as effector cells with an effector:target ratio of 1:2. After 24 hours of co-culture, 100 uL of culture supernatant was analyzed by IL2 ELISA in triplicate. Alternatively, target cells were stimulated, labeled with CFSE, washed, plated, and proliferation was assessed after 72 hours of co-culture by flow cytometry using ModFit LT for data analysis.

D. Results

$CD4^+CD25^+$ Treg cells support a productive infection in vitro.

We have previously documented productive infection of $CD4^+CD25^+$ Treg cells by FIV (12, 13). To confirm that our in vitro method of infection was successful, $CD4^+CD25^+$ T cells

were FACS purified from peripheral lymph nodes of SPF cats, infected at a MOI of 2.5 and analyzed for virus replication. Six days post infection (PI), high virus copy number was detected in $CD4^+CD25^+$ cells by real-time PCR (Fig. 1A), and the presence of p24 in the culture supernatant was detected by FIV p24 specific ELISA (Fig. 1B), demonstrating a productive infection. As a control, FIV was UV irradiated and shown to be noninfectious for the highly susceptible feline $CD4^+$ cell line CD4E after various exposure times (Fig 1C). All subsequent experiments were performed using cells infected with at a MOI of 2.5 and, as negative controls, the equivalent MOI of virus exposed to UV light for 30 minutes (mock infected), or uninfected cells.

FIV-infected $CD4^+CD25^+$ cells display an activated phenotype.

To determine if virus infection alone was capable of inducing an activation phenotype in Treg cells, $CD4^+CD25^+$ cells were infected with FIV or mock infected with UV irradiated virus and analyzed at 3 and 6 days post infection for surface expression of GARP, TGFβ, and FoxP3 protein by flow cytometry or for mRNA by real time RT-PCR. As a positive control for activation, purified $CD4^+CD25^+$ were stimulated with LPS (10ug/ml) and recombinant human IL2 (rhIL2) (100U/ml) as described previously (11). Figure 2A shows that FIV infection of sorted $CD4^+CD25^+$ Treg cells resulted in up-regulation of GARP mRNA at 6 days post infection compared to uninfected controls and the levels were similar to LPS/rhIL2 activated cells. Both the percent of cells expressing GARP surface protein and the level of protein expression (mean fluorescence intensity) were significantly elevated as a result of FIV infection (Fig. 2C and 2E). Interestingly, GARP protein levels were increased at three

days post infection, prior to the observed increase in mRNA, suggesting sequestration of intracellular GARP protein in the absence of stimulation as described for GARP in human Treg cells (21). The $GARP^{+}CD4^{+}CD25^{+}$ population also co-expressed FoxP3 protein in the FIV infected cells at 6 days post infection (Fig. 2G), supporting the classification of this population as Treg cells. No changes in GARP protein or mRNA expression occurred in the mock infected cells, suggesting that direct infection of the cells, rather than exposure to viral antigens, is necessary for activation (Fig. 2C and 2E).

FoxP3 message was increased in in vitro FIV-infected $CD4^{+}CD25^{+}$ cells by 3 days PI but returned to normal levels by 6 days (Fig. 2B). This rapid increase is similar to that seen in vivo, as Mexas et al. (2008) demonstrated increased FoxP3 mRNA as early as one week post FIV infection. As described for in vivo studies of FIV and HIV infections (7, 8, 11, 22), the percentage of cells expressing FoxP3 protein remained constant regardless of treatment (Fig. 2D). However, the intracellular protein level as measured by MFI increased at 3 days post FIV infection (Fig. 2F), consistent with the measured increase in FoxP3 transcription. No change in FoxP3 message or protein was observed following UV inactivated virus treatment (Fig. 2D and 2F).

A number of studies have reported that TGFβ is expressed on the surface of activated Treg cells and plays a role in contact-dependent Treg-mediated suppression (2, 7, 17, 18). Further, we have demonstrated that GARP forms a complex with membrane TGFβ (mTGFβ) on activated Treg cells (18). As direct FIV infection induced GARP expression on $CD4^{+}CD25^{+}$

cells (Fig. 2), we also analyzed these cells by flow cytometry for surface expression of TGFβ. mTGFβ expression was up-regulated following FIV infection of $CD4^+CD25^+$ lymphocytes (Fig. 2H), consistent with GARP up-regulation. Taken together, these data suggest that FIV infection is sufficient to induce an activated Treg cell phenotype as measured by the up-regulation of GARP, FoxP3, and mTGFβ, whereas exposure to noninfectious FIV antigens alone is not.

FIV-infected $CD4^+CD25^+$ Treg cells suppress IL2 production and proliferation of activated $CD4^+CD25^-$ T helper cells through a GARP:TGFβ dependent mechanism.

Having demonstrated that FIV infection directly induces an activation phenotype in purified $CD4^+CD25^+$ Treg cells, we then asked whether these cells are functionally activated using an in vitro suppression assay. $CD4^+CD25^+$ cells were purified from FIV negative cats and either activated by LPS/rhIL2 as a positive control or by infection with FIV for 3 days prior to co-culture with ConA (5ug/ml) activated $CD4^+CD25^-$ cells. After 24 hours, culture supernatant was collected and assayed for IL2 by ELISA. For analysis of proliferation, CFSE-labeled $CD4^+CD25^-$ target cells were co-cultured as described for 72 hours prior to flow cytometric analysis and the percent suppression was calculated. As shown in figure 3A, $CD4^+CD25^+$ Treg cells infected in vitro with FIV for 3 days were able to suppress IL2 production by activated T helper cells to the same degree as LPS/rhIL2 activated CD4+CD25+ Treg cells. Importantly, treatment of $CD4^+CD25^+$ cells with UV inactivated virus for three days prior to co-culture did not result in activation of regulatory function as no significant change in IL2 levels were observed in these cultures. Depletion of GARP expressing cells from the FIV-

infected Treg pool reduced the suppressor capability of these effector cells, consistent with our previous studies demonstrating that $GARP^+$ Treg cells are highly efficient suppressors (18). In parallel with decreased IL2 production, FIV-activated $CD4^+CD25^+$ cells suppressed proliferation of ConA-activated $CD4^+CD25^-$ T helper cells, and depletion of the $GARP^+$ Treg cells abrogated suppression (Fig. 3B).

E. Discussion

AIDS-lentiviral infections are characterized by progressive immune dysfunction. Our lab has previously reported that Treg cells isolated from chronically FIV-infected cats are activated and capable of suppressing $CD4^+$ T helper cells in vitro. In support of this, researchers have reported the in vivo activation of $CD4^+CD25^+$ Treg cells during HIV and SIV infection, as well (22-24). Additionally, we have reported that $CD4^+CD25^+$ Treg cells are susceptible to FIV infection and support a productive infection as measured by release of infectious virions from provirus-bearing Treg cells (11-13, 25). However, a specific link between infection and activation has not been established.

We first validated our method of infection by demonstrating that purified $CD4^+CD25^+$ Treg cells infected at an MOI of 2.5 carried proviral FIV as measured by PCR. We also confirmed that this was a productive infection by measuring FIV p24-gag peptide in the culture medium at 6 days PI. To our knowledge, no other reports of productive Treg infection by FIV have been published with the exception of our own previous work on this subject (12, 13, 25).

Very little data is currently available regarding direct infection of human Treg cells by HIV but one report has provided evidence that Treg cells are susceptible to HIV infection and the degree of susceptibility is dependent on virus strain (26). Following productive infection, we evaluated the phenotypic and functional activation status of the $CD4^+CD25^+$ cells. We demonstrated that the Treg activation markers GARP and mTGFβ were upregulated following FIV infection. GARP:TGFβ complexes play an important role in Treg-mediated suppression as demonstrated in our previous publications and in related human-Treg studies (18, 27, 28). As such, we hypothesized that the expression of these markers on the Treg cell surface would correlate with suppressor function. Using an in vitro assay for suppression, we demonstrated that FIV-infected Treg cells suppressed activated $CD4^+$ Th cells. Importantly, we did not identify any such effects in Treg cells exposed to UV-inactivated virus.

In this study, we have demonstrated that lentiviral infection directly activates Treg cells. These data suggest that FIV infection directly activates $CD4^+CD25^+GARP^+$ Treg cells rendering them capable of suppressing $CD4^+CD25^-$ T helper cells. This virus-induced activation of a suppressor population suggests a novel method for chronic activation of Treg cells during infection and further demonstrates the significance of Treg FIV-susceptibility in shaping the immune response to this pathogen. The inability of UV treated virus to induce Treg activation suggests that it is not simply the presence of antigen but a specific mechanism during reverse transcription, retroviral incorporation or virion formation which triggers this response. .

F. Figures

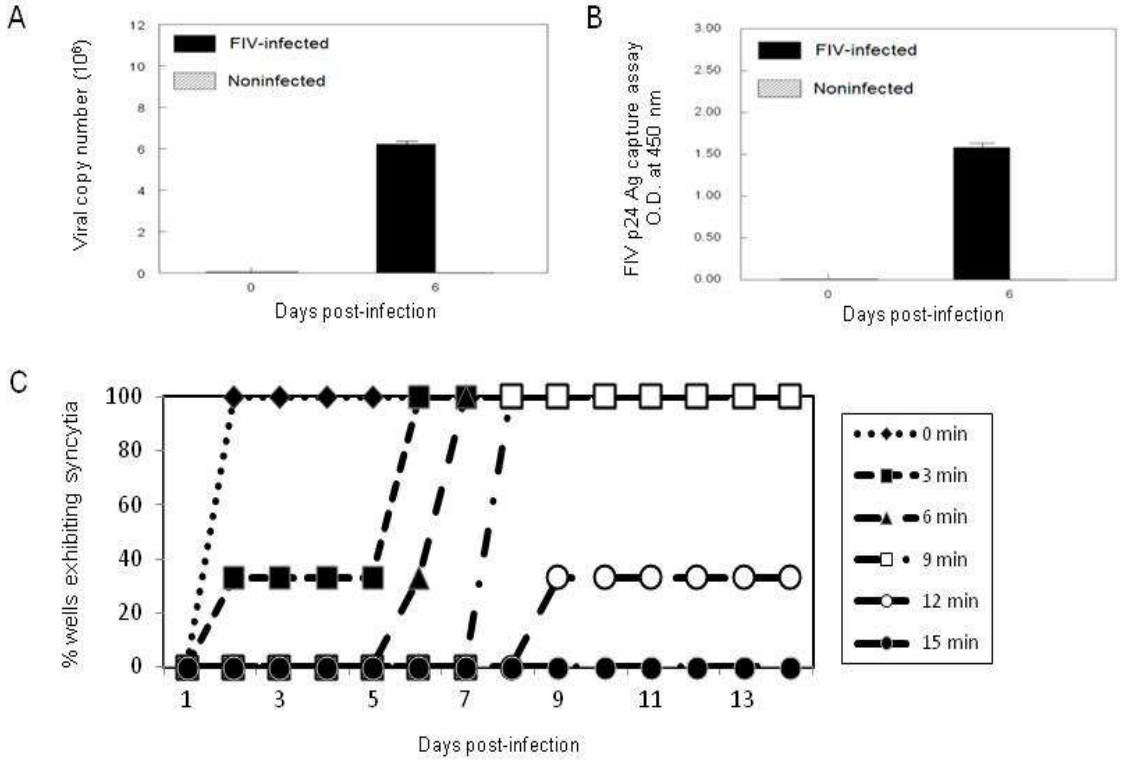


FIGURE 1. **Validation of $CD4^+CD25^+$ in vitro infection methods.** Purified feline $CD4^+CD25^+$ cells were infected with the $NCSU_1$ isolate of FIV at an MOI of 2.5. Cells and culture supernatant were harvest at 6 days post infection and analyzed for **A**. virus copy by real time RT-PCR and **B**. p24 antigen by ELISA. Each bar represents the mean +/- SEM of 3 replicates. **C.** UV inactivation of FIV was confirmed using the FIV-susceptible feline T cell line FCD4-E. Virus was exposed to 1 microjoule of UV light for various times then added to FCD4-E cells at an MOI of 2.5; cultures were observed daily up to 14 days for the formation of syncytia.

FIGURE 2. **In vitro FIV infection of purified $CD4^+CD25^+$ cells produces an activation phenotype.** FACS purified $CD4^+CD25^+$ cells were either untreated, in vitro infected with FIV at an MOI of 2.5, treated with UV inactivated virus, or were in vitro activated by 4 day LPS and IL2 stimulation. After treatment, expression of **A.** GARP or **B.** FoxP3 mRNA were analyzed by RT-PCR and expression of **C**. cell surface GARP or **D**. intracellular FoxP3 proteins were assessed by flow cytometry. **E.** GARP or **F.** FoxP3 detection by flow cytometry was also analyzed by MFI. **G**. A representative dot plot for GARP and FoxP3 flow cytometry analysis of FIV infected cells at 6 days PI is shown with percent co-expressing GARP and FoxP3 in the upper right corner. **H.** mTGFβ expression was assessed by flow cytometry and percent of cells expressing TGFβ is shown. (N = 4 for each treatment group, * $p < .05$ Mann-Whitney test for significance).

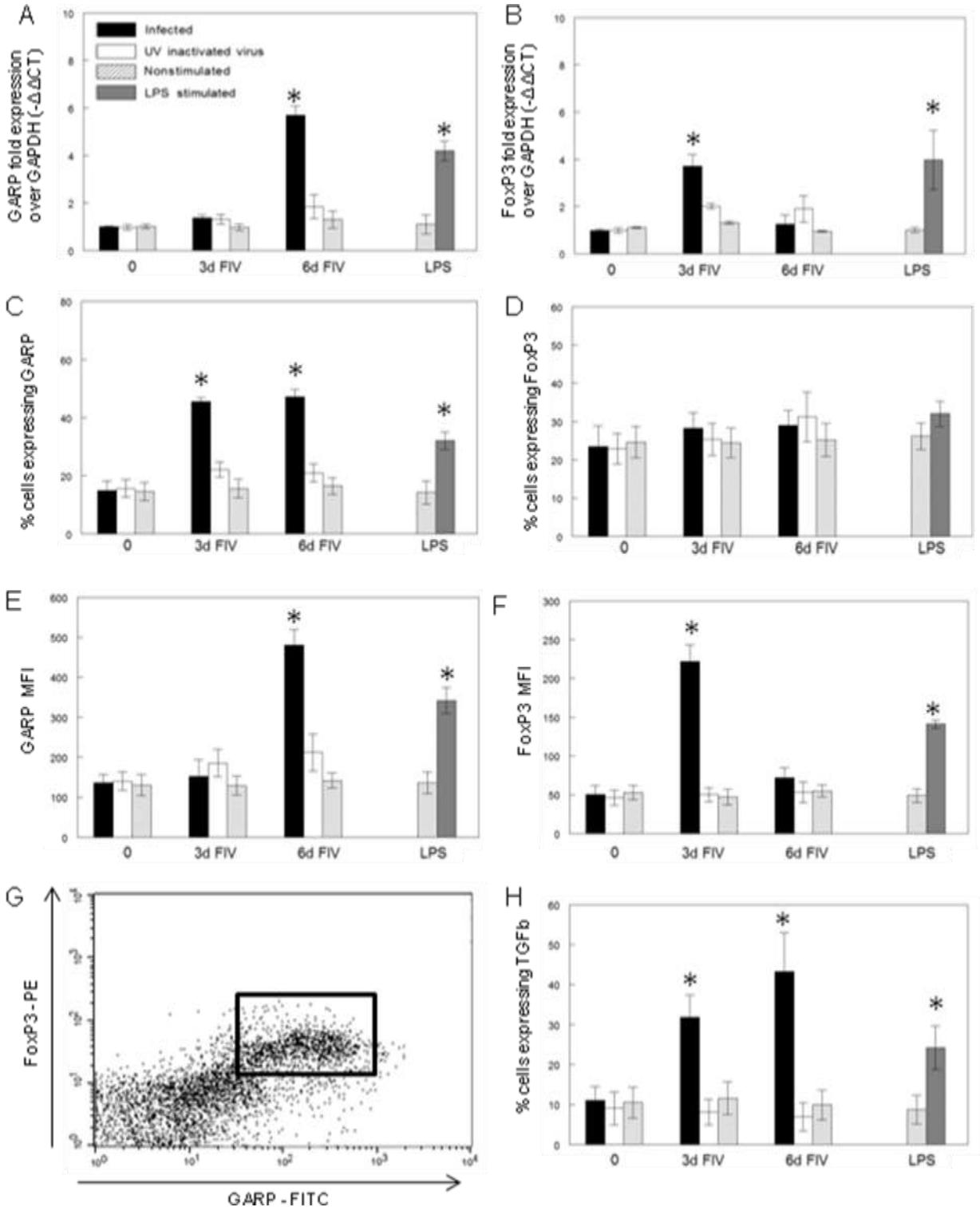
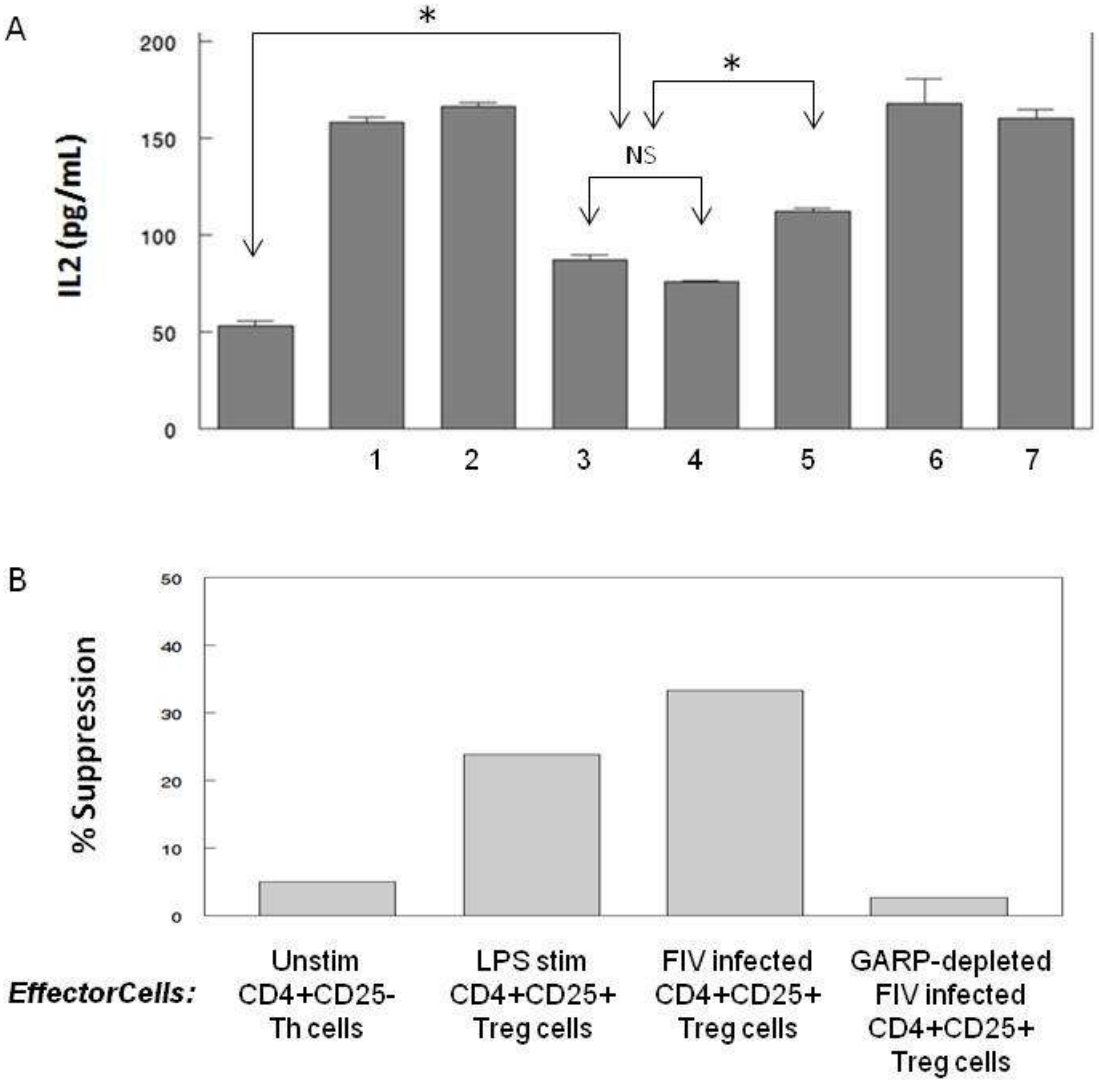

A
Infected
UV inactivated virus
Nonstimulated
LPS stimulated
GARP fold expression over GAPDH (-ΔΔCT)
0
3d FIV
6d FIV
LPS
B
FoxP3 fold expression over GAPDH (-ΔΔCT)
C
% cells expressing GARP
D
% cells expressing FoxP3
E
GARP MFI
F
FoxP3 MFI
G
FoxP3 - PE
GARP - FITC
H
% cells expressing TGFb

FIGURE 3. **FIV-activated Treg cells suppress $CD4^+CD25^-$ T helper cells.** $CD4^+CD25^-$ cells were FACS sorted from FIV negative cat PBMCs and used as target cells. APCs were prepared by FACS depletion of CD4 and CD8 expressing lymphocytes and added at a 1:1 ratio with target cells. Cells were stimulated for 1 hour with ConA (5ug/mL), washed, CFSE labeled and then plated. Effector (suppressor) cells were added at an effector to target cell ratio of 1:2. **A.** Supernatant was collected after 24 hours of co-culture and IL2 analyzed by ELISA in triplicate. Bars represent ConA-stimulated Th cells co-cultured with: (1) no effector cells, (2) nonstimulated $CD4^+CD25^-$ cells, (3) LPS activated Treg cells (4), FIV infected Treg cells, (5) GARP-depleted FIV infected Treg cells, (6) UV-FIV treated Treg cells, (7) GARP-depleted UV-FIV treated Treg cells. The unlabeled bar represents non-stimulated Th cells used as a negative assay control group. **B.** Proliferation was measured by Flow cytometry of CFSE labeled cells after 72 hours in co-culture with various effector groups. Proliferation index was analyzed for each group using ModFit LT and percent suppression was calculated as follows: [[(PI of stimulated $CD4^+CD25^-$ cells alone) – (PI of stimulated $CD4^+CD25^-$ cells with effector cells)]/ (PI of stimulated $CD4^+CD25^-$ cells alone)] x 100.


A
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EffectorCells:
Unstim CD4+CD25- Th cells
LPS stim CD4+CD25+ Treg cells
FIV infected CD4+CD25+ Treg cells
GARP-depleted FIV infected CD4+CD25+ Treg cells

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5. $CD4^+CD25^+$ T Regulatory cells activated in vivo during chronic FIV infection convert $CD4^+CD25^-$ Th Cells in vitro into phenotypic and functional Treg cells by a mTGFβ mechanism

This chapter represents a joint project. All conversion/proliferation assays involving [^{3}H]thymidine and the FoxP3 PCR analysis in Figure 5D were performed by Dr. Christopher Petty as part of his graduate work under the direction of Dr. Wayne Tompkins. The remaining assays were performed by Michelle Miller.

A. Abstract

Suppression of $CD4^+CD25^-$ T helper (Th) cell function by $CD4^+CD25^+$ T regulatory (Treg) cells is crucial for regulation of immune responses to self and foreign antigens. We have previously reported that TGFβ (mTGFβ) on the surface of activated Treg cells mediates suppressor function. More recently, we reported that Glycoprotein A Repetitions Predominant (GARP) anchors active mTGFβ on Treg cells and antibodies to GARP or TGFβ block Treg suppressor function. We have also demonstrated the expansion of $GARP^+TGF\beta^+$ Treg cells during the course of FIV infection. As Treg cells are anergic and cannot proliferate, we asked whether Treg homeostasis during lentiviral infection is maintained by recruitment from the $CD4^+$ Th cells pool and if GARP-bound TGFβ is essential to this recruitment. Here, we demonstrate that immunosuppressive Treg cells from FIV^+ cats express GARP and mTGFβ and are capable of converting Th cells to $CD4^+CD25^+GARP^+$ $TGF\beta^+FoxP3^+$ suppressor T cells. Th to Treg conversion was abrogated by anti-TGFβ or anti-GARP treatment of Treg cells or by anti-TGFβ-RII treatment of Th target cells, suggesting that Treg homeostasis and suppressor function are both mediated by TGFβ/TGFβ-RII signaling and that cell surface GARP plays a major role in this process.

B. Introduction

Thymus-derived natural T regulatory (Treg) cells are a distinct population of immunosuppressive $CD4^+$ lymphocytes identified by constitutive expression of CD25 (IL-2Rα-chain), GITR, CTLA-4 and the nuclear transcription factor, FoxP3 (1-6). In addition to the well described Treg cells involved in self-tolerance, a population of pathogen-induced Treg cells have been described which express biologically active membrane TGFβ (mTGFβ) when activated and play a major role in modulating immune responses to a variety of infectious agents by suppressing pathogen-induced effector cells (1, 2, 7, 8). The Treg specific expression of surface-bound TGFβ has recently been attributed to the glycoprotein A repetitions predominant (GARP) protein which is specifically expressed in the lymphoid compartment on regulatory cells and binds latent TGFβ to the Treg cell membrane; however, whether GARP functions in the conversion of latent TGFβ to biologically active TGFβ is not known (9-16). We recently reported that TGFβ is anchored to the Treg cell surface by GARP and that GARP-anchored TGFβ is biologically active and capable of suppressing Th cell function (9). Although there is considerable knowledge as to how $mTGF\beta^+$ Treg cells mediate suppression, there is less knowledge of the mechanism(s) that maintain their numbers and function in the peripheral immune compartment as they are anergic and cannot proliferate (17, 18). Chen et al (19) reported that TCR-engaged $CD4^+CD25^-$ T cells stimulated with TGFβ converted to a Treg phenotype, suggesting a mechanism of Treg homeostasis. For conversion of Th to Treg cells to occur, both Treg cells and target Th cells must be activated through TCR stimulation or by mitogenic stimulation (20). We previously reported that feline $CD4^+CD25^-$ Th cells could be converted to a Treg phenotype

($CD25^+$mTGFβ$^+$FoxP3 mRNA$^+$) by treatment with ConA followed by soluble TGFβ (21). These converted cells displayed immunosuppressive function against ConA stimulated $CD4^+CD25^-$ Th cells, suggesting that they possessed both the functional and phenotypic characteristics of activated Treg cells. To provide a mechanism for Th to Treg conversion, we demonstrated that ConA treatment of $CD4^+CD25^-$ Th cells up-regulates expression of TGFβ-RII on their surface, rendering them susceptible toTGFβ-mediated conversion to Treg cells (21). We also reported that anti-TGFβ-RII treatment of ConA-stimulated Th cells abrogated the Th to Treg conversion, supporting a role for TGFβ/TGFβ-RII signaling in this conversion process (21). Recent studies indicate that peripheral Treg cells, once activated, express both mTGFβ and GARP on their surface and that both molecules are instrumental in Treg cell suppressor function. It is not known if this TGFβ/GARP complex plays a role in recruitment of Treg cells from the Th cell pool but evidence suggests that it may be the key to contact-mediated TGFβ signaling in this context (12, 13).

The in vivo activation of Treg cells and subsequent suppression of $CD4^+$ Th cells has been demonstrated in HIV and FIV infection and thus represents an important component of lentiviral-induced immune suppression (1-3, 5, 6). We have previously demonstrated that $CD4^+CD25^+$ Treg cells are preferentially infected and activated during FIV infection and have identified GARP bound mTGFβ to be upregulated on the activated Treg cell surface (22-25). As the percentage of $CD4^+CD25^+$ cells among the $CD4^+$ fraction is consistent despite the overall decline in $CD4^+$ cell numbers over the course of HIV or FIV infection, a mechanism must exist by which $CD4^+CD25^+$ Treg cells are generated to maintain the

regulatory cell population (17, 18, 26-31). Here, we utilize activated $CD4^+CD25^+$ Treg cells from FIV-infected cats to determine if in vivo activated Treg cells displaying TGFβ:GARP on their surface could convert $CD4^+CD25^-$ Th cells to $CD4^+CD25^+$ Treg cells and if TGFβ:GARP played a role in this conversion. Data presented herein support this hypothesis by first demonstrating that TGFβ and GARP are up-regulated on the surface of $CD4^+CD25^+$ Treg cells and TGFβ-RII is up-regulated on $CD4^+$ Th cells from FIV-infected cats. Further, we show that co-culture of these two lymphocyte populations results in conversion of $CD4^+$ Th cells to phenotypic and functionally immunosuppressive $CD4^+CD25^+mTGF\beta^+GARP^+$ $FoxP3^+$ Treg cells. This Th to Treg conversion was blocked by either pretreatment of Treg cells with anti-TGFβ or anti-GARP antibodies or by pretreatment of target $CD4^+$ Th cells with anti-TGFβ-RII antibodies. These results further support the hypothesis that peripheral Treg cell homeostasis and suppressor function can be maintained in vivo by recruitment from the $CD4^+$ Th cell pool in a TGFβ:GARP dependent manner. In the case of lentivirus infections, where both Treg and Th cells are chronically activated, maintenance of $CD4^+CD25^+$ Treg numbers and function may be favored at the expense of the Th pool. Chronic, progressive recruitment of immunosuppressive Treg cells from the Th cell pool thus contributes to the Th cell immune deficiency characteristic of lentiviral infection.

C. Materials and Methods

Cats and FIV infection

Specific pathogen-free cats were obtained from Liberty Labs (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA) and housed at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Cats were inoculated with the $NCSU_1$ isolate of FIV, a pathogenic clade A virus, as described by Bucci et al (32). FIV-infection was confirmed by immunoblot analysis and provirus detection by PCR using primers specific for the FIV-p24 GAG sequence. At the time samples were taken, cats had been infected with FIV for at least 5 years and were clinically asymptomatic. Non-infected control cats ranged in age from 3 to 6 years and were housed separately from FIV-infected cats. Protocols were approved by the North Carolina State University Institutional Animal Care and Use Committee.

Sample Collection and Preparation

Whole blood (28 ml/cat) was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). PBMC were isolated by Percoll density gradient centrifugation (Sigma-Aldrich, St. Louis, MO) as previously described (33) or by Ficoll-Histopaque-1077 density gradient centrifugation (Sigma-Aldrich, St-Louis, MO) following the manufacturer's guidelines. Single-cell suspensions were prepared from lymph nodes obtained through biopsies by gently and repeatedly injecting sterile PBS into the tissue using 18G needle until the cells were released from the tissue. Cell counts and viability were determined by trypan blue dye exclusion and viability was always >90%.

Reagents and Antibodies

Recombinant human IL2 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Maurice Gately, Hoffmann - La Roche Inc. LPS and Concanavalin A (ConA) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-mouse IgG coated magnetic Dynabeads® M-450 were purchased from Dynal (Great Neck, NY). Streptavidin–PerCP was purchased from BD Biosciences PharMingen (San Diego, CA). Anti-TGFβ1 (MAB240) was purchased from R&D Systems (Minneapolis, MN) and conjugated to allophycocyanin (APC) or left unconjugated for blocking studies; PE-conjugated anti-TGFβ-RII (FAB241P), neutralizing anti-TGFβRII (AF-241-NA), and recombinant human TGFβ1 (240-B) were also purchased from R&D Systems. Mouse anti-feline CD25 (mAb 9F23) was kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan). Anti-CD21 was purchased from Serotec (Raleigh, NC). Mouse anti-feline CD4 (mAb 30A) and CD8 (mAb 3.357) were developed in our laboratory (34). PE conjugated rat anti-mouse FoxP3 (FJK-16s) was purchased from eBioscience (San Diego, CA). FITC-conjugated anti-GARP IgG2b monoclonal antibody (LRRC32, Plato-1) was purchased from Enzo life Sciences (Ann Arbor, MI).

Flow Cytometric Analysis

For comparison of TGFβ and TGFβ-RII in chronic FIV-infected or uninfected cats, at least $5x10^5$ PBMC were stained for surface expression of CD25, CD4 and TGF-β or CD25, CD4 and TGFβ-RII. Three-color flow cytometry was performed on a FACSCaliber flow cytometer (BD Biosciences, Mountain View, CA). Lymphocytes were gated based on

forward vs. side scatter, and 20,000 gated events were acquired and stored list-mode fashion for analysis using CellQuest software. For phenotyping studies, sorted and/or cultured cells were stained for surface expression of CD4, CD25, TGFβ, GARP and TGFβ-RII and analyzed on the FACSCaliber. For intracellular staining of FoxP3 and Helios, cells were stained for CD4 and CD25 expression, washed in PBS, incubated with 4% PFA for 10 minutes, and washed twice more. Cells were then incubated in 0.1% Triton x-100 for 30 minutes, washed with PBS + 4% FBS, resuspended in 100 uL of PBS and incubated with FoxP3-specific antibody at room temperature for 20 minutes. Cells were washed overnight in PBS + 4% FBS and analyzed on an LSRII flow cytometer (BD Biosciences, Mountain View, CA). Lymphocytes were gates on forward vs. side scatter and 20,000 gated events were acquired and stored list-mode fashion for analysis using BD FACSDiva software. All gating was determined by isotype controls.

Purification of lymphocyte populations

$CD4^+CD25^+$ and $CD4^+CD25^-$ cell populations were purified as previously described (3, 21). Briefly, for FACS purification, PBMCs or LN cells were stained with anti-CD4, anti-CD25 and anti-CD8. $CD4^+CD25^+$, $CD4^+CD25^-$ and $CD4^-CD8^-$ (APCs) cell subsets were purified using a high-speed, high-purity fluorescence activated cell sorter (MoFlo, DakoCytomation). The purity of FACS sorted cell populations was always > 95%. $CD4^+$ $CD25^-$ T cells for use as target cells in proliferation assays were enriched using biomagnetic bead separation using goat anti-mouse IgG-coated beads as described by Bucci et al (32). Briefly, $CD21^+$ B cells, $CD8^+$, and $CD25^+$ T cells were depleted in successive steps using magnetic beads coated with

anti-CD21, anti-CD8 and anti-CD25 antibody respectively. Purity of the magnetic bead enriched $CD4^+CD25^-$ T cells was > 90%, as verified by flow cytometric analysis.

Treg cell-induced conversion assay

Converter cells consisted of purified $CD4^+CD25^+$ T cells from either control cat or FIV-infected cat PLN. In the case of control cats, the converter cells were activated by culturing ($4x10^6$ per well, 24-well plate) for 4 days in the presence of LPS (10 ng/mL) plus IL2 (100 U/mL) then washed twice and labeled with Vybrant DiD (Molecular Probes) fluorescent dye. In the case of FIV-infected cats, as the $CD4^+CD25^+$ T cells are constitutively activated, the converter cells were freshly isolated and labeled with Vibrant DiD (see Fig. 2). Converter cell control groups consisted of $CD4^+CD25^-$ or $CD4^+CD25^+$ cells from control cats incubated in IL2 (100 U/ml) alone for 4 days then labeled with DiD. The positive control for conversion of $CD4^+CD25^-$ Th was a 5d treatment of sorted cells with 5 μg/mL ConA and 10 ng/mL soluble TGFβ which has been described in previous conversion experiments (21). The target cells for testing conversion cell immunosuppressive function consisted of autologous unlabeled $CD4^+CD25^-$ T cells freshly purified from peripheral blood and stimulated with 5 μg/mL ConA and 100U/ml IL2 for 4 h. The target cells were washed and added to the converter cell cultures at a converter to target cell ratio of 1:2. Cultures were kept at 37°C for 5 days in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10U/ml IL2. After 5 days, DiD positive cells were FACS depleted and the remaining cells were used in suppression assays, cell surface molecule analysis, and

mRNA analysis. The purity of DiD-depleted cells was >98% as verified by flow cytometric analysis. For conversion blocking experiments, 100 µg/ml anti–TGF-β was added to the converter cell population, or 100 µg/ml anti–TGF-βRII was added to the target cell population just prior to co-culture. Experimental design is outlined in Figure 2.

In vitro T cell IL2 Suppression Assay

PLN cells from FIV-negative cats were FACS purified into $CD4^+CD25^-$ target cells and $CD4^-CD8^-$ Antigen Presenting Cells (APCs), combined at a 1:1 ratio, stimulated for 4 h with 5 µg/ml Con A, washed twice in RPMI 1640, and plated at 2 x 10^6 cells/mL in 12-well plates. DiD negative cells from the conversion assays described above were added as suppressor cells at a suppressor to target cell ratio of 1:2. Effector cell controls for the suppressor assay consisted of $CD4^+CD25^+$ cells from control cats that had been stimulated for 4 days with 10 µg/ml LPS and 100 U/ml rhIL2 (positive control) or nonstimulated $CD4^+CD25^-$ cells from control cats (negative control) as suppressor cells. A positive control consisted of ConA stimulated $CD4^+CD25^-$ cells plus APCs without effector cells and a negative control consisted of non-stimulated $CD4^+CD25^-$ cells plus APCs without effector cells. After 24 hours, 100uL of the supernatant from each well was analyzed in triplicate by IL2 ELISA using the Feline IL2 DuoSet DY1890 (R&D Systems, Minneapolis, MN) as per manufacturer's protocol.

In vitro T cell Proliferation Suppression Assay

Enriched $CD4^+CD25^-$ target cells (10^6 cells/ml) were stimulated for 4 h with 5 µg/ml Con A, washed twice in RPMI 1640, and plated at 5 x 10^4 viable cells/well in 96-well U bottom plates. DiD negative cells from the conversion assays described above were added as suppressor cells at suppressor to target cell ratios ranging from 0.125:1 to 1:1. Controls for the suppressor assay consisted of $CD4^+CD25^+$ cells from control cats that had been stimulated for 4 days with 10 µg/ml LPS and 100 U/ml rhIL2 (positive control) or non-stimulated $CD4^+CD25^-$ cells from control cats (negative control) as suppressor cells. Effector and target cells were co-cultured at 37°C for 72 hrs, pulsed with 1 µCi of [^{3}H]TdR/well for the last 18 h and harvested using a Filtermate Harvester (Packard Bioscience, Meriden, CT). [^{3}H]thymidine incorporation was measured using a Top Count NXT Microplate scintillation counter (Packard Bioscience). Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ target cells alone and calculated as follows:

$$\textit{percent inhibition} = [(CD4^+CD25^- \textit{ alone} - CD4^+CD25^+) \textit{ cpm}/(CD4^+CD25^- \textit{ alone}) \textit{ cpm}] \times 100.$$

For suppressor activity blocking experiments, the suppressor cells were pretreated with 100 µg/ml anti-TGF-β for 30 minutes then washed, counted, and added to the target cells. Assays were run in triplicate.

Reverse Transcription PCR analysis of FoxP3

FoxP3 mRNA was detected by RT-PCR using feline specific primers for FoxP3 as described previously (21). GAPDH mRNA expression was used as a normalizing control. Briefly, total

RNA was isolated from $1x10^6$ $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells using RNeasy Protect Mini Kit (Qiagen, Valencia, CA). Reverse transcription was carried out using a reverse transcription system kit from Promega as per the manufacturer's protocol, followed by PCR using HotStar Taq polymerase (Qiagen, Valencia CA). PCR products were resolved on agarose/ethidium bromide gels and visualized using GelRed (Biotium, Hayward CA).

Statistical Analysis

The Mann-Whitney U test (t test for nonparametric data) was used for pair wise comparison of parameters. Differences were considered to be significant at $p < 0.05$.

D. Results

Membrane-bound TGF-β (mTGF-β) and GARP on the surface of $CD4^+CD25^+$ Treg cells and TGFβ-RII expression on $CD4^+CD25^-$ Th cells are increased during chronic FIV-infection

We have reported that ConA/TGFβ stimulated $CD4^+CD25^-$ Th cells were converted to immunosuppressive $CD25^+FoxP3^+mTGFβ^+$ Treg cells and that this conversion could be abrogated by pre-treatment of ConA-stimulated Th target cells with anti-TGFβ-RII (21). As Vahlenkamp, et al. (4) reported that $CD4^+CD25^+$ Treg cells from FIV^+ cats are constitutively activated in vivo, we asked if these activated $CD4^+CD25^+$ Treg cells from FIV^+ cats upregulate the activation markers GARP and mTGFβ in response to infection. PBMC were isolated from 6 FIV negative control cats and 6 chronically FIV-infected cats then analyzed

for surface expression by flow cytometry. As shown in Figure 1, a significant increase in the percent of $CD4^+CD25^+$ T cells expressing both GARP and mTGFβ was observed in FIV-infected cats when compared to FIV negative control cats (Fig. 1A and 1B). Given that long-term FIV infection is associated with chronic T cell activation and with increased Treg suppressor function (4, 35, 36), we have hypothesized that mTGFβ on activated Treg cells mediates suppressor function against Th cells expressing TGFβ-RII. To investigate the potential for TGFβ-mediated suppression, we analyzed the expression of TGFβ-RII on the surface of $CD4^+CD25^-$ cells from FIV^+ cats. As shown in Figure 1C, there is a significant increase in the percent of both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from FIV^+ cats expressing TGF-βRII compared to control cats, consistent with our hypothesis.

$CD4^+CD25^+$ Treg cells from FIV^+ cats convert $CD4^+CD25^-$ Th cells to a Treg phenotype.

Chen et al. (19) demonstrated that TGFβ treatment coupled with TCR co-stimulation of Th cells induces Treg phenotype and function. We also reported a similar Th to Treg conversion of feline $CD4^+CD25^-$ cells stimulated in vitro with ConA and TGFβ (21). Increased expression of TGFβ on Treg cells and TGFβ-RII on Th cells could promote interaction between these two T cell subsets and convert Th cells to Treg cells. To test this, rhIL-2/LPS-activated $CD4^+CD25^+$ Treg cells from SPF control cats or freshly isolated $CD4^+CD25^+$ Treg cells from FIV^+ cats were membrane labeled with Vybrant DiD then co-cultured with ConA-activated, non-labeled $CD4^+CD25^-$ Th target cells as illustrated in Figure 2. After 5 days of co-culture, the DiD positive cells were depleted by FACS and the phenotype and suppressor

function of the remaining cells, referred to herein as induced Treg (iTreg) cells, were analyzed. A positive control for conversion consisted of $CD4^+CD25^-$ cells from FIV negative cats cultured with ConA and soluble TGFβ as described previously (21). Negative controls consisted of $CD4^+CD25^-$ cells cultured in medium alone or in medium supplemented with either 5 ug/mL ConA or 100 U/mL IL2.

Following culture of $CD4^+CD25^+$ PLN cells from FIV-infected cats with $CD4^+CD25^-$ cells from FIV negative cats, iTreg cells were analyzed by flow cytometry for the expression of CD25, GARP, TGFβ, TGFβ-RII and FoxP3. Figure 3A shows representative dot plots from one conversion assay and Figure 3B shows the averages of 3 experiments. Non-stimulated $CD4^+CD25^-$ Th cells do not express the Treg-associated surface proteins CD25, GARP, or TGFβ and less than 10% of these cells express TGFβ-RII, consistent with a resting Th population. Treatment of Th cells with ConA or IL2 alone resulted in an increase percent of cells expressing CD25 and TGFβ-RII, suggesting Th cell activation, but there was no increase in cells expressing the Treg markers GARP, mTGFβ or FoxP3. Only culturing in the presence of ConA and TGFβ, as we have previously demonstrated, or culturing with activated Treg cells from FIV^+ cats resulted in the expression of GARP, mTGFβ, and FoxP3, as well as CD25 and TGFβ-RII on approximately a third of the Th cells. As the forkhead transcription factor FoxP3 is the defining marker of $CD4^+CD25^+$ Treg cells and is required for both homeostasis and suppressor function (37), we also analyzed FoxP3 mRNA expression in Th cells converted to iTreg cells. Similar to the flow cytometry analysis, only Th cells cultured in the presence of ConA and TGFβ or with activated Treg cells lead to the

expression of FoxP3 or GARP mRNA (Fig. 3C and 3D). This phenotype ($CD25^+GARP^+mTGF\beta^+FoxP3^+$) is consistent with Treg cells and suggests that activated Treg cells are capable of converting Th cells to a Treg phenotype.

$CD4^+CD25^+$ T cells from FIV-infected cats convert $CD4^+CD25^-$ Th cells into iTreg cells capable of suppressing Th cell IL2 production and proliferation.

As $CD4^+CD25^-$ Th cells can be converted in vitro into phenotypic iTreg cells, we asked if these iTreg cells were functional suppressor cells. ConA stimulated $CD4^+CD25^-$ target cells were co-cultured with iTreg effector cells at a 1:2 effector to target ratio for 24 hours, then culture supernatant was collected and analyzed for IL2 by ELISA. As shown in Figure 4A, IL2 produced by the activated $CD4^+CD25^-$ cells was significantly reduced by the addition of LPS-activated $CD4^+CD25^+$ effectors but no change was observed by the addition of $CD4^+CD25^-$ cells. Importantly, addition of iTreg cells that were converted by co-culture resulted in a significant reduction of IL2 to a level consistent with activated Treg cells. iTreg suppressor function was also assayed by a standard proliferation assay of ConA-activated $CD4^+CD25^-$ T cells. The suppressor function of the converted iTreg cells was similar to suppression by isolated $CD4^+CD25^+$ Treg cells from FIV^+ cats (Fig. 4B). These results demonstrate that in addition to displaying the phenotypic characteristics of a regulatory cell population, the iTreg cells produced from co-culture of Th cells with Treg cells from FIV^+ cats have functional characteristics of Treg cells.

To confirm that conversion of $CD4^+CD25^-$ cells into iTreg cells was due to activated Treg cells and not an artifact of co-culture or FIV infection, Th cells were co-cultured with DiD stained $CD4^+CD25^+$ or $CD4^+CD25^-$ cells from normal cats treated as follows prior to co-culture: IL2/LPS, shown previously to activate Treg cells in vitro (4, 9), or IL2 alone as a negative control for Treg activation. After the 5 day co-culture, the DiD "converter" population was depleted and the remaining cells were assayed for effector function using the $[H^3]$-Thymidine assay for proliferation of activated Th target cells. Similar to what we observed with in vivo activated $CD4^+CD25^+$ Treg cells from FIV^+ cats, in vitro activated $CD4^+CD25^+$ cells convert Th cells into functional iTreg cells as measured by inhibition of proliferation of ConA stimulated Th target cells (Figure 4C, open squares). In contrast, neither $CD4^+CD25^+$ cells treated with IL2 alone (Fig. 4C, open triangles) nor $CD4^+CD25^-$ cells treated with IL2/LPS (Fig. 4C, open circles) or IL2 alone (Fig. 4C, closed circles) were capable of converting Th cells into functional iTreg cells.

Blockade of TGFβ/TGFβ-RII Interaction Abrogates $CD4^+CD25^+$ Treg-Induced Conversion of $CD4^+CD25^-$ Th cells to iTreg cells.

To confirm that $CD4^+CD25^+$ Treg cells mediate conversion of $CD4^+CD25^-$ Th to iTreg cells by a TGFβ/TGFβ-RII signaling pathway, DiD labeled $CD4^+CD25^+$ Treg cells from FIV-infected cats were pre-treated with anti-TGFβ or $CD4^+CD25^-$ Th target cells were pre-treated with anti-TGFβ-RII antibodies prior to the 5 day co-culture. To confirm that these antibodies successfully blocked surface proteins, flow cytometric analysis for TGFβ and TGFβ-RII surface expression was performed following pretreatment of cells with blocking antibodies.

Pretreatment of $CD4^+CD25^+$ cells with anti-TGFβ (Fig. 5A) or of $CD4^+CD25^-$ cells with anti-TGFβ-RII (Fig. 5B) for 15 minutes prior to flow analysis prevented the binding of detection antibodies, demonstrating efficient blocking of surface proteins. After blocking TGFβ on DiD-labeled $CD4^+CD25^+$ cells or TGFβ-RII on $CD4^+CD25^-$ cells, cells were co-cultured for 5 days. DiD^+ cells were then depleted and the remaining cells analyzed for phenotypic and functional characteristics of iTreg cells. Flow analysis revealed that anti-TGFβ pre-treatment of $CD4^+CD25^+$ T cells, anti-GARP pre-treatment of $CD4^+CD25^+$ T cells, and/or anti-TGFβ pre-treatment of $CD4^+CD25^-$ cells prior to the 5 day co-culture significantly reduced the expression of TGFβ on the surface of the $CD4^+$ Th cells as compared to the isotype control treated cell cultures (Fig. 5C) and prevented the expression of FoxP3 mRNA (Fig. 5D), suggesting that mTGFβ on Treg cells and TGFβ-RII on Th cells are crucial for the Treg-mediated conversion of $CD4^+CD25^-$ Th cells to a $mTGF\beta^+$ iTreg phenotype. In keeping with a lack of phenotypic conversion the blockade of mTGFβ on Treg converter cells or blockade of TGFβ-RII on Th target cells before the 5 day co-culture abrogated the suppressor function of the target cells (Fig. 5E). To confirm that the suppression by Treg cells requires TGFβ, neutralizing anti-TGFβ was added into the media during the proliferation assay. Blocking TGFβ effectively eliminated the ability of iTreg cells to inhibit the proliferation of activated $CD4^+CD25^-$ Th target cells (Fig. 5E). In figure 5F, suppressor function of iTregs was evaluated by IL2 suppression assay following conversion cultures. Here, $CD4^+CD25^+$ converter cells were left untreated or treated with TGFβ or GARP blocking antibodies and/or $CD4^+CD25^-$ Th target cells were treated with TGFβ-RII blocking antibodies prior to the conversion assay. Blocking TGFβ or TGFβ-RII

separately during conversion reduced the ability of iTregs to suppress activated Th targets (5F, treatments 4 and 5). Blocking both TGFβ on Treg converter cells and TGFβ-RII on Th target cells during the converter assay or blocking GARP during the converter assay completely eliminated the suppressor capacity of these cells (5G, treatments 6 and 7). Thus, both the conversion of Th cells to a Treg phenotype and subsequent suppressor function of the converted cells are dependent upon GARP-bound TGFβ and TGFβ-RII signaling.

E. Discussion

While it is well established that pathogen-induced Treg cells play a central role in maintaining the balance between immunity and immunopathology to infectious agents in the peripheral immune compartment (1, 2, 8, 17), mechanisms regulating their homeostasis and suppressor function, particularly in the case of AIDS lentivirus infection remain unclear. It is essential that there exist a mechanism for maintaining Treg cell peripheral homeostasis and suppressor function, as they comprise a small fraction (5-10%) of the total $CD4^+$ T cell population in the blood despite the fact that they do not produce IL2, are anergic and cannot expand without Th cell help (4). As it has been established that Treg cells are chronically activated and constitutively immunosuppressive in vivo throughout the course of FIV and HIV infections (4, 22, 38, 39), we examined the possibility that these activated Treg cells in FIV-infected cats are capable of converting $CD4^+$ Th cells into a Treg phenotype, thereby maintaining population numbers. Some data suggests that Treg cell homeostasis can be maintained in the periphery by expansion of the existing Treg population during responses to

several pathogens including SIV, HIV, and FIV (40, 41), and molecules such as LPS and IL2 have been shown to induce Treg proliferation (20, 42). However, Chen et al. (19) reported that $CD4^+CD25^-$ Th cells could be converted into $FoxP3^+$ immunosuppressive Treg cells in vitro by stimulation with TGFβ in combination with TCR engagement, suggesting an additional mechanism of Treg peripheral homeostasis that is not dependent on expansion of existing Treg cells.

Recent studies in our laboratory (21) demonstrated that stimulation of $CD4^+CD25^-$ Th cells with a combination of ConA and TGFβ converted them into $CD25^+mTGF\beta^+FoxP3^+$ suppressor cells. These finding are supported by other research identifying that soluble TGFβ treatment of TCR-activated Th target cells expressing TGFβ-RII in mice will induce regulatory activity (43, 44). In support of this, we were able to demonstrate that, in contrast to control cells, a significant number of Treg cells in FIV^+ cats are mTGFβ positive and that a fraction of Th cells, as well as Treg cells express the TGFβRII. To test the possibility that Th to Treg conversion could occur in the peripheral lymphoid tissue of FIV^+ cats, an ex vivo model for conversion was designed. First, $CD4^+CD25^+$ Treg cells from FIV^+ cats were co-cultured with $CD4^+CD25^-$ cells from FIV negative cats for 5 days and the latter analyzed by flow cytometry for Treg-like phenotype and assayed for suppressor activity as evidence for conversion into functional iTreg cells. Following this co-culture the converted cells displayed the Treg-cell markers CD25, GARP, TGFβ, and FoxP3, consistent with a regulatory cell phenotype. The expression of GARP was unexpected as these genes are reportedly restricted to natural thymus-derived Treg cells in the humans and mice (45). Here

we identified these markers on iTregs developed through ConA/sTGFβ treatment as well as through co-culture of Th cells with FIV^+ Treg cells. These findings highlight a key difference between feline Treg cells and murine or human Treg cells. Additionally, the expression of GARP and mTGFβ on these cells indicates that these are activated iTreg cells. This conclusion is supported by IL2 suppression assay data utilizing the iTreg cells as effector cells. Following conversion and in the absence of any additional stimulation, these iTreg cells were capable of suppressing IL2 production from activated $CD4^+CD25^-$ Th cells in vitro at a level comparable to activated Treg cells. The data presented herein clearly show that co-culturing of Treg cells from FIV^+ cats with naive Th cells converts the latter into immunosuppressive iTregs. These results support the observation of Dieckmann et al. (20) that activated $CD4^+CD25^+$ Treg cells are capable of inducing a Treg phenotype from activated Th cells, and suggest that Treg cells may maintain homeostasis by recruitment from the Th cell pool.

In addition to up-regulation of TGFβ on $CD4^+CD25^+$ Treg cells from FIV^+ cats, significantly more $CD4^+CD25^+$ Treg cells were found to express the TGFβ-RII than $CD4^+CD25^-$ Th cells. We know of no such reports of TGFβ-RII on $CD4^+CD25^+$ T cells in other models of Treg activation. $mTGF\beta^+$ Treg cells could interact with heterologous $TGF\beta\text{-}RII^+$ Treg cells but we hypothesize that positive feedback could be occurring in which $TGF\beta\text{-}RII^+$ Treg cells engage in autocrine signaling, which would be important to maintaining peripheral Treg anergy as well as suppressor function and homeostasis. Additional studies will be required to answer these questions.

Marie et al. (46) reported that FoxP3 expression in $CD4^+$ T cells and the size of the Treg cell compartment, as well as suppressor function, are dependent on signals induced by TGFβ in the periphery, consistent with our previous observation that TGFβ stimulation of ConA-activated Th cells converts them to a Treg phenotype (21). The data presented in this study support this conjecture and demonstrate that engagement of mTGFβ on Treg cells from FIV^+ cats with TGFβ-RII^+ Th cells converts the latter to Treg cells. The role of TGFβ/TGFβ-RII signaling was confirmed by the demonstration that pre-treatment of mTGFβ^+ Treg cells with anti-TGFβ antibodies, or pre-treatment of TGFβ-RII^+ Th target cells with anti- TGFβ-RII antibodies prior to co-culture abrogated conversion of Th cells to a Treg phenotype.

As others have reported that TGFβ regulates the expression of FoxP3 in Treg cells (47) and as FoxP3 is required for Treg homeostasis and suppressor function (31, 46), we also examined FoxP3 expression in the converted Th cells. These experiments revealed that Th cells from FIV^+ cats co-cultured with autologous Treg cells markedly up-regulated FoxP3 mRNA expression. These data collectively demonstrate that peripheral Treg homeostasis may be regulated by recruitment from the Th cell pool by mTGFβ/TGFβ-RII signaling, and that FIV infection may disrupt the normal balance between Th and Treg cells by chronic activation of both Treg and Th cells, shifting the equation in favor of Treg cells.

Importantly, it appears that in the case of AIDS lentivirus infection, the processes which may be a normal component of the immune response to pathogens could be skewed in favor of

Treg cells with iTreg recruitment at the expense of the Th cell pool and may contribute to the $CD4^+$ Th immune deficiency associated with these infections. Early and long-lasting T cell immune dysfunction in AIDS lentivirus and other chronic viral infections may be the result of Th to Treg conversion mediated by activated $mTGF\beta^+CD4^+CD25^+$ Treg cells that limit expansion and effector function of anti-viral $CD4^+$ T cells by converting them to $CD25^+mTGF\beta^+FoxP3^+$ Treg cells. This is an important concept, as the relative balance between $CD4^+$ immune responses and $CD4^+CD25^+$ Treg immune suppression during the acute stage of virus infection may help to determine the ultimate virus set point and the long-term ability to control viremia, and in effect predict disease progression. A more in depth understanding of conversion events and the emergence of iTreg populations during acute infection would be instrumental in understanding this process. The data presented in this manuscript takes this argument a major step forward in demonstrating that the FIV-AIDS lentivirus infection is capable of activating $CD4^+CD25^+$ Treg cells in vivo that are in turn capable of interacting with $TGF\beta\text{-}RII^+CD4^+$ Th cells by $mTGF\beta$ engagement of $TGF\beta$-RII and thus converting the Th cells into Treg cells. This mechanism could not only explain Treg homeostasis and function, but provide a plausible explanation for AIDS lentivirus-induced $CD4^+$ T cell immune dysfunction.

F. Figures

FIGURE 1. **PBMCs from FIV-infected cats display increased surface expression of TGFβ and GARP on $CD4^+CD25^+$ lymphocytes and TGFβ-RII on $CD4^+CD25^-$ lymphocytes.** PBMCs from chronically FIV-infected or control cats were analyzed for surface expression of CD4, CD25, TGFβ, GARP and TGFβ-RII by flow cytometry using specific antibodies. Cells were first gated on $CD4^+CD25^+$ or $CD4^+CD25^-$ populations and analyzed for percent expression of either **A.** TGFβ, **B.** GARP or **C.** TGFβ-RII. Box-whisker plots are shown representing the 5^{th} and 95^{th} percentiles (whisker), 25^{th} and 75^{th} percentiles (box), and median of percent $CD4^+CD25^+$ and $CD4^+CD25^-$ expression from 6 FIV-positive and 6 FIV-negative cats. Symbols represent individual cats. $CD4^+CD25^+$ cells from FIV^+ cats express more TGFβ and GARP when compared to $CD4^+CD25^+$ cells from FIV^- cats. $CD4^+CD25^-$ cells from FIV^+ cats express more TGFβ-RII than $CD4^+CD25^-$ cells from FIV^- cats. ($p < 0.05$, Mann-Whitney test for significance)

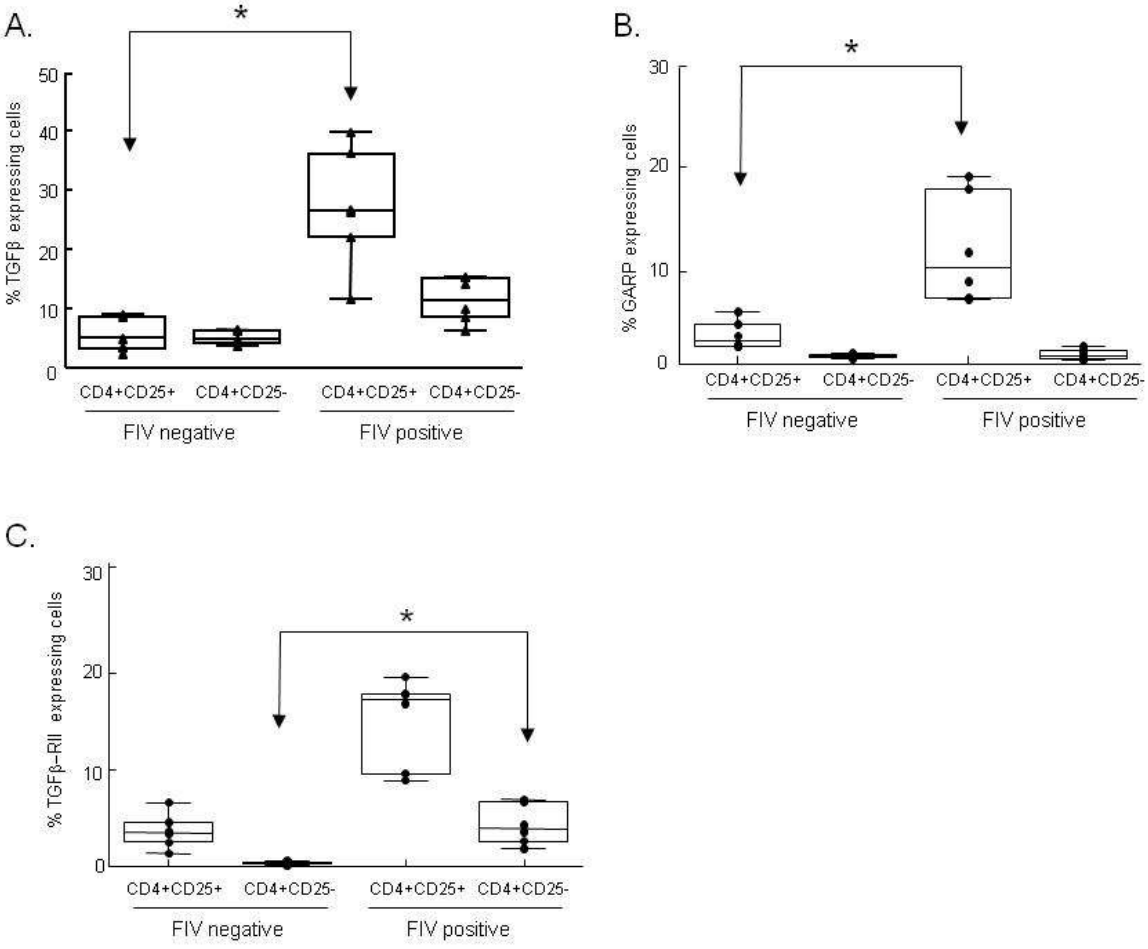

A.
*
% TGFβ expressing cells
50
40
30
20
10
0
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
FIV negative
FIV positive
B.
*
% GARP expressing cells
30
20
10
0
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
FIV negative
FIV positive
C.
*
% TGFβ–RII expressing cells
30
20
10
0
CD4+CD25+
CD4+CD25-
CD4+CD25+
CD4+CD25-
FIV negative
FIV positive

FIGURE 2. **Experimental design for $CD4^+CD25^+$ Treg cell-mediated conversion of feline $CD4^+CD25^-$ T cells to iTreg cells.** Single cell suspensions were prepared from PLNs collected from FIV-infected or SPF control cats. Cells were stained with feline anti-CD25 FITC and anti-CD4 PerCP labeled monoclonal antibodies and $CD4^+CD25^+$ T cell populations were purified by FACS. Converter $CD4^+CD25^+$ cells from control cats were stimulated with LPS/IL2 for 4 days, whereas $CD4^+CD25^+$ cells from FIV-infected cats were not stimulated prior to co-culture. PBMCs from FIV negative cats were FACs purified into CD4+CD25- Th target cells or CD4-CD8- APCs and stimulated for 4 hours with ConA (5 ug/mL) plus IL2 (100 U/mL). Converter cells were labeled with Vybrant DiD membrane dye and co-cultured with the purified $CD4^+CD25^-$ Th cells at a converter to target cell ratio of 1:2 in medium supplemented with 100 U/mL IL2. After 5 days, DiD membrane negative cells were FACS purified from the co-culture and analyzed by flow cytometry and PCR for expression of Treg specific proteins or genes. These "iTreg" cells were also used as effector cells in functional assays to determine suppressor capacity. PBMCs from FIV negative cats were again sorted into $CD4^+CD25^-$ and $CD4^-CD8^-$ populations, stimulated with ConA (5 ug/mL) and IL2 (100 U/mL) for 4 hours, then co-cultured with the iTregs at various effector:target cell ratios. Supernatant from cultures were analyzed by IL2 ELISA after 24 hours and $CD4^+CD25^-$ target cell proliferation was assessed by a standard 72 hour [^{3}H] thymidine assay.

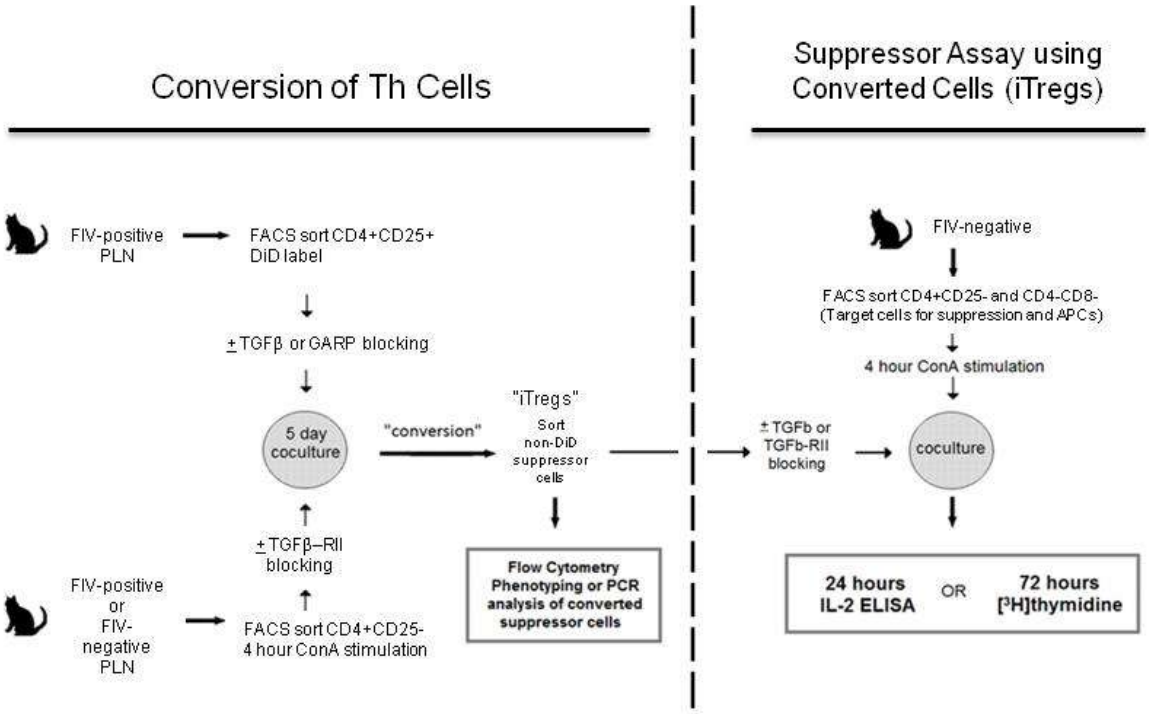
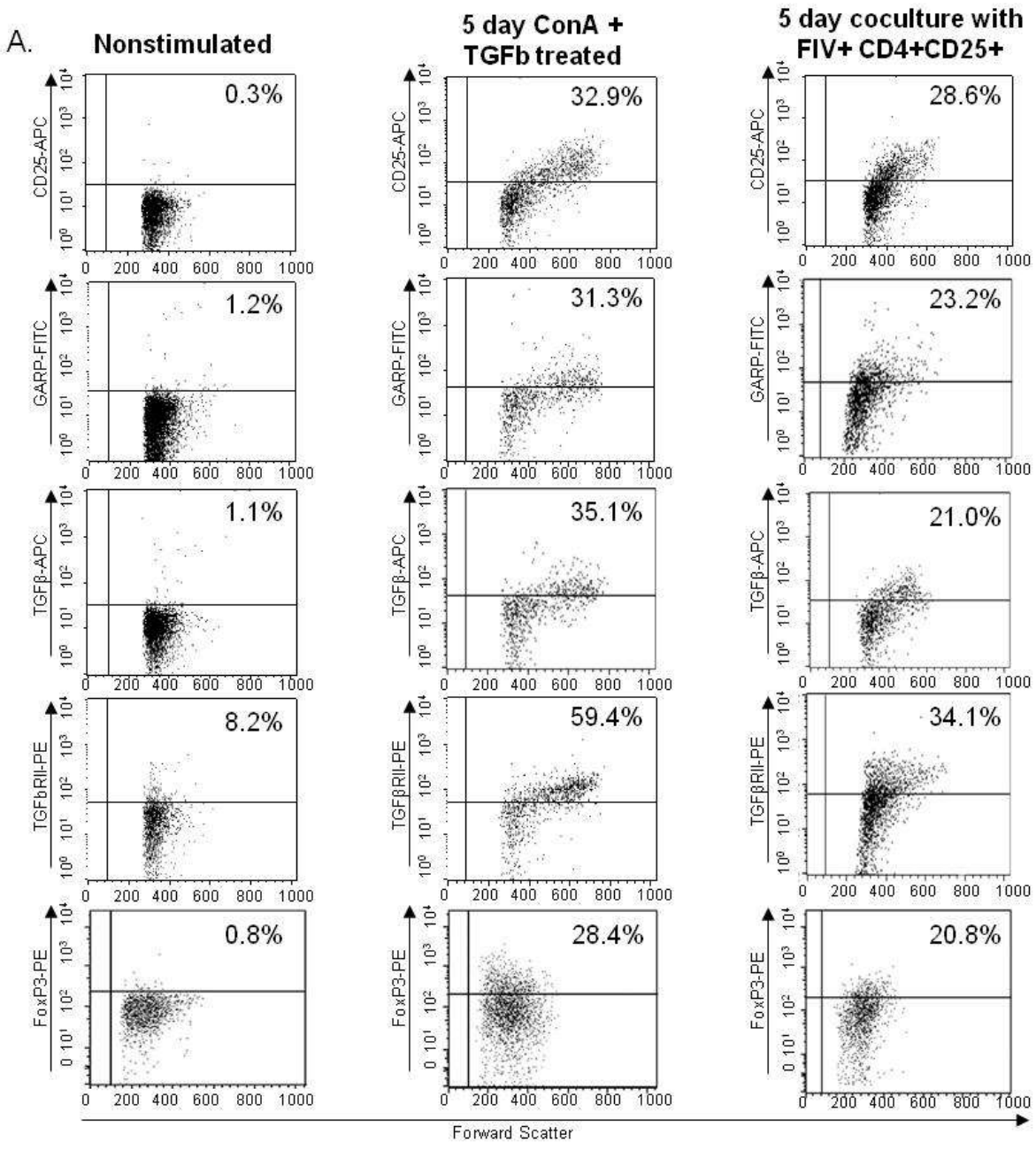
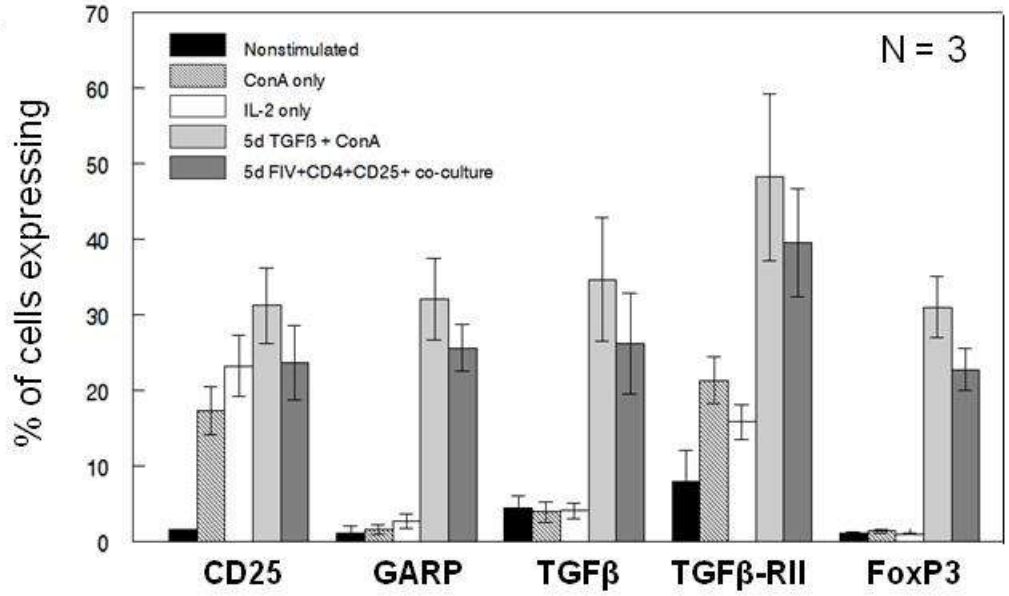

Conversion of Th Cells
Suppressor Assay using Converted Cells (iTregs)
FIV-positive PLN
FACS sort CD4+CD25+ DiD label
±TGFβ or GARP blocking
5 day coculture
"conversion"
"iTregs" Sort non-DiD suppressor cells
±TGFβ–RII blocking
FIV-positive or FIV-negative PLN
FACS sort CD4+CD25- 4 hour ConA stimulation
Flow Cytometry Phenotyping or PCR analysis of converted suppressor cells
FIV-negative
FACS sort CD4+CD25- and CD4-CD8- (Target cells for suppression and APCs)
4 hour ConA stimulation
±TGFb or TGFb-RII blocking
coculture
24 hours IL-2 ELISA
OR
72 hours [3H]thymidine

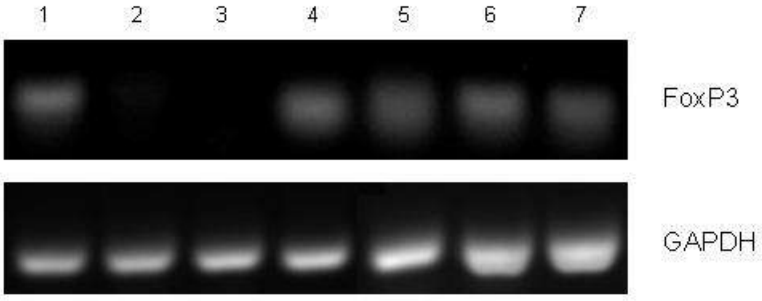
FIGURE 3. **$CD4^+CD25^+$ Treg cells from FIV-infected cats are capable of converting $CD4^+CD25^-$ Th cells from FIV-negative cats into phenotypic iTreg cells.** Nontreated $CD4^+CD25^-$ T cells, soluble TGFβ and ConA treated $CD4^+CD25^-$ cells or $CD4^+CD25^-$ cells converted by co-culture with FIV^+ Treg cells were analyzed by flow cytometry for Treg specific surface markers CD25, GARP, TGFβ and TGFβ-RII then fixed and permeabilized for detection of the intranuclear transcription factor FoxP3. **A.** Representative dot plots are shown for each treatment and each surface marker examined. The percent of cells expressing each marker is given to the top right of each dot plot. **B.** The percent of cells expressing each marker is given as the average $\pm$ SEM of three experiments. **C and D.** Target Th cells from two FIV^+ cats were analyzed in separate experiments for FoxP3 (C) or GARP (D) mRNA expression by PCR following 5 days in culture with either IL2 alone, sTGFβ (10 ng/mL) and ConA (5 ug/mL), or autologous $CD4^+CD25^+$ converter cells. Freshly isolated CD4+CD25+ cells from Cat A (lane 1) or freshly isolated $CD4^+CD25^-$ cells from FIV^+ Cat A (lane 2) or FIV^+ Cat B (lane 3) did not express FoxP3 or GARP mRNA. As a positive control for conversion, $CD4^+CD25^-$ cells were treated with soluble TGFβ (10ng/mL) and ConA (5ug/mL) for 5 days and cells from both Cat A (lane 4) and Cat B (lane 5) were shown to express FoxP3 and GARP. Purified $CD4^+CD25^+$ and ConA-activated $CD4^+CD25^-$ lymphocytes were subjected to the outlined co-culture conversion assay and the target $CD4^+CD25^-$ cells were isolated after 5 days for PCR analysis. These converted cells were shown to express FoxP3 and GARP, consistent with a regulatory phenotype and independent of sample source (lanes 6 and 7).


A.
Nonstimulated
5 day ConA + TGFb treated
5 day coculture with FIV+ CD4+CD25+
CD25-APC
GARP-FITC
TGFβ-APC
TGFbRII-PE
FoxP3-PE
0.3%
32.9%
28.6%
1.2%
31.3%
23.2%
1.1%
35.1%
21.0%
8.2%
59.4%
34.1%
0.8%
28.4%
20.8%
Forward Scatter

B.


% of cells expressing
70
60
50
40
30
20
10
0
Nonstimulated
ConA only
IL-2 only
5d TGFß + ConA
5d FIV+CD4+CD25+ co-culture
N = 3
CD25
GARP
TGFβ
TGFβ-RII
FoxP3

C.


1
2
3
4
5
6
7
FoxP3
GAPDH

D.

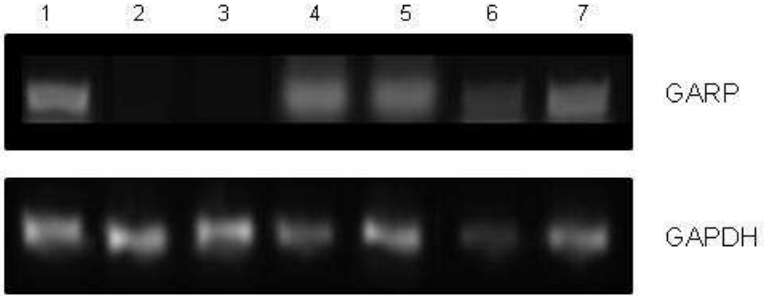

1
2
3
4
5
6
7
GARP
GAPDH

FIGURE 4. **Activated feline $CD4^+CD25^+$ T cells from FIV-infected cats convert $CD4^+CD25^-$ Th cells into functional iTreg cells.** $CD4^+CD25^-$ cells were converted into iTreg cells by co-culture with $CD4^+CD25^+$ T cells from FIV-infected cats as outlined in figure 2 and assayed for effector function. For all suppression assays, $CD4^+CD25^-$ Th cells and $CD4^-CD8^-$ APCs were FACS purified from control cat PBMCs to be used as targets. APCs were added at a 1:1 ratio with Th cells and, where indicated, these cells were stimulated for 1 hour with 5 ug/uL of ConA, washed and then plated. **A.** Nonstimulated $CD4^+CD25^-$ (negative effector control), LPS/rhIL2 activated $CD4^+CD25^+$ (positive effector control), or iTreg cells were used as effectors at an 1:2 effector:target ratio. Supernatant was collected after 24 hours of co-culture and IL2 analyzed by ELISA in triplicate. Data shown is representative of two separate experiments. Error bars represent the standard error of the mean (*$p < .05$ Mann-Whitney). **B.** $CD4^+CD25^-$ target cells were labeled with ^{3}H-thymidine prior to 72 hour co-culture with converted iTreg cells (open square), freshly isolated $CD4^+CD25^+$ cells from an FIV^+ cat (closed square), or freshly isolated $CD4^+CD25^-$ cells (inverted triangle) at various E:T ratios. **C.** $CD4^+CD25^-$ target cells for the iTreg conversion assay were first cultured for 5 days with DiD-labeled $CD4^+CD25^+$ cells from an FIV negative cat that had been treated with IL2 alone (open triangle, negative conversion control) or activated with 5 day IL2/LPS treatment (open square), or with DiD-labeled $CD4^+CD25^-$ cells from an FIV negative cat which had been treated with IL2 alone (closed circle, negative conversion control) or with 5 day IL2/LPS treatment (open circle, negative conversion control). Following the 5 day conversion assay co-culture, DiD-labeled cells were depleted and the remaining cells were used as effectors in a standard 72 hour proliferation assay with ^{3}H-thymidine-labeled $CD4^+CD25^-$ target cells at various E:T ratios. 5 day IL2/LPS activated $CD4^+CD25^+$ cells were used as a positive control effector population (closed square) and Nonstimulated $CD4^+CD25^-$ cells were used as a negative control effector population (inverted triangle). **B and C.** The percent suppression of proliferation was calculated and bars represent the mean ± standard deviation of three separate experiments.

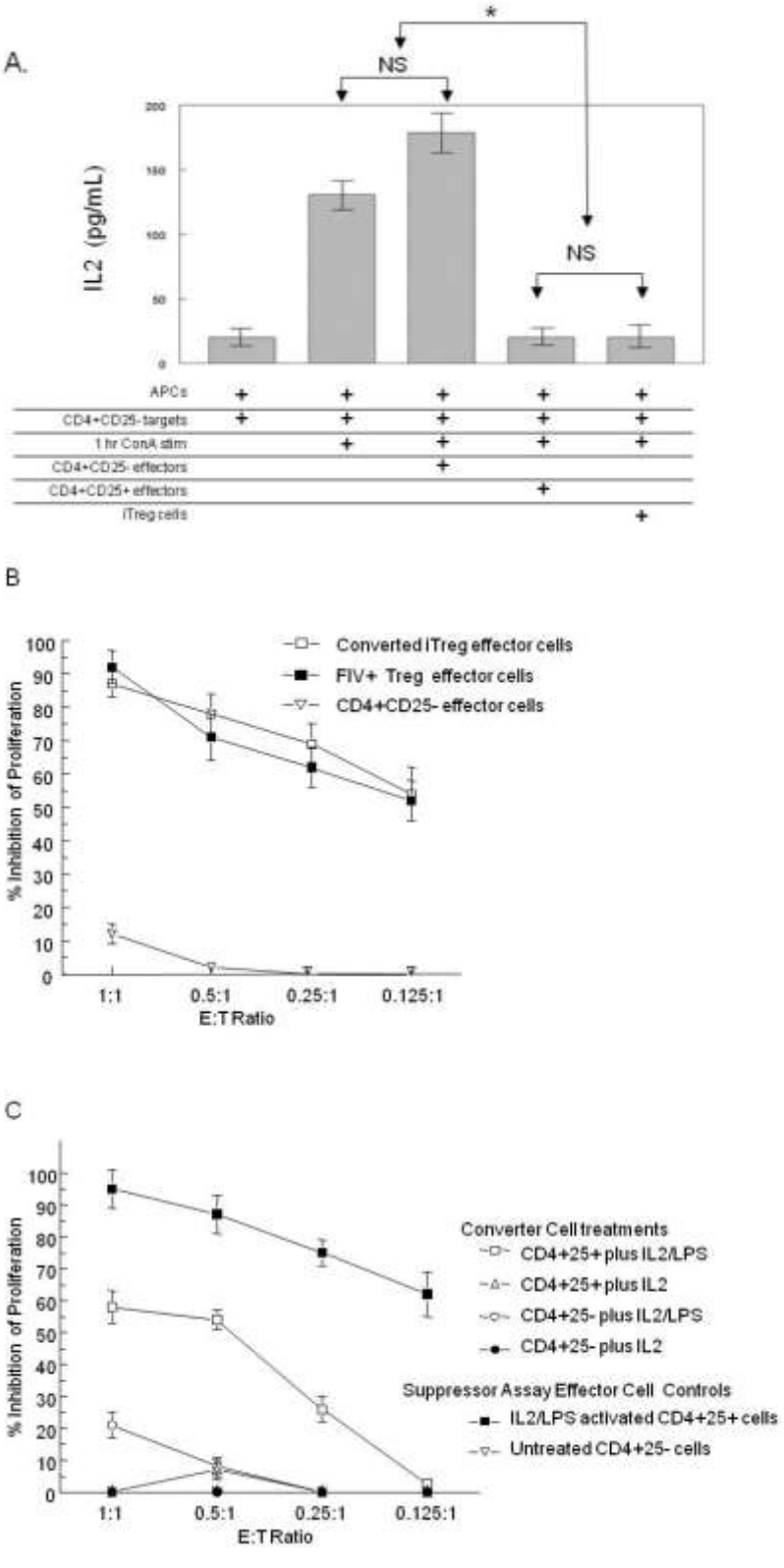
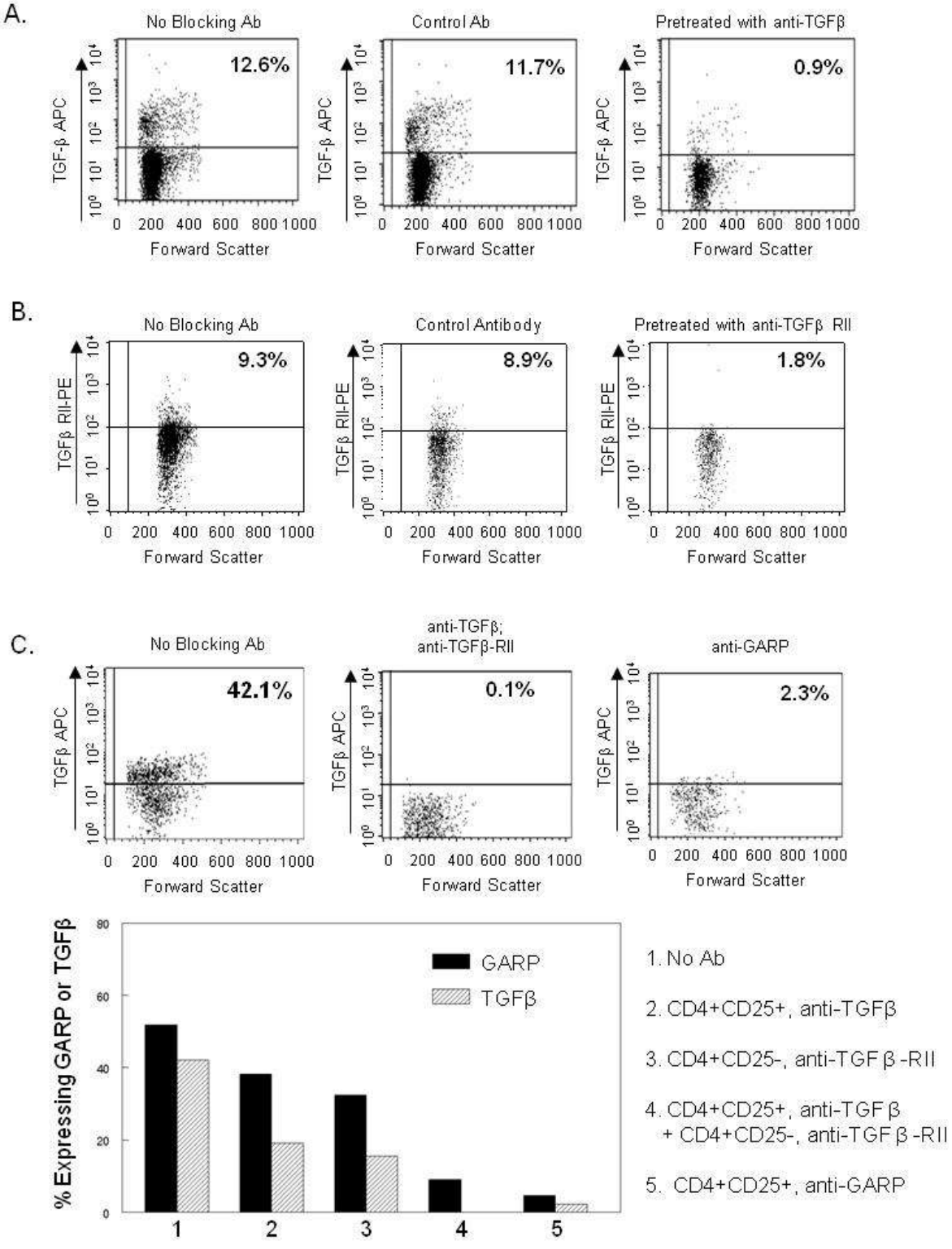
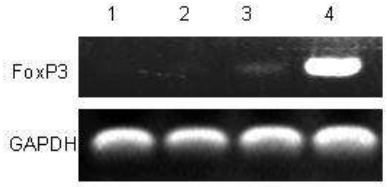

A.
*
NS
NS
IL2 (pg/mL)
200
150
100
50
0
APCs
CD4+CD25- targets
1 hr ConA stim
CD4+CD25- effectors
CD4+CD25+ effectors
iTreg cells
B
% Inhibition of Proliferation
Converted iTreg effector cells
FIV+ Treg effector cells
CD4+CD25- effector cells
E:T Ratio
1:1
0.5:1
0.25:1
0.125:1
C
% Inhibition of Proliferation
Converter Cell treatments
CD4+25+ plus IL2/LPS
CD4+25+ plus IL2
CD4+25- plus IL2/LPS
CD4+25- plus IL2
Suppressor Assay Effector Cell Controls
IL2/LPS activated CD4+25+ cells
Untreated CD4+25- cells
E:T Ratio
1:1
0.5:1
0.25:1
0.125:1

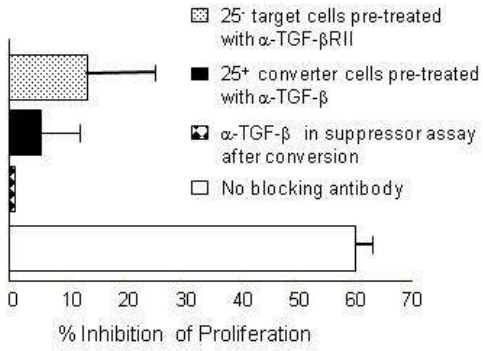
FIGURE 5. **Feline $CD4^+CD25^+$ T cell mediated iTreg conversion occurs by a TGFβ/TGFβ-RII dependent mechanism.** Prior to the iTreg conversion assay, ConA-activated $CD4^+CD25^-$ Th target cells were pre-treated with anti-TGFβ-RII antibodies and $CD4^+CD25^+$ Treg cells were pre-treated with anti-TGFβ or anti-GARP antibodies for 30 min and washed. **A.** To confirm efficient blocking of TGFβ on the $CD4^+CD25^+$ T cells, flow cytometry was performed on isolated Treg cell populations prior to blocking, after blocking with a control antibody of the same murine isotope, or after blocking with the TGFβ-specific antibody. **B.** $CD4^+CD25^-$ T cells were also analyzed for TGFβ-RII expression following treatment with anti-TGFβ-RII or a control antibody. A complete reduction in TGFβ on $CD4^+CD25^+$ and or TGFβ-RII on $CD4^+CD25^-$ cells was observed following these specific blocking treatments. Data shown in **A** and **B** are representative of 3 experiments. **C-F.** After 5 day conversion culture, the target cells for conversion were analyzed by flow cytometry for TGFβ expression **(C)**, by PCR for FoxP3 mRNA expression **(D)**, or were used as effector cells in a standard 72 hour proliferation assay with ^{3}H-thymidine-labeled $CD4^+CD25^-$ target cells or in an IL2 suppression assay with activated $CD4^+CD25^-$ target cells at a 1:2 E:T ratios **(E,F)**. Conversion of Th cells to suppressor cells was reduced by blocking TGFβ on the $CD4^+CD25^+$ cells or TGFβ-RII on the $CD4^+CD25^-$ cells prior to conversion as measured by reduced proliferation **(E)** or IL2 suppression **(F).** Suppressor function was completely abrogated by anti-TGFβ treatment of the converted Treg-like cells prior to suppressor assay **(E)**. Conversion was completely abrogated when TGFβ on the $CD4^+CD25^+$ and TGFβ-RII on the $CD4^+CD25^-$ were blocked simultaneously during conversion or when GARP was blocked during conversion culture **(F)**. (data in E represents mean ± standard deviation of three separate experiments; data in F is from one experiment.)


A.
No Blocking Ab
12.6%
Control Ab
11.7%
Pretreated with anti-TGFβ
0.9%
TGF-β APC
Forward Scatter
B.
No Blocking Ab
9.3%
Control Antibody
8.9%
Pretreated with anti-TGFβ RII
1.8%
TGFβ RII-PE
Forward Scatter
C.
No Blocking Ab
42.1%
anti-TGFβ;
anti-TGFβ-RII
0.1%
anti-GARP
2.3%
TGFβ APC
Forward Scatter
% Expressing GARP or TGFβ
GARP
TGFβ
1. No Ab
2. CD4+CD25+, anti-TGFβ
3. CD4+CD25-, anti-TGFβ-RII
4. CD4+CD25+, anti-TGFβ
+ CD4+CD25-, anti-TGFβ-RII
5. CD4+CD25+, anti-GARP

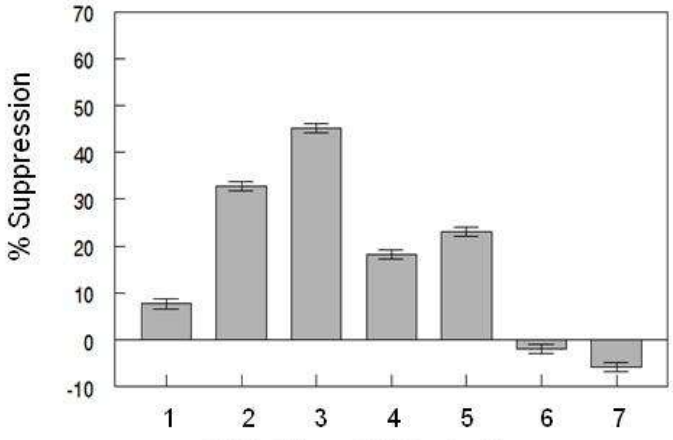
D.



E.



F.



1. Nonstimulated Th
2. Activated Treg
3. iTreg (No Ab)
4. iTreg (anti-TGFβ)
5. iTreg (anti-TGF β –RII)
6. iTreg (anti-TGF β/TGF β –RII)
7. iTreg (anti-GARP)

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6. SUMMARY

Our laboratory was the first to identify the in vivo activation of $CD4^+CD25^+$ Treg cells in FIV-infected cats. Since that time, we have investigated the contributions of this immunosuppressive T cell subset to immune dysfunction during AIDS-lentiviral infection. We have established that activated Treg cells from FIV-infected cats suppress CD4 Th cells and CD8 CTLs in a TGFβ-dependent manner in vitro. We have further identified that feline $CD4^+CD25^+$ Treg cells are able to support a productive FIV infection in vitro and cell associated viremia indicates that they support infection in vivo as well. The aims of this dissertation were to expand these findings by identifying a mechanism for mTGFβ expression and contact-dependent suppression by Treg cells, to investigate the mode of Treg activation during infection and to determine whether Treg homeostasis could be maintained by TGFβ conversion of Th cells during infection.

In chapter 3, we described a novel protein, Glycoprotein A Repetitions Predominant, or GARP, which forms a complex with mature TGFβ and is expressed on the surface of activated $CD4^+CD25^+$ Treg cells. Treg cells expressing this functional Treg surface marker are highly efficient suppressor cells which function in a TGFβ-specific manner as the addition of blocking antibodies to GARP or TGFβ abrogated the ability of $GARP^+$ Treg cells to suppress activated $CD4^+CD25^-$ Th cells in vitro. We further demonstrated that chronically FIV-infected cats have a higher percent of $GARP^+$ Treg cells than FIV-negative cats, suggesting that these suppressor cells are activated during infection and can contribute to immune dysfunction. In support of this, we demonstrated that freshly isolated $GARP^+$ Treg

cells from FIV-infected cats were able to suppress $CD4^+CD25^-$ Th cells in vitro. These experiments collectively identify GARP:TGFβ expressing Treg cells as an important suppressor cell population during FIV-infection and provide a mechanism by which TGFβ is anchored to the Treg cell membrane rather than secreted as a soluble protein.

In chapter 4, we asked if the activation of $GARP^+$ Treg cells was a consequence of direct FIV infection or a response to general immune activation. We had previously identified that $CD4^+CD25^+GARP^+$ Treg cells are expanded during the first few weeks of acute FIV infection in vivo and that the peak in GARP-expressing PBMCs occurs in concert with the peak in plasma viremia (Appendix I). This observation and the previously demonstrated in vivo activation of Treg cells during infection led us to hypothesize that FIV infection itself was capable of activating Treg cells. We first demonstrated that our methods for FIV infection were successful by infecting FACS purified $CD4^+CD25^+$ Treg cells from an FIV-negative cat and evaluating proviral load and gag production six days later. Our results demonstrate that these cells supported a productive viral infection and we asked whether this infection induced phenotypic and functional activation of the Treg cells. Flow cytometry analysis for surface protein expression of GARP and TGFβ or intracellular expression of FoxP3 revealed that all three of these Treg-specific proteins were upregulated in the purified Treg cells following FIV-infection. PCR for GARP and FoxP3 mRNA demonstrated that this upregulation occurs at the level of gene expression as well. To demonstrate that these cells were functionally active, we used in vitro FIV-infected $CD4^+CD25^+$ Treg cells as effector cells in an IL2 suppression assay or a CFSE proliferation assay with activated

CD4+CD25- Th target cells. Both $CD4^+$ Th cell IL2 production and proliferation were suppressed by co-culture with FIV-infected Treg cells, indicating that these cells were both phenotypically and functionally activated. Importantly, UV inactivated virus did not induce Treg activation under the same culture conditions, suggesting that the productive infection of Treg cells themselves and not the presence of FIV antigen was causing this activation phenomenon.

We have previously established that soluble TGFβ treatment of activated $CD4^+CD25^-$ Th cells can convert these cells into functional suppressors. In chapter 5, we hypothesized that the upregulation of GARP:TGFβ on Treg cells during FIV infection in combination with the upregulation of TGFβ-RII on Th cells during infection would support the conversion of Th into induced Treg (iTreg) cells by this same signaling cascade. To test our hypothesis, we devised an in vitro model by which $CD4^+CD25^-$ Th cells were cultured for 5 days with DiD-labeled APCs and Treg cells from an FIV-infected cat. Following this "conversion" co-culture, the iTreg cells were isolated and evaluated for effector phenotype by PCR and flow cytometry, or for effector function in [H3]-thymidine proliferation or IL2 suppression assays using activated Th cells as targets. We first demonstrated that culturing activated Th cells under these conversion conditions did result in the production of iTreg cells. These converted cells upregulated the Treg specific markers, FoxP3, GARP and mTGFβ and suppressed both proliferation and IL2 production of Th targets. We then asked if this conversion was mediated by GARP:mTGFβ on the surface of the converter $CD4^+CD25^+$ Treg cells from FIV-infected cats and TGFβ-RII on the surface of $CD4^+CD25^-$ Th target

cells. To answer this question, we pretreated the converter cells with neutralizing antibodies to GARP or TGFβ, and/or pretreated the Th target cells with neutralizing antibody to TGFβ-RII. Blocking the TGFβ/TGFβ-RII signaling during co-culture abrogated the conversion process as no GARP or mTGFβ were induced on the target cell surface and these cells were no longer able to suppress activated Th cells. These data support our hypothesis that during FIV infection, $CD4^+CD25^+$ Treg cells are activated, express mTGFβ and are thereby able to recruit new suppressor cells from the $CD4^+CD25^-$ Th pool.

Taken together, these three studies suggest that $GARP^+$ Treg cells represent an important aspect of FIV-induced immune dysfunction. We have demonstrated that these highly efficient suppressor cells are susceptible to FIV infection which renders them active and capable of suppressing $CD4^+CD25^-$ Th cells. We have demonstrated that in addition to suppressing Th function, the $CD4^+CD25^+$ Treg cells can induce the conversion of Th into regulatory cells by a mTGFβ-dependent mechanism. Identification of these new aspects of Treg-mediated immune dysfunction contribute to the overall understanding of how these regulatory cell shape the pathogenesis of AIDS-lentiviral infection.

7. FUTURE DIRECTIONS

The experiments in chapter 3 will be repeated but using CD8 CTLs as target in place of $CD4^+CD25^-$ cells. Having previously demonstrated that CD8 cells express TGFβ-RII and that this is upregulated during FIV infection (1, 2), we hypothesize that $GARP^+$ Treg cells are efficient suppressors of the CD8 T cell subset as well. We have performed one such experiment already (Appendix II). Here, the CD8 targets were co-cultured with Treg suppressor cells at a 1:2 E:T ratio. In our previous suppression assays using CD8 cells as targets we have discovered that a 1:1 E:T ratio is most effective and we will use this approach in the future (1). In this initial study, we did not have success with blocking $GARP^+$ Treg suppression using an anti-GARP antibody. In the following studies we will optimize the blocking protocol by increasing the preincubation time and then adding all effector cells into the ELISPOT plate before addition of activated $CD8^+$ target cells to maximize the contact time between cells. These changes to our methods should improve the detection of differences in suppression between effector groups.

Also in chapter 3, we discussed the uncertainty of the GARP bound TGFβ activation status on the surface of Treg cells. Our data suggests that mTGFβ is active as $GARP^+$ Treg cells are capable of suppressing Th cells in vitro. To further evaluate this subject, we will include western blots for detection of SMAD phosphorylation in our CD8 target cells during these follow-up studies. If phosphorylation is detected following co-culture with $GARP^+$ Treg cells but not with $GARP^-$ Treg cells, this will support our theory that $GARP^+$ Treg cells express activated mTGFβ.

In chapter 4, we used UV inactivated virus as a control for FIV infection. We demonstrated that FIV infection activates $CD4^+CD25^+$ Treg cells but exposure to UV inactivated virus antigens does not. As these were sorted $CD4^+CD25^+$ cell cultures, FIV peptides would not have been processed and presented in the context of antigen presenting cells. Previous research has suggested that T cell stimulation with peptide in the absence of APCs leads to an anergic phenotype in these cells as measured by their inability to proliferate or produce cytokines in response to secondary TCR and PMA stimulation (3). Therefore, UV inactivated virus antigen in pure Treg cultures may not be the most appropriate control group in these experiments as T cell to T cell presentation may be a complicating factor. We should repeat the UV inactivated virus culture with APCs to present virus within the normal activating presentation pathways for measurement of Treg activation in the presence of antigen only versus infectious virus.

The specific mechanism by which the FIV-infected Treg cells in chapter 4 become activated is also enigmatic. Future experiments should be designed to investigate this phenomenon. First, we suggest that in vitro infected cells should be evaluated for specific cell signaling pathways. TCR stimulation of Treg cells results in the upregulation of GSK-3, an enzyme which inhibits NFAT activity and measuring the activity of this enzyme would demonstrate activation through direct signaling pathways (4). PI3K/mTOR and STAT3/5 pathways should also be considered as the balance between these is important during Treg activation (5). TLR-7 recognition of ssRNA viruses has been demonstrated during Treg activation as

well and measuring MyD88 and NFκB activation will help to determine if this pathway has been activated (6).

An additional theory for Treg activating following infection is that the location of proviral insertion may alter the transcription of cellular genes. As FIV provirus LTRs contain binding sites for ubiquitous cellular transcription factors including AP-1 and NFκB, the recruitment of these factors could increase availability to proximal gene promoters. This phenomenon has been demonstrated during Moloney murine leukemia virus with insertion of provirus leading to the upregulation of oncogenic factors (7). Future studies should be designed to determine patterns in the site of FIV lentiviral integration and the relationship of proviral location to Treg activation.

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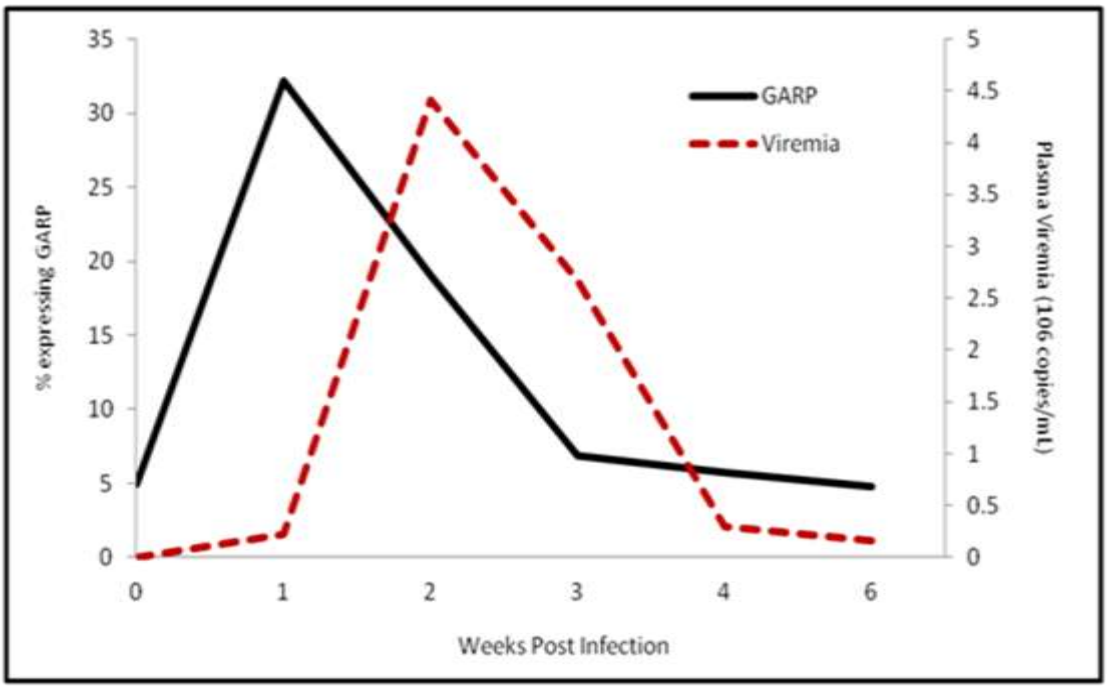
APPENDICES

Appendix I

GARP+CD4+CD25+ Treg cells increase during acute FIV infection and correlate with plasma viremia. After characterizing the GARP protein in feline Treg cells as described in chapter 3, we asked if GARP expression was altered during acute infection in vivo. We hypothesized that acute infection would activate Treg cells and this activation would be characterized by an upregulation of surface GARP expression on these lymphocytes. SPF cats were infected with FIV $NCSU_1$ and PBMCs were analyzed at one week intervals by flow cytometry for GARP expression or by PCR for FIV-Gag to quantify viral loads. The percent of GARP+ Treg cells out of total CD4+ cells was increased at 1 week post-infection but returned to baseline levels by 6 weeks PI. Plasma viremia exhibited a similar time course, reaching a peak at two weeks PI and returning to nearly undetectable levels by 6 weeks PI. This data is pooled from two separate studies. The first study contained three FIV-infected cats which were analyzed for GARP expression only and the second study consisted of five FIV-infected cats which were analyzed for GARP expression and viral loads. These data suggest that Treg activation occurs early during acute infection and may be correlated with viral loads.



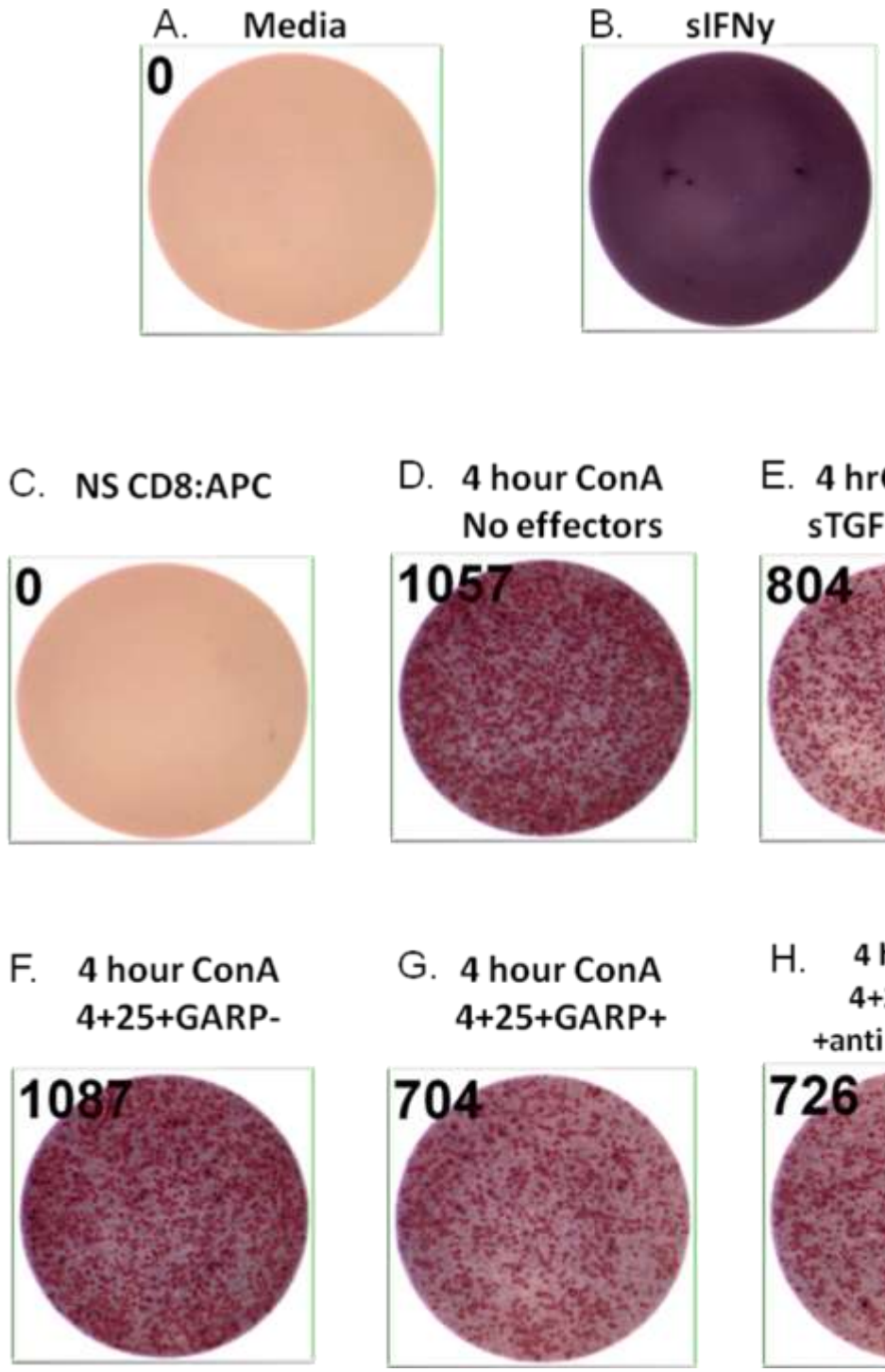
APPENDIX I. **GARP+CD4+CD25+ Treg cells increase during acute FIV infection and correlate with plasma viremia.** SPF cats were infected with FIV $NCSU_1$ and PBMCs were analyzed at one week intervals by flow cytometry for GARP expression (left Y axis) or by PCR for FIV-Gag to quantify viral loads (right Y axis). (N = 8 GARP, N = 5 Viremia)

Appendix II

GARP+ Treg cells suppress IFNγ production from activated CD8+ T cells in vitro.

While the focus of this dissertation was CD4+CD25+ Treg suppression of CD4+CD25- Th cells, we have previously shown that Treg cells also suppress CD8 CTLs in a TGFβ-dependent manner. We therefore hypothesized that GARP+ Treg cells would suppress CD8+ T cells during in vitro co-culture and this could be measured by IFNγ ELISPOT. CD8+, CD4-CD8- (APCs), CD4+CD25+GARP- and CD4+CD25+GARP+ cells were FACS purified from a chronic FIV-infected cat PLN. CD8+ cells and APCs were combined at a 1:1 ratio, stimulated for 4 hours with ConA (5ug/mL) and washed prior to addition to ELISPOT plate at a final concentration of 4e5 CD8+ T cells per well. Nonstimulated CD8+ cells and APCs were used as a negative control. As a positive control for TGFβ-mediated suppression, 10 ng/mL of soluble TGFβ was added to ConA stimulated CD8+ cells and APCs. Effector cells were added to the wells at a 1:2 Effector:Target ratio and plates were incubated at 37°C for 24 hours then processed according to manufacturer's instruction (R&D systems, #EL-764). Effector populations were GARP-CD4+CD25+ cells, GARP+CD4+CD25+ cells or GARP+CD4+CD25+ cells pretreated with 10ug of blocking anti-GARP antibody. GARP expressing Treg cells suppressed CD8+ CTLs as measured by a decrease in IFNγ-producing cells during the in vitro culture. sTGFβ treatment led to a similar reduction in IFNγ producing cells. CD8 cells cultured with GARP- Treg cells had similar SFUs to CD8 cells cultured without effector cells, suggesting that GARP- Treg cells are unable to suppress CD8 CTLs. The addition of blocking antibody to GARP did not alter GARP+ Treg suppression.

APPENDIX II. **GARP+ Treg cells suppress IFNγ production from activated CD8+ T cells in vitro.** A feline specific IFNγ ELISpot assay was performed with **A.** Media only or **B.** soluble IFN-γ as negative and positive assay controls, respectively. Chronic FIV-infected PBMCs were FACS purified into CD4+CD25+GARP+, CD4+CD25+GARP-, CD8+ or CD4-CD8- (APC) populations. **C.** Nonstimulated CD8+ cells and APCs were plated at a 1:1 ratio as a negative control for IFN-γ stimulation. **D.** CD8+ cells and APCs that were stimulated with ConA (5ug/mL) for 4 hours were used as a positive control for IFN-γ stimulation. **E.** sTGFβ was added at 10 ng/mL to ConA stimulated CD8+ cells and APCs to serve as a positive control for TGFβ-mediated signaling. **F-H.** CD4+CD25+GARP- **(F)** or CD4+CD25+GARP+ **(G-H)** effector cells were added to the ConA activated CD8+ cells at a 1:2 E:T ratio. **H.** CD4+CD25+GARP+ cells were pretreated with GARP-blocking antibody (10ug) prior to addition. Co-cultures were incubated in a feline-specific IFN-γ ELISPOT plate (R&D systems, #EL-764) overnight at 37°C and then processed according to manufacturer's instructions.


A. Media
0
B. sIFNy
C. NS CD8:APC
0
D. 4 hour ConA
No effectors
1057
E. 4 hrConA CD8 +
sTGFb (10ng/ml)
804
F. 4 hour ConA
4+25+GARP-
1087
G. 4 hour ConA
4+25+GARP+
704
H. 4 hour ConA
4+25+GARP+
+anti GARP (10ul)
726