

## ABSTRACT

ROGERS, MELINDA CADD. Differential Thrombospondin Expression on T Lymphocytes in a Feline Immunodeficiency Virus Model. (Under the direction of Mary B. Tompkins.)

CD4+CD25+ T regulatory cells represent an important subset of lymphocytes whose function is to suppress autoimmune disease and control normal immune responses. There is much research indicating a direct role for TGF- $\beta$  expressed on the surface of Tregs in the suppressor function of these cells. TGF- $\beta$ , whether bound to the cell surface or secreted, is produced as part of a complex that renders the cytokine inactive.

Thrombospondin is the primary activator of biologically latent TGF- $\beta$ . This research demonstrates that thrombospondin is expressed on the surface of T lymphocytes isolated from the blood and lymph nodes of normal and FIV positive felines. Thrombospondin is expressed at significantly higher levels on CD4+CD25+ T lymphocytes, but can be induced in culture by activating T helper cells in the presence of LPS and IL2. We also observed that the CD4+CD25- T helper cells isolated from FIV negative control lymph nodes were able to markedly upregulate surface thrombospondin expression compared with similarly stimulated CD4+CD25- T helper cells from FIV positive sources. These findings are initial steps in working to understand the mechanism behind latent TGF- $\beta$  activation on CD4+CD25+ T regulatory cells and the role this cell type plays in FIV/HIV pathogenesis.

**Differential Thrombospondin Expression on T Lymphocytes  
in a Feline Immunodeficiency Virus Model**

by  
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## **DEDICATION**

*For my parents*

## **BIOGRAPHY**

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## LIST OF ABBREVIATIONS

**AIDS** – Acquired Immunodeficiency Disorder

**APC** – Allophycocyanin

**APCs** – Antigen Presenting Cells

**CMV** - Cytomegalovirus

**ConA** – Concavalin-A

**CTLA-4** – Cytotoxic T Lymphocyte Antigen 4

**FITC** – Fluorescein Isothiocyanate

**FIV** – Feline Immunodeficiency Virus

**HIV** – Human Immunodeficiency Virus

**HSV** – Herpes Simplex Virus

**IL2** – Interleukin 2

**IPEX** – Immunodysregulation Polyendocrinopathy Enteropathy X-linked

**LAP** – Latency Associated Peptide

**LLC** – Large Latent Complex

**LN** – Lymph Node

**LPS** – Lipopolysaccharide

**LTBP** – Latent TGF- $\beta$  Binding Protein

**PBMC** – Peripheral Blood Mononuclear Cells

**PBT** – Peripheral Blood T cell

**PE** – Phycoerythrin

**PHA** – Phytohemagglutinin

**PPD** – Purified Protein Derivative/Tuberculin

**TCR** – T Cell Receptor

**TGF- $\beta$**  - Transforming Growth Factor Beta

**TGF- $\beta$ RII** – Transforming Growth Factor Beta Receptor II

**T<sub>h</sub>** – CD4+CD25- T helper cell

**TLR4** – Toll Like Receptor 4

**Treg** – CD4+CD25+ T regulatory cell

**TSP** – Thrombospondin

## **BACKGROUND AND SIGNIFICANCE**

### **A. What are CD4+ CD25+ T Regulatory Cells (Tregs)?**

The T regulatory cell (Treg) population is a well-characterized subpopulation of CD4+CD25+ T cells that function in the maintenance of self-tolerance and the regulation of immune responses to pathogens. Tregs comprise 5-10% of the total peripheral CD4+ T cells population in healthy human, murine and feline species (Powrie, 2003; Vahlenkamp, 2004). These cells were first identified in mice as a specific subpopulation of circulating T cells vital to controlling a wide variety of autoimmune disorders (Asano, 1996; Sakaguchi, 1995). Phenotypically, Tregs were initially described as CD4+ T cells that express CD25, the alpha chain of the IL2 receptor (Sakaguchi, 1995; Suri-Payer, 1998). It was later discovered that this unique lineage of regulatory T cells are more accurately described as expressing foxp3, a forkhead transcription factor involved in the development and maintenance of Treg function and homeostasis (Fontenot, 2003).

Two distinct subsets of T regulatory cells have been described as thymic-derived naturally occurring Tregs and peripheral Tregs. Natural, or professional, Tregs develop in the thymus during neonatal development and continuously throughout life (Maggie, 2005). These cells are polyclonal with the ability to recognize as diverse an array of self and non-self antigens as circulating naive T cells. Natural Tregs are long-lived and serve to suppress self-reactive T cells that have the potential to cause severe autoimmune disease and regulate normal immune responses against foreign antigens (Powrie, 2003).

Adaptive Tregs develop independently of the thymus in response to inflammation and chronic immune stimulation. This subset of pathogen-induced or adaptive Tregs can be derived from CD4<sup>+</sup> CD25<sup>+</sup> or CD4<sup>+</sup> CD25<sup>-</sup> T cells present in the periphery to constitute a population of Tregs specific to a single pathogen (Apostolou, 2002; Liang, 2005). It is now well documented that CD4<sup>+</sup>CD25<sup>-</sup> T helper cells can be converted into foxp3<sup>+</sup> suppressor Tregs *in vitro* by TCR engagement and co-stimulation with TGF- $\beta$ , suggesting a mechanism of Treg homeostasis in the periphery, independent of the thymus (Chen, 2003; Marie, 2005). Adaptive and natural Tregs are phenotypically and functionally indistinguishable. Both have a similar phenotype when activated, constitutively expressing CTLA-4, GITR, certain toll-like receptors, CD62L, CD103, CD69, CD45RO, foxp3, LAP (latency associated peptide) and membrane TGF- $\beta$  (Sakaguchi, 2004; Shimizu, 2002; Caramlho, 2003; Maggi, 2005; Oida, 2003).

Functionally, Tregs are classified by their anergy, as defined by the inability to produce interleukin 2 (IL2) and proliferate in response to mitogenic stimulation and TCR engagement (de la Rosa, 2004). Tregs are dependent on exogenous IL2, and express high levels of cell surface CD25 necessary for maintenance in the periphery as well as function. Mice deficient in IL2 or CD25, its receptor, have decreased numbers of Tregs in the thymus and the periphery (Papiernik, 1998; Malek, 2002). IL2 is essential for Treg viability and homeostasis but has no effect on Treg function (de la Rose, 2004; Thornton, 1998). High doses of IL2 abrogate the suppressive effects of Tregs *in vitro* by causing the T helper population to expand and cause the Treg population to exit its anergic state and proliferate. It is also important to note that culturing CD4<sup>+</sup>CD25<sup>-</sup> T helper cells in

the presence of IL2 will induce expression of CD25 but not necessarily confer regulatory activity to these cells, making CD25 an unreliable marker for the regulatory T cell population (Thornton, 1998; Baecher-Allan, 2005).

Several groups have reported the forkhead transcription factor, *foxp3*, to be a more reliable marker involved in Treg function and homeostasis (Fontenot, 2003; Smyk-Person, 2003; Sakaguchi, 2006). This discovery was brought about by examining the underlying cause of scurfy in mice and immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) in the human population. In these models, a genetic mutation in *foxp3* completely ablates development of the CD4<sup>+</sup> CD25<sup>+</sup> Treg population, causing rapid and fatal onset of autoimmune disease, which can be reversed by adoptive transfer of CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells (Smyk-Pearson, 2003; Brunkow, 2001; Wildin, 2001). These studies emphasize the critical role Tregs play in maintaining normal function of the immune system and demonstrate a central role for *foxp3* in the development and function of regulatory T cells.

### **B. Do Tregs play a role in AIDS Lentiviruses pathogenesis?**

While it has been clearly demonstrated that Treg function is essential to prevent autoimmune disease in healthy animal models, there are also ample data to suggest a potentially subversive role for Tregs in an immune response against pathogens. This characteristic has been observed during the immune response to malaria parasites.

Walther et al., (2005) demonstrated that increased numbers of CD4<sup>+</sup>CD25<sup>+</sup> Tregs and

production of TGF- $\beta$  correlated with an increase in parasite growth *in vivo* in humans. The parasite's ability to utilize Tregs in its evasion of the host immune response was further characterized by Hisaeda et al., (2004) who showed that infecting mice with *Plasmodium yoelii* activated Tregs specific for *P. yoelii* antigen. When isolated, these Tregs had increased immunosuppressive activity compared to Tregs from naïve mice, potentially allowing the parasites to persist in the host and establish a chronic infection (Hisaeda, 2004). Tregs have also been shown to allow the persistence of the parasite *Leishmania major* by a similar mechanism to that demonstrated with malaria. *L. major*-specific Tregs traffic to the site of primary infection where they become activated and expand, suppressing the natural protective immune response. This response leads to chronic persistence of the parasite in the host (Belkaid, 2002). The full effect of this type of immune modulation was demonstrated by depleting the CD4+CD25+ T regulatory cell in models of *L. major* and herpes simplex virus (HSV) (Suvas, 2003). Depletion of the CD4+CD25+ Treg subset enhanced the immune response against *L. major*, ultimately leading to complete eradication of the parasite and enhancing the cell-mediated response to both HSV and unrelated antigens (Suvas, 2003; Belkaid, 2002).

Research into the failure of the immune response to eliminate certain pathogens and thus allowing a chronic antigenemia suggest that activated Tregs are capable of suppressing protective CD4+ and CD8+ T cell responses to the detriment of the host. Another body of evidence clearly demonstrates the protective role of Tregs against the development of autoimmune disease (Suri-Payer, 1998; Zheng, 2004). This complicated and often unresolved balance between protective and injurious roles for Tregs in modulating

immune responses is no clearer when we consider the function of Tregs in Feline Immunodeficiency Virus (FIV) and Human Immunodeficiency Virus (HIV) models. The role of T regulatory cells relating to the pathogenesis of FIV/HIV is currently an area of intense research and much controversy.

Feline Immunodeficiency Virus and HIV are lentiviruses, the etiological agents of Acquired Immunodeficiency Disorder (AIDS) in their respective hosts (Pedersen, 1989; Chermann, 1983; Sarnhadharan, 1984). These viruses infect host CD4<sup>+</sup> T cells by binding the CD4<sup>+</sup> T cell receptor and to co-receptors CCR-5 or CXCR-4 on feline T lymphocytes (Nielsen, 2005; Willett, 2002). Clinically, these infections are manifested by an early acute phase characterized by a decline in total CD4<sup>+</sup> T cell numbers, a CD8<sup>+</sup> lymphocytosis, high virus titers, a transient low-grade fever and generalized lymphadenopathy (Cooper, 1985). A long-term asymptomatic phase follows with a gradual decline of total CD4 T cells numbers and an inversion of the CD4:CD8 T cell ratio. Disease progression is characterized by a chronic loss of CD4<sup>+</sup> T cell numbers leading ultimately to the AIDS phase, which has been detailed in both humans and cats (Cooper, 1985; Pederson, 1989). Ultimately, plasma viremia increases and the decrease in overall CD4<sup>+</sup> T cell numbers and generalized immunodeficiency leads to the development of a variety of disorders including opportunistic infections and cachexia (Wahren, 1987; Conner, 1993; Levy, 2006; Burkhard, 2003).

Virus replication in activated CD4<sup>+</sup> T cells leads to the destruction of those cells. This process accounts for the correlation between immune activation, increased plasma

viremia and the chronic decline in CD4+ T cell numbers (Hazenberg, 2003; Leng, 2001; Connor, 1993). However, it has now been shown that immune dysregulation occurs prior to any appreciable loss in CD4+ T cell numbers. A failure of CD4+ and CD8+ T cells to produce IL2 and proliferate in response to HIV antigen has been documented in the early acute stage of infection by a number of investigators (Wahren, 1987; Reddy, 1987; Kelker, 1992). This loss of T cell immune responsiveness appears to be specific to HIV antigens as it has been shown that CD4+ and CD8+ T cells from these same individuals respond to other antigens such as PPD, tetanus, CMV, HSV and mitogenic PHA but at reduced levels compared to HIV negative controls (Wahren, 1987; Reddy, 1987; Kelker, 1992). In parallel to the HIV-specific immune hyporesponsiveness, there is a state of chronic T cell hyperactivation characterized by a consistent loss of CD62L, upregulation of activation markers CD49d and CD44, and of co-stimulatory molecules CD80, CD86 and CTLA-4 (Kochli, 1999; Gebhard, 1999; Tompkins, 2002). This chronic T cell activation could be explained by the chronic antigenemia seen with HIV/FIV infections; however, the mechanism underlying T cell hyporesponsiveness to both antigenic and mitogenic stimulation has yet to be fully described.

Possible mechanisms suggested to explain this lentivirus-associated T cells immune hyporesponsiveness include cytokine dysregulation (decreased IL2, increased IL10 and IFN $\gamma$  production compared to uninfected samples), activation-induced apoptosis and clonal anergy (Murray, 1985; Clerici, 1997). These explanations, while accurate observations of FIV and HIV pathogenesis, do not adequately explain the viral antigen-specific immune deficiency observed in patients (Arrode, 2005; Wahren, 1987). Clonal

anergy, as defined by a lack of responsiveness to antigen engagement of TCR and a failure to produce IL2, could be one mechanism of viral antigen specific T cell hyporesponsiveness.

Our laboratory has focused on CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells as possible mediators of T cell clonal anergy in FIV infection. We were the first to identify CD4<sup>+</sup>CD25<sup>+</sup> Treg cells in the cat and describe their phenotypic and functional activation in the FIV infection model for human AIDS (Vahlenkamp, 2004; Joshi, 2005; Joshi, 2004; Joshi, 2005). Freshly isolated CD4<sup>+</sup> CD25<sup>+</sup> Tregs from FIV<sup>+</sup> cats demonstrated significant up-regulation of co-stimulatory molecules CD80, CD86 and CTLA4 on the cell surface and were able to suppress concanavilin A (Con-A) activated CD4<sup>+</sup>CD25<sup>-</sup> autologous T cells in a dose-dependent manner, suggesting the Treg cells are constitutively activated *in vivo* as a result of FIV infection. CD4<sup>+</sup>CD25<sup>+</sup> Tregs isolated from FIV<sup>+</sup> cats were anergic and failed to produce IL2 or proliferate in response to Con-A or LPS stimulation (Vahlenkamp, 2004). Tregs isolated from control cats, as well as mice and humans, express TLR4 and when activated with LPS will suppress proliferation and IL2 production of autologous CD4<sup>+</sup>CD25<sup>-</sup> T cells in a dose-dependent manner (Vahlenkamp, 2004; Caramalho, 2003).

There is ample evidence to indicate that the Treg population is activated during FIV/HIV infection and is able to suppress the proliferation of activated CD4<sup>+</sup> T cells. Given that we also understand CD4<sup>+</sup> T cell activation to be the best indicator of disease progression, it would stand to reason that the Treg population could control disease progression by

hampering immune activation (Leng, 2001; Hazenberg, 2003). In support of this hypothesis, several reports documented that depletion of the regulatory T cells from PBMC of HIV patients causes the CD4+ T cell population to become highly activated in HIV+ individuals followed shortly by onset of chronic viremia (Eggena, 2005; Baker, 2007). Further studies describe cases in which increased numbers of Tregs in peripheral blood of HIV-infected individuals is associated with favorable clinical markers and lower plasma viremia (Kinter, 2004). These data enforce the idea that Tregs act during lentivirus infection to reduce CD4+ T cell immune activation and therefore delay disease progression by limiting the number of targets for viral spread within the host.

These data suggest an overall positive role for Tregs in decreasing virus burden and slowing disease progression. However, there are also data indicating a potentially harmful role for Tregs in the progression of disease and treatment of lentivirus infections. Our laboratory, as well as others, identified Tregs as targets for FIV/HIV infection (Joshi, 2004; Joshi, 2005; Joshi, 2005; Oswald-Ritcher, 2004). CD4+CD25+ Tregs require only low levels of exogenous IL2 and virus input to be productively infected while CD4+CD25- require much higher levels of IL2 or high virus titer to establish a productive FIV infection, while otherwise serving as a site for latent infection (Joshi, 2005; Shen, 2003). Given the chronic activation status of the Treg population in lentivirus infection, these cells serve as a reservoir for virus throughout the course of disease (Joshi, 2004). As another consequence of the activated Treg population during FIV/HIV infection, the suppressive function of this population could potentially abrogate protective CD4+ and CD8+ T cell responses to viral antigens early in infection,

contributing to the development of AIDS. There is no direct evidence for such a theory, but given the activation state of the Treg population during the early acute phase of disease, it can be hypothesized that these activated Tregs suppress a protective antiviral response before an ample defense is mounted.

As previously described, Tregs play an integral role in protecting the host against autoimmune disease and in controlling the magnitude and duration of the immune response against pathogens, thus preventing collateral damage to the host. This scenario presents several possible complications and benefits to the host in the case of AIDS as detailed above. To better understand the potential consequence of the T regulatory cell population and FIV, our laboratory focused on the generation of peripheral Tregs and evaluating the mechanism of Treg suppression.

### **C. By what mechanisms do Tregs suppress T helper cell proliferation?**

Early reports on Treg-mediated immune suppression focused on various potential cytokine mediators including IL-4, IL-10 and TGF- $\beta$  (Shevach, 2001; Zhang, 2004).

While there is still much disagreement as to the actual mediators of suppression, there is consensus that the mechanism is cell-cell contact dependent (Shevach, 2001; Maggi, 2005; Nakamura, 2001; Zheng, 2004; Vahlenkamp, 2004). A role for TGF- $\beta$  in deriving adaptive Tregs in the periphery has been described by several groups who demonstrated that treating CD4<sup>+</sup> CD25<sup>-</sup> T cells with TGF- $\beta$  induces these cells to express the regulatory T cell specific transcription factor foxp3 (Nakamura, 2004; Marie, 2005;

Davidson, 2007; Zheng, 2004; Chen, 2003). Fantini et al., (2004) demonstrated that treating activated CD4<sup>+</sup> CD25<sup>-</sup> T cells with TGF- $\beta$ -induced expression of foxp3 in both human and murine T cells. This group went on to report that TGF- $\beta$  induced foxp3<sup>+</sup> Tregs downregulated expression of TGF- $\beta$  inhibitory molecule Smad7, thus allowing for a positive autoregulatory loop of TGF- $\beta$  signaling in the adaptive Tregs (Fantini, 2004). These TGF- $\beta$  converted adaptive Tregs express a similar phenotype to thymic-derived naturally occurring T regulatory cells, including the ability to suppress an immune response (Nakamura, 2004; Marie, 2005; Davidson, 2007; Zheng, 2004; Chen, 2003).

TGF- $\beta$  is clearly an important cytokine for the development and maintenance of the Treg population; however its role as a mediator of Treg suppressor function is highly contested. Nakamura et al., (2001) first reported that activated Treg cells express membrane bound TGF- $\beta$  (mTGF- $\beta$ ) on their surface and indicated a potential role for the cytokine in mediating Treg immune suppression. Several groups subsequently demonstrated that treatment of Tregs with a blocking antibody against TGF- $\beta$  at least partially, but consistently, neutralized the contact-dependent suppressor function of the Tregs in a dose-dependent manner (Nakamura, 2001; Annunziato, 2002; You, 2007). Ostroukhova et al., (2006) attempted to replicate this suppressive activity by culturing CD4<sup>+</sup> CD25<sup>-</sup> T helper cells with exogenous soluble TGF- $\beta$  but observed no anti-proliferative effects, lending support to the model for cell-cell contact mediated suppression.

Dispute over a TGF- $\beta$ -mediated mechanism is exhibited by reports showing that treatment of Tregs *in vitro* with blocking antibodies to TGF- $\beta$  and TGF- $\beta$ RII did not inhibit suppression. Also, T regulatory cells derived from TGF- $\beta$  null mice maintain suppressor function *in vitro* (Kullberg, 2005; Piccirillo, 2002). It should be noted however, that TGF- $\beta$  knockout mice exhibit a similar phenotype to foxp3 deficient murine models with severe lymphoproliferative disease resulting in death by 3-4 weeks of age (Suri-Payer, 2001; Thornton, 1998). The similarity between these gene knockout models is consistent with the loss of immunoregulatory activity, therefore lending support to a TGF- $\beta$ -dependent model of Treg mediated immunosuppression.

This apparent conflict regarding a role for TGF- $\beta$  in CD4+CD25+ Treg mediated immunosuppression might be resolved by considering the overwhelming biological significance of TGF- $\beta$ . It is reasonable that redundant pathways exist to compensate for a scenario where TGF- $\beta$  expression is suppressed or completely abrogated. This would reconcile the knowledge that Tregs from TGF- $\beta$  null models still exhibit some regulatory activity while maintaining support for the hypothesis that a TGF- $\beta$  mediated contact-dependent Treg suppressor mechanism is responsible for immune modulation in wild-type control biological models.

Transforming growth factor  $\beta$  is a pluripotent cytokine that modulates cell growth, inflammation, apoptosis and extracellular matrix synthesis (Annes, 2003). TGF- $\beta$  is synthesized as a homodimeric 75kDa latent complex referred to as the large latent complex (LLC). The LLC is composed of the mature 24kDa TGF- $\beta$  protein, latent TGF-

$\beta$  binding protein (LTBP) and the latency associated peptide (LAP), see **Figure 1**. The LTBP serves to target secreted forms of the LLC to the extracellular matrix. LAP binds TGF- $\beta$  during processing. These bonds are cleaved in the golgi and LAP remains non-covalently associated with TGF- $\beta$ , rendering the cytokine biologically inactive (Feng, 2005; Annes, 2003).

#### **D. Latency Associated Peptide and Treg-mediated immune suppression**

To further reinforce the hypothesis that TGF- $\beta$  plays a central role in Treg immune suppression, Nakamura et al. went on to demonstrate that treating Tregs with a blocking antibody against recombinant latency-associated peptide (rLAP) abrogated Treg suppressor function in both human and mouse models (Nakamura, 2004). Several papers demonstrate a link between LAP expression and regulatory activity in a CD4<sup>+</sup> CD25<sup>-</sup> T cell population (Ochi, 2006; Oida, 2003). These groups showed LAP expression to be a better cell surface marker of the Treg population than CD25, and that treating Tregs with a blocking antibody specific to LAP abrogated Treg suppression of CD4<sup>+</sup> T helper cells (Ochi, 2006; Oida, 2003).

Yet, another body of evidence exists in dendritic cell research suggesting an immunoregulatory role for TGF- $\beta$  present on the surface of immature dendritic cells in association with LAP. Dendritic cells play a major role in both initiating an immune response by activating the resting T cell population and in maintaining T cell tolerance to innocuous and self antigens (Lanzavecchia, 2001; Banchereau, 1998; Steinman, 2003;

Roncarolo, 2001; Enk, 2005). Gandhi et al., (2007) reported that immature dendritic cells are able to regulate naïve and effector T cell activation in a TGF- $\beta$ -dependent manner. These immature human dendritic cells expressed surface bound TGF- $\beta$  in association with LAP and were also able to induce the differentiation and survival of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs via expression of TGF- $\beta$  (Gandhi, 2007).

#### **E. What role does thrombospondin-1 play in Treg-mediated immune suppression?**

The above references report surface expression of TGF- $\beta$  in association with expression of LAP but fail to address the mechanism by which TGF- $\beta$  is dissociated from LAP into its biologically active form. Several mechanisms exist by which TGF- $\beta$  can become activated. The integrin  $\alpha_v\beta_6$ , proteases, reactive oxygen species and low pH can cause LAP to dissociate from TGF- $\beta$ , however thrombospondin-1 (TSP) is widely regarded as the primary molecule that confers bioactivity onto latent TGF- $\beta$  (Annes, 2003; Crawford, 1998; Young, 2004; Abdelouahed, 2000). Masli et al., (2006) addressed this issue by analyzing the effect of TSP production by antigen-presenting cells (APCs) on their tolerance inducing capacity. They demonstrated that TGF- $\beta$  treated APCs from TSP deficient mice failed to suppress an immune response to OVA, while TGF- $\beta$  treated APCs from wild type mice effectively abrogated an OVA-specific response (Masli, 2006). Yehualaeshet et al., (1999) reported a similar role for TSP in activating latent TGF- $\beta$  expressed by alveolar macrophages involved in regulating inflammation in the lungs by demonstrating a decrease in activate TGF- $\beta$  in anti-TSP treated macrophages

compared to controls. These reports suggest a potential role for TSP in conferring immunoregulatory activity to TGF- $\beta$  expressing APCs.

Thrombospondins are a small family of extracellular matrix glycoproteins produced by a variety of cells and having pluripotent functions. This discussion will focus entirely on thrombospondin-1 (TSP), a 145kDa homo-trimeric protein originally identified as thrombin-sensitive protein, given that it is released by thrombin-activated platelets. In this context, TSP is involved in platelet aggregation and wound healing. TSP is capable of binding calcium, heparan sulfate proteoglycans, integrins  $\alpha_v\beta_3$ ,  $\alpha_4\beta_1$ , and  $\alpha_5\beta_1$ , the integrin-associated protein and cell surface proteins CD36 and CD47. The function of TSP depends on the cell type that produces it, whether it is secreted or retained on the cell surface and conditions of the proximal environment. Thrombospondin can function as an inhibitor or positive regulator of endothelial cell growth and aggregation, a chemoattractant for smooth muscle cells, neutrophils, and monocytes. TSP has also been shown to have anti-angiogenic properties making it a possible negative regulator of tumor growth that depends on neovascular development for metastasis (Bornstein, 2001; Guo, 1997; Chandrasekaran, 2000; Jiménez, 2000; Streit, 1999; Bornstein, 2004; Li, 2000; Li, 2006).

Surprisingly, TSP-null mice exhibit only mild epithelial hyperplasia, increased leukocytic infiltration, with acute and chronic inflammatory changes in the lungs and pancreas (Bornstein, 2001). A simple explanation for this contradiction would be that given the extreme biological significance of functions like tissue repair; several redundant

mechanisms exist with which the body can accomplish such tasks. However, another explanation which more clearly delineates why the loss of a chemoattractant and platelet aggregator results in increased cell migration and inflammation relies on the relationship between TSP and TGF- $\beta$ .

Thrombospondin is a major physiological regulator of the activation of latent TGF- $\beta$  (Ribeiro, 1999; Annes, 2003). As previously described, TGF- $\beta$  exists in two forms, as an active molecule or in a latent state as part of the large latent complex (LLC) (Annes, 2003). The KRFK amino acid sequence of TSP interacts with the LSKL amino acid sequence on LAP to release the active form of TGF- $\beta$  from the LLC, see **Figure 1** (Ribeiro, 1999; Young, 2004). It has been shown that integrin  $\alpha_v\beta_6$ , some proteases and low pH can activate latent TGF- $\beta$ , however there is data to suggest that TSP is primarily responsible for much of active TGF- $\beta$ 's effects *in vivo* (Adbelouahed, 2000; Annes, 2003).

TSP knockout mice exhibit a similar, although less severe, phenotype to TGF- $\beta$  knockout mice (Lawler, 1998; Crawford, 1998). Considering TSP's role in activating TGF- $\beta$ , the TSP null mice would theoretically have less activate TGF- $\beta$  and therefore could be more prone to experience unchecked inflammation. While TGF- $\beta$  knockout mice die within the first four weeks, their early pathologic changes, especially in lung and pancreatic tissue, is similar to the inflammatory changes observed in the TSP null mouse model (Crawford, 1998). Crawford et al., (1998) demonstrated that treating TSP knockout mice with a KRFK peptide, the amino acid sequence necessary for latent TGF- $\beta$  activation,

caused remission of the observed phenotype, comparable to wild type mice. These findings clearly indicate that TSP is responsible for TGF- $\beta$  activity *in vivo*.

Given the convincing evidence for upregulation of CD4+CD25+ regulatory T cell activity and increased expression of membrane-bound TGF- $\beta$  (mTGF- $\beta$ ) on the surface of these Tregs during FIV infection, our studies addressed the following questions: is thrombospondin expressed on the surface of feline T lymphocytes and to what degree does this vary between CD4+CD25+ Tregs and CD4+CD25- T helper cells (Joshi, 2005; Vahlenkamp, 2004; Petty, 2006). The studies described here focus primarily upon the surface expression of TSP-1 on the CD4+CD25+ T regulatory cell and CD4+CD24- T helper cell populations from both FIV positive and FIV negative feline models *ex vivo* and under various *in vitro* culture conditions. It is well documented that thrombospondin can have multiple effects on T lymphocytes. T cells have been shown to produce endogenous TSP which was first described as being responsible for regulating T cell motility within the extracellular matrix (Li, 2005; Li, 2006). Thrombospondin interacts on the cell surface with its receptor, CD47, and calreticulin to induce T cell migration in the extracellular matrix (Li, 2005). Li et al. also demonstrated that TSP expression on the surface of quiescent T lymphocytes undergoes rapid turnover and activation of the T lymphocytes through the TCR appears to inhibit this turnover and upregulates stable TSP surface expression (Li, 2006).

Considering the indisputable evidence of the ability of TSP to activate TGF- $\beta$  *in vivo* and the role of TGF- $\beta$ /LAP in APC and Treg cell suppressor function, we asked first if feline

T lymphocytes are capable of expressing endogenous TSP on their surface. We hypothesize that feline T cell expression of TSP is likely to be the link between membrane bound latent TGF- $\beta$ /LAP and the active form of TGF- $\beta$  capable of mediating T regulatory cell suppressor function.

## MATERIALS AND METHODS

### **Animals**

The cats used in this study were specific pathogen free (SPF) cats obtained from Harlen Laboratories (Indianapolis, IN). Cats were inoculated with with the NCSU<sub>1</sub> isolate of FIV (English, 1994), as described by Bucci et al. (Bucci, 1998). PCR with specific primers for FIV-gag mRNA and ELISA for anti-FIV p24 antibodies were used to confirm FIV infection. These animals were housed at the Laboratory Animal Resource Facility at the North Carolina State University College of Veterinary Medicine. Samples were collected from FIV+ cats that were infected with the virus for more than one year and control samples were obtained from uninfected SPF cats. Both groups ranged from 2 to 4 years of age. FIV-infected and control cats were housed separately.

### **Protein sequence analysis**

Human thrombospondin mRNA sequence (GenBank accession number NM\_003246) was used to determine human thrombospondin protein sequence. Other species' protein sequences were determined by searching the NCBI Entrez protein database

(<http://www.ncbi.nlm.nih.gov/sites/entrez?db=Protein>).

Thrombospondin protein sequences were compared using the ClustalW program

(<http://www.ch.embnet.org/software/ClustalW.html>) and Boxshade 3.21 program

([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

### **Sample Collection**

Whole blood was collected into EDTA Vacutainer tubes (Sigma-Aldrich, St. Louis, MO) by jugular venipuncture. Lymph node (LN) cells were collected by LN biopsies of submandibular and popliteal peripheral lymph nodes. Single-cell suspensions were prepared by gently injecting RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, 1% ciprofloxacin, and 1mM HEPES buffer repeatedly into the tissue using an 18 gauge needle until the cells were released from the tissue. Cell numbers and viability were determined by trypan blue dye exclusion.

### **Serum free medium**

Serum free medium used for stimulations was sterile filtered using Cell M Ab Medium Serum Free (Becton, Dickson and Company, Sparks, MD) containing 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, 1% ciprofloxacin, and 1mM HEPES buffer.

### **CD4+ T cell subset separation**

To obtain CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cell subsets lymphocytes from LN suspensions were sorted on a MoFlo cell sorter (DakoCytomation, Fort Collins, CO). Purity was determined by flow cytometric analysis and was greater than 95%. Viability of T cell subsets was determined by trypan blue dye exclusions and was always greater than 90%. All assays were based on viable cell numbers.

### **Flow cytometry analysis**

Between  $5 \times 10^5$  and  $10^6$  PBMCs or lymphocytes were stained for surface expression of CD25, CD4 and/or CD8 using FITC-conjugated anti-CD25 mAb (mAb 9F23), biotinylated anti-CD4 (mAb CAT30A) and PE-conjugated anti-CD8 (mAb 3.375) (Tompkins, 1990). Monoclonal antibody for TSP (mouse anti-human, Ab-3 clone A6.1, Calbiochem, San Diego, CA). We obtained monoclonal antibody for TGF- $\beta$ 1 (mouse anti-human, mAb 240, clone 9016) from R and D Systems. Isotype control for TSP and TGF- $\beta$  monoclonal antibodies was from Southern Biotech, RPE and APC-conjugated mouse IgG<sub>1</sub> isotype controls (clone 15H6).

Data were acquired on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Lymphocytes were gated on forward vs. side scatter and 20,000 gated events were acquired then analyzed using Cell Quest Pro software. All samples were performed in duplicate.

### **T cell stimulation assays**

Unsorted lymphocytes or T cell subsets were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, 1% ciprofloxacin, and 1mM HEPES buffer. Between  $5 \times 10^5$  and  $10^6$  viable cells were cultured in round-bottom 96-well plates in the presence or absence of 100U/ml recombinant human IL2 (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD) and/or 10ng/ml LPS, *E. coli*055:B5 (Sigma Chemical Company). All assays were run in duplicate. After four

days of incubation at 37°C cells were washed and stained, as above, for flow cytometry analysis.

### **Statistical Analysis**

T-test for unpaired data was performed using Slide Write Pro to determine significance of differences between mean fluorescence intensity of TSP expression in freshly isolated and cultured lymphocytes. P-values are noted where used.

## RESULTS

### *Feline T lymphocytes express thrombospondin on their surface*

**Figure 1** depicts a proposed structure of biologically inactive TGF- $\beta$  in the large latent complex. This figure also displays the amino acid interaction between the KRFK sequence found on thrombospondin and the LSKL sequence of LAP necessary to induce a proposed conformational change in the complex and release TGF- $\beta$  from latency.

It has been reported that human peripheral blood T cells express endogenous thrombospondin on their cell surface (Li, 2006). To address whether T lymphocytes from cats also express TSP on their cell surface, we first asked whether a monoclonal antibody specific for human TSP could be used to detect this protein on feline cells. Since the protein sequence of TSP in cats has yet to be elucidated, we instead compared the human TSP protein sequence against all other available TSP sequences to observe the degree of conservation among species. As shown in **Figure 2**, the TSP protein is very highly conserved among all seven available sequences. This degree of conservation provided us with a high degree of confidence that the monoclonal antibody would bind specifically to feline TSP in our flow cytometry analysis.

We then analyzed T lymphocytes from peripheral blood and from peripheral lymph nodes of FIV positive and control cats for surface expression of thrombospondin. Between 0.3 – 1.07 % CD8+ T cells from peripheral blood were positive for surface expression of

thrombospondin as compared to isotype control in FIV+ cats and between 0.13 and 1.00% positive in FIV negative control cats (**Figure 3d**). CD4+ T cells from peripheral blood ranged from 0.31 – 2.63% expressing TSP in FIV-uninfected control animals and 0.09 – 1.26% TSP positive in FIV positive cats (**Figure 4a**). The proportion of CD4+TSP+ T cells isolated from peripheral lymph nodes were not significantly greater in total numbers compared to T cells from the peripheral blood expressing TSP on the cell surface with 0.59 – 2.49% positive in controls and 0.27 – 3.05% TSP+ in FIV positive cats (**Figure 4b**).

#### ***CD4+ CD25+ T lymphocytes express higher levels of surface thrombospondin***

To further characterize TSP expression in the CD4+ T lymphocyte population, we asked how surface expression of thrombospondin correlated with surface expression of CD25 on CD4+ T cells isolated from peripheral lymph nodes of FIV positive and uninfected cats. We chose this marker so that we could identify the T regulatory cell population (CD4+CD25+) in freshly isolated lymphocytes. **Figure 5** depicts representative dot plots from lymph nodes of an FIV negative and FIV positive cat, as well as the respective percentages of TSP expression on the surface of the T helper cell (T<sub>h</sub>) population (CD4+CD25-) compared with the Treg population (CD4+CD25+) in freshly isolated lymphocytes from FIV+ and uninfected cats. We then asked what percentage of the CD4+CD25+ population is expressing surface thrombospondin. **Figure 6** demonstrates that only a small percentage, 0.01 – 0.2%, of CD4+CD25+ Tregs are expressing surface

thrombospondin with no difference between Tregs isolated from peripheral lymph nodes of FIV negative or FIV+ cats.

To further describe thrombospondin expression on the surface of feline T lymphocytes, we asked if the mean fluorescence intensity of TSP expression differed between the CD4+CD25<sup>-</sup> T<sub>h</sub> cell populations and CD4+CD25<sup>+</sup> Treg populations from uninfected and FIV+ cats. **Figure 7** illustrates the mean fluorescence intensity (MFI) of TSP expression on the T<sub>h</sub> population compared with the Treg population in freshly isolated lymphocytes from FIV+ and control animals. While the CD4+CD25<sup>-</sup> T<sub>h</sub> cell population had an increased proportion of cells expressing surface thrombospondin, the CD4+CD25<sup>+</sup> Treg populations, in both FIV+ and control groups, displayed significantly higher fluorescence for surface thrombospondin expression than did the respective T<sub>h</sub> cell populations (**Figure 5d and Figure 7**).

#### ***CD4+ CD25- T lymphocytes upregulate TSP expression upon activation***

Previous experiments from our laboratory indicated that stimulating CD4+CD25<sup>+</sup> Tregs with LPS and IL-2 for 96 hours significantly increased their ability to suppress T<sub>h</sub> cell proliferation in a direct co-culture assay (Vahlenkamp, 2004). It has also been reported that TGF- $\beta$  expressed on the surface of the Tregs is responsible for mediating this suppression (Nakamura 2001, Petty, 2006). Given the role of TSP in activating latent TGF- $\beta$ , we asked if the previously mentioned stimulation would have an effect on surface TSP expression in either the Treg or T<sub>h</sub> cell populations.

First, we placed bulk, unsorted lymphocytes from peripheral lymph nodes into 96-well culture plates with or without LPS and IL2, then analyzed their CD4 CD25 phenotype as well as TSP expression by flow cytometry after 96 hours in culture. This protocol led to no change in either percentage or intensity of TSP expression in any treatment groups compared with freshly isolated cells from FIV+ (**Figure 8a and b**) or FIV negative control populations (**Figure 9a and b**). Given that we did not determine CD25 expression until after 96 hours in culture and that CD25 is not only a marker for the T regulatory cell population, but also an indicator of T<sub>h</sub> cell activation, we were unable to draw any meaningful conclusions from these results as this method did not reliably distinguish between the Treg and T<sub>h</sub> cell subsets. To address this problem, we evaluated the phenotype of the Treg and T<sub>h</sub> cell populations upon isolation and prior to stimulation.

Prior to conducting the experiments with sorted cell populations, we needed to address what the potential source(s) of the thrombospondin detecting with our flow cytometry analysis was. It is well established that serum can contain biological products such as LPS and TGF- $\beta$ . To determine if the thrombospondin detected by flow cytometry on the surface of our T lymphocytes was being produced endogenous by the cells themselves, or if it was being derived from the fetal bovine serum present in our medium and merely adhering to the lymphocyte membranes, we repeated our stimulations on unsorted lymphocytes in serum free medium. **Figure 10** is a representative dot plot of T lymphocytes from the peripheral lymph node of an FIV-infected cat that were cultured for 96h in the presence of LPS and IL2 then analyzed for CD4, CD25 and TSP

expression by flow cytometry. As reported in **Figure 10b**, 1.29% of CD4+CD25+ T lymphocytes, phenotype determined post-96h stimulation, express TSP with a mean fluorescence intensity of 63.08. The CD4+CD25- T cells had a mean fluorescence intensity of thrombospondin expression of 25.64 with 1.85% of this cell population positive for surface TSP (**Fig. 10b**). These numbers fall well within the range for peripheral lymph node T lymphocytes from FIV+ cats cultured in serum-containing medium. Given the duration of the culture conditions, we observed greatly increased incidence of cell death with serum-free medium than with the medium containing fetal bovine serum. For this reason, we continued to use serum in our media to increase the cell numbers available for flow cytometry analysis.

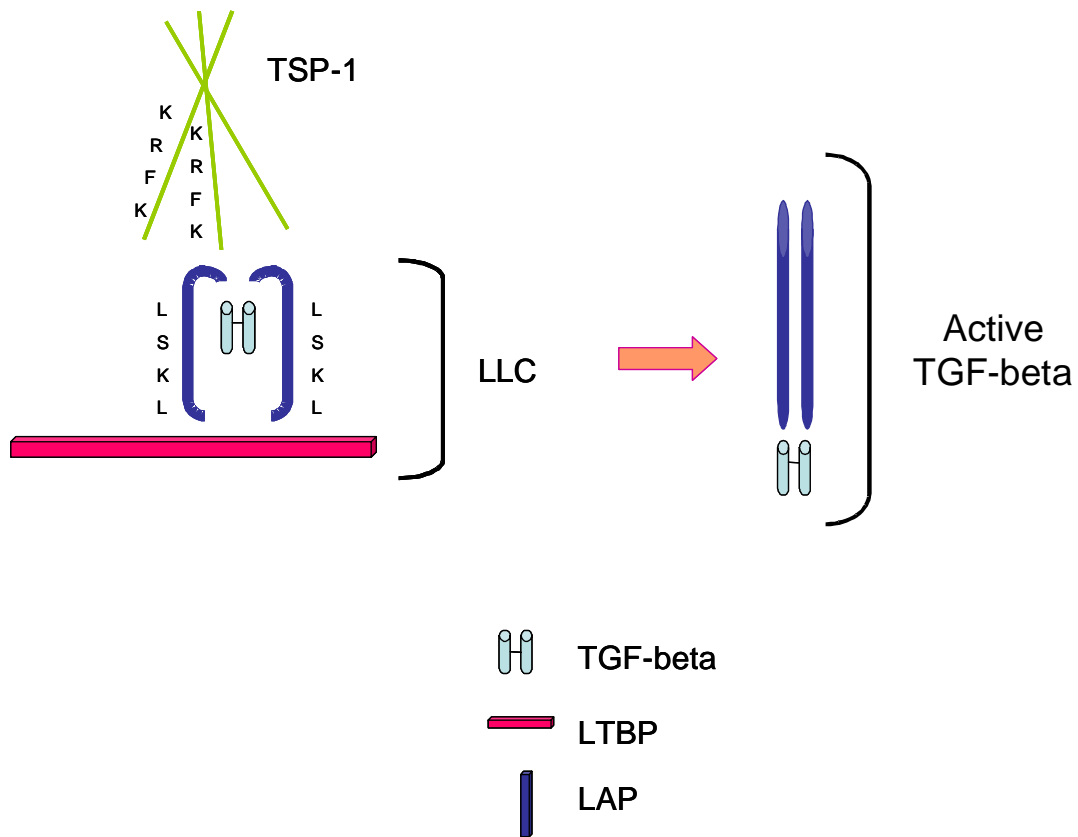
To better answer the question; is thrombospondin expressed at different levels on CD4+CD25+ Tregs and CD4+CD25- T helper cells, we classified the CD4 CD25 phenotype of our lymphocytes prior to stimulation. Upon isolation, lymphocytes from peripheral lymph nodes were stained with biotinylated CD4 and FITC-conjugated CD25. These cells were sorted into CD4+CD25+ and CD4+CD25- populations with a MoFlo cell sorter. Each population was incubated with 100U/ml recombinant IL2 and/or 10 µg/ml LPS for 96 hours then analyzed again for expression of CD4, CD25 and TSP by flow cytometry. The CD4+CD25+ Treg population showed no significant change in surface TSP expression with any treatment (**Figure 11**). The CD4+CD25- population however, showed significant increases in surface TSP expression when treated with LPS and IL2 both added at day 0 and with LPS added at day 0 with IL2 added at day 2 of the 96h culture (**Figure 12**). These data are only from FIV positive cats as we were unable to

collect more than  $1.5 \times 10^6$  CD4+CD25+ cells from any single FIV negative cat. Since a minimum of  $5 \times 10^5$  cells is required to perform a single stimulation, too few cells were available to perform all necessary controls and experimental stimulations.

The flow analysis of LPS and IL2 activated sorted CD4+CD25- T cells from FIV+ cats revealed that some of these cells had upregulated CD25 expression on their surface, consistent with an activated T helper cell phenotype (Thornton, 1998). To further analyze what was occurring in the CD4+CD25- T helper population as a result of stimulation with LPS and IL2, we compared the MFI of thrombospondin expression between the sorted cells that remained CD25- and those that had converted to a CD25+ phenotype (**Figure 13**). In a representative sample, approximately equal proportions of CD25- and CD25+ cells were expressing TSP, 0.46 and 0.44% respectively, but the CD25+ population expressed much higher intensity of TSP on their surface, 64.3 MFI compared with 8.8 MFI in the CD25- population (**Figure 13b**). **Figure 14** compares the two populations of LPS and IL2 activated sorted CD4+CD25- T helper cells. The converted CD25+ cells express significantly higher levels of surface TSP than the CD25- cells. The treatment of LPS added at day 0 with IL2 also added at day 0 had significantly higher TSP expression than the treatment populations where IL2 was added at day 2. From this, we concluded that TSP expression was not variable in the Treg population after stimulation with LPS plus IL2; however, TSP expression was upregulated on CD4+CD25- T helper cells that were activated as a result of LPS and IL2 treatment.

*FIV negative CD4+ CD25- T lymphocytes have a greater ability to upregulate surface expression of TSP*

Next, we asked whether there was a difference in the ability of CD4+CD25- T<sub>h</sub> cells from FIV positive compared with control cats to upregulate surface thrombospondin. As previously discussed, we observed no significant difference in TSP expression between CD4+CD25- cells from FIV infected and control cats, or in the CD4+CD25+ Treg populations as a result of the cats FIV status when freshly isolated (**Figure 7**). After LPS plus IL2 stimulation however, we observed more marked upregulation of TSP on CD4+CD25- T<sub>h</sub> cells from FIV negative, control, cats compared to the equivalent population from FIV+ animals. We obtained similar results for cells stimulated with both LPS and IL2 at day 0 (**Figure 15**) and LPS at day 0 with IL2 at day 2 (**Figure 16**).



**Figure 1. Proposed structure of how thrombospondin converts latent TGF-beta complex into biologically active form.** TGF-beta is associated with the latency-associated peptide (LAP) and the latent TGF-beta binding protein (LTBP) to form the large latent complex (LLC), left. The KRFK amino acid residues present on thrombospondin interact with the LSKL residues on LAP to convert TGF-beta into its biologically active form (right), capable of binding and signaling through TGF-beta receptor II.

**Figure 2. Thrombospondin protein conservation.** Black shading indicates identical amino acid residues compared with the human thrombospondin sequence. Gray shading indicates functionally similar amino acids. White boxes depict functionally different residues and (–) indicate areas where sequences do not align.

Human 1 --MGLAWGLGVLEFLMHVCGSNRIPESGGDNCVFDIFELTG---AARKGSGRRLLVKGPDPS  
mouse 1 --MELLRGLGVLEFLLHMCNRIIPESGGDNCVFDIFELIG---GARRGPGRRLLVKGQDLS  
rat 1 --MELLRGLGVLEFLLHVCGSNRIPESGGDNCVFDIFELIG---GARKVPGRRLLVKGQDLS  
cow 1 --MGLAWGLGVLEFLLHACNRIIPESGGDNCVFDIFELTG---AARKRSGRRLLVKGPDPS  
zebrafish 1 ---MKSTAFELLLMLWNCEARVAESRDNSVVDLIFELVQVP---RKNHGVTLLVKGDDPY  
dog 1 --MGLAWALGVLEFLLRVCASSRIIPESGGDNCVFDIFELTG---AARRGSGRRLLVKGPDPS  
chicken 1 MGPAAVVLLLLALGGPEAKRTAESRGDDSDVFDIFELTVRKAGARRAPGVHLLVKGPDTS

Human 56 SPAFRIEDANLIPVPPDDKFDQLVDVAVRAEKGFLLLASLRQMKKTRGTLTLLAERKDHSGQ  
mouse 56 SPAFRIENANLIPAVPDDKFDQLLDVAVWADKGFIFLASLRQMKKTRGTLTLLAERKDNIGQ  
rat 56 SPAFRIENANLIPVPPDDKFDQLLDVAVWADKGFIFLASLRQMKKTRGTLTLLAERKDNISGQ  
cow 56 SPAFRIEDANLIPVPPDDKFDQLVDVAVRAEKGFLLLASLRQMKKTRGTLTLLAERKDHSGQ  
zebrafish 55 SPAYKILNPDLIPVPPESAFRDLIDSIHAEKGFLLLVNEKQFKRTRGSLTIVKNDGSGP  
dog 56 SPAFRIEDANLIPVPPDDKFDQLVDVAVRAEKGFLLLASLRQMKKTRGTLTLLAERKDHSGQ  
chicken 61 SPAYRIEDASRIPAVSDSKFDQLLDIITHAEKGFILQATLRQAKKSRGTLTLLAERKDGSGH

Human 116 VFSVVSNGKAGTLDLSTLVQKQHVVSVEEALLATGQWKSITLFLVQEDRAQLYIDCEKME  
mouse 116 IFSVVSNGKAGTLDLSTLSPGKQOVVSVEEALLATGQWKSITLFLVQEDRAQLYIDCDKME  
rat 116 IFSVVSNGKAGTLDLSTLSPGKQOVVSVEEALLATGQWKSITLFLVQEDRAQLYIDCDKME  
cow 116 VFSVVSNGKAGTLDLSTLVQKQHVVSVEEALLATGQWKSITLFLVQEDRAQLYIDCEKME  
zebrafish 115 VFEIVSNGKANTLDIVFSTENKQOVVSIEEADLAVGHWNITLFLVQEDRVQFYVGCBEVN  
dog 116 VFSVVSNGRAGTLDLSTLVQGMCHVVSVEEALLATGQWKSITLFLVQEDRAQLYIDCEKME  
chicken 121 VFSLVSNGKAGTLDLSTLSDGKQQLVSVEDALLATGHWNITLFLVQEDRAQLYVGCBEKME

Human 176 NAELDVPIQSVFTRDLASVARLRVAKGVDNDFQGVLQNVRFVFGTTPEDILRNKGCSSS  
mouse 176 SAELDVPIQSVIFTRDLASVARLRVAKGVDNDFQGVLQNVRFVFGTTPEDILRNKGCSSS  
rat 176 SAELDVPIQSVIFTRDLANVARLRVAKGVDNDFQGVLQNVRFVFGTTPEDILRNKGCSSS  
cow 176 NAELDVPIQSVIFTRDLASVARLRVAKGVDNDFQGVLQNVRFVFGTTPEDILRNKGCSSS  
zebrafish 175 VAELDASTHILITQEIPGAKMRIGKGAVDKRFMGLVQNVRFVFGTTPEDILRNKGCQNS  
dog 176 NAELDVPIQSVIFTRDLASVARLRVAKGVDNDFQGVLQNVRFVFGTTPEDILRNKGCSSS  
chicken 181 NAELDIPIONIFTRDLASSARLRVAKGVDNDFQGLLQNVRFVVRTTLETILRNKGCSSS

Human 236 TS-VLITLDNIVVNGSSPAIRTNVYIGHKTKDLQAICGISCDELSSMVLELRGLRTIVTTL  
mouse 236 ATNVLITLDNIVVNGSSPAIRTNVYIGHKTKDLQAICGLSCDELSSMVLELRGLRTIVTTL  
rat 236 TN-VLITLDNIVVNGSSPAIRTNVYIGHKTKDLQAICGLSCDELSSMVLELRGLRTIVTTL  
cow 236 TS-VFVITLDNIVVNGSSPAIRTDYIGHKTKDLQAICGISCDELSSMVLELRGLRTIVTTL  
zebrafish 235 MT--DIITLDNIPVNGSSPAIRTDYIGHKTKDLQMICGFSCEDLAAMFKELKGLGVVQQL  
dog 236 TN-VLITLDNIVVNGSSPAIRTNVYIGHKTKDLQAICGISCDELSSMVLELRGLRTIVTTL  
chicken 241 TS--AIIITLDKPMNGSSPAIRTNVYIGHKTKDLQAVCGFSCDELINMFVELQGLRSMVTTTL

Human 295 QDSIRKVTEENKELANELRRPE-LCYHNGVQYRNNEEWTVDSCTECHCONSVTICKKVSC  
mouse 296 QDSIRKVTEENRELVSELKRPE-LCFHNGVQYRNNEEWTVDSCTECHCONSVTICKKVSC  
rat 295 QDSIRKVTEENRELASELRRPE-LCFHNGVQYRNNEEWTVDSCTECHCONSVTICKKVSC  
cow 295 QDSIRKVTEENKELANELRRPE-LCYHNGVQYRTGDEWTVDSCTECRCONSVTICKKVSC  
zebrafish 293 SNEIRKVTDDKNMLMNQMGIRAGVCLHNGIVHKNKEEWTVDCTECTCONSATVCRKISC  
dog 295 QDSIRKVTEENKELAIELRRPE-LCYHNGVQYRNNEEWTVDSCTECRCONSVTICKKVSC  
chicken 299 QDRVRKVTEENELIAKVQITPQVCIHNGILHKNKEEWTVDSCTECTCONSATICRKVSC

Human 354 PIMPCSNATVPDGECCPRCWF-SDSADDGWSPWSEWTSCSATCGNGIQQRGRSCDSLNNR  
mouse 355 PIMPCSNATVPDGECCPRCWF-SDSADDGWSPWSEWTSCSATCGNGIQQRGRSCDSLNNR  
rat 354 PIMPCSNATVPDGECCPRCWF-SDSADDGWSPWSEWTSCSATCGNGIQQRGRSCDSLNNR  
cow 354 PIMPCSNATVPDGECCPRCWF-SDSADDGWSPWSEWTSCSATCGNGIQQRGRSCDSLNNR  
zebrafish 353 PLIPCANATVPDGECCPRCWF-SDSAEDGWSPWSEWTHCSVSCGRGIQQRGRSCDRINNV  
dog 354 PIMPCSNATVPDGECCPRCWF-SDSADDGWSPWSEWTSCSATCGNGIQQRGRSCDSLNNR  
chicken 359 PIMPCSNATVPDGECCPRCWF-SDYADDGWSPWSEWTSCSATCGNGIQQRGRSCDSLNNR  
Human 413 CEGSSVQTRTCHIQECDKRFQDGGWSHSPWSSCSVTCCDGVITRIRLNCNSPSPQMNGK  
mouse 414 CEGSSVQTRTCHIQECDKRFQDGGWSHSPWSSCSVTCCDGVITRIRLNCNSPSPQMNGK

rat 413 CEGSSVQTRTCHIQECDKRFKQDGGWSHWSPWSSCSVTCCDGVITRIRLNCNSPSPQMNGK  
 cow 413 CEGSSVQTRTCHIQECDKRFKQDGGWSHWSPWSSCSVTCCDGVITRIRLNCNSPSPQMNGK  
 zebrafish 413 CEGTSVQTRTCHIQECDKRFKQDGSWSHWSPWSSCSVTCCAGVITRIRLNCNSPTPQMDGK  
 dog 413 CEGSSVQTRTCHIQECDKRFKQDGGWSHWSPWSSCSVTCCDGVITRIRLNCNSPSPQMNGK  
 chicken 418 CEGSSVQTRTCHIQECDKRFKQDGGWSHWSPWSSCSVTCCDGMITRIRLNCNSEVLPQINGK

Human 473 PCEGEARETKACKKDACPINGGWGWPSPWDICSVTCGGGVQRRSRLCANNPTPQFGGKDCV  
 mouse 474 PCEGEARETKACKKDACPINGGWGWPSPWDICSVTCGGGVQRRSRLCANNPTPQFGGKDCV  
 rat 473 PCEGEARETKACKKDACPINGGWGWPSPWDICSVTCGGGVQRRSRLCANNPTPQFGGKDCV  
 cow 473 PCEGEARETKACKKDACPINGGWGWPSPWDICSVTCGGGVQRRSRLCANNPTPQFGGKDCV  
 zebrafish 473 DCQEGCRQTERCEKSPCPINGGWGWPSPWDICSVTCGGGVQNRKRLCANNPVPKHGGKECV  
 dog 473 PCEGEARETKACKKDACPINGGWGWPSPWDICSVTCGGGVQRRSRLCANNPTPQFGGKDCV  
 chicken 478 PCEGEARETKSCKKDPCPINGNWGWPSPWDICTVTCCGGVQRRSRLCANNPEPQYGGKACV

Human 533 GDVTENQICNKQDCPIDGCLSNPCFAGVKCTSYPDGSWKCGACPPPGYSNGIQCCTDVDEC  
 mouse 534 GDVTENQVCNKQDCPIDGCLSNPCFAGAKCTSYPDGSWKCGACPPPGYSNGIQCKDQVDEC  
 rat 533 GDVTENQVCNKQDCPIDGCLSNPCFAGAKCTSYPDGSWKCGACPPPGYSNGIQCCKDQVDEC  
 cow 533 GDVTENQICNKQDCPIDGCLSNPCFAGVQCTSYPDGSWKCGACPPPGYSGDGVECKDQVDEC  
 zebrafish 533 GDAKVSQICNKQACPVDGCLSSPCFEGAQCTSEPDGSWKCGCPTGYTNGINGCKDQVNEC  
 dog 533 GDATENQICNKQDCPIDGCLSNPCFAGVKCTSYRDPGSWKCGCCTPPPGYSNGIQCCKDQVDEC  
 chicken 538 GEAKGTQVCNKQDCPIDGCLSNPCFAGTTCTSSPDGSWKCGACPPAGYHNGIHCQDIDEC

Human 593 KEVPDACFNHNGEHRCENTDPGYNCLPCPPRFTGSQPFGRGVEHATANKQVCKPRNPCTD  
 mouse 594 KEVPDACFNHNGEHRCKNTDPGYNCLPCPPRFTGSQPFGRGVEHAMANKQVCKPRNPCTD  
 rat 593 KEVPDACFNHNGEHRCKNTDPGYNCLPCPPRFTGSQPFGRGVEHAMANKQVCKPRNPCTD  
 cow 593 KEVPDACFNHNGEHRCENTDPGYNCLPCPPRFTGSQPFGRGVEHATANKQVCKPRNPCTD  
 zebrafish 593 KEVPDACFEFNGVHRCENTVPGYNCLPCPTRYTGPQPFGRGVEDAAAKKQVCTPRNPCTD  
 dog 593 KEVPDACFNHNGEHRCENTDPGYNCLPCPPRFTGSPQPFGRGVEHATAHKQVCKPRNPCTD  
 chicken 598 KEVPDACFVFNNGVHRCENTDPGYNCLPCPPHFTGSQPFGRSVEDATANKQVCKPRNPCTD

Human 653 GTHDCNKNAKCNLYLGHYSDPMYRCECKPGYAGNGIICGEDTDLDGWPNEMLVCVANATYH  
 mouse 654 GTHDCNKNAKCNLYLGHYSDPMYRCECKPGYAGNGIICGEDTDLDGWPNEMLVCVANATYH  
 rat 653 GTHDCNKNAKCNLYLGHYSDPMYRCECKPGYAGNGIICGEDTDLDGWPNEMLVCVANATYH  
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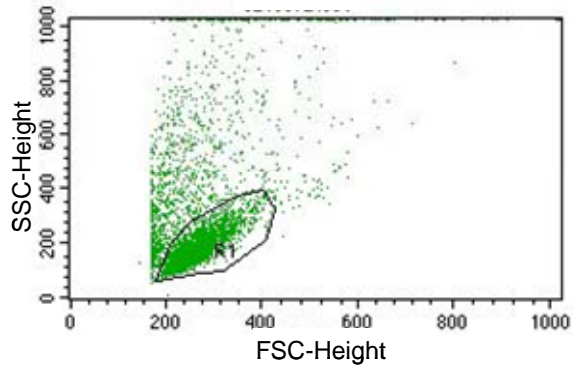
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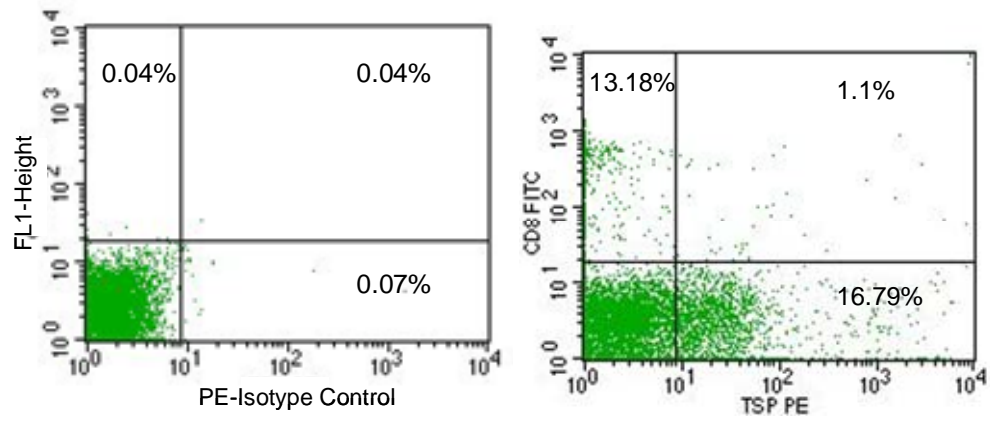
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dog	1133	ADSGPIYDKTYAGGRLGLFVFSQEMVFFSDLKYECRDS
chicken	1138	ADSGPIYDKTYAGGRLGLFVFSQEMVFFSDLKYECRDP

**Figure 3. Percent CD8+ TSP+ from the total peripheral blood lymphocyte population.** (A) Forward vs. side scatter used to isolate the live lymphocyte population and set the R1 gate from which all other analysis is performed. (B and C) PE-isotype control for thrombospondin antibody used to set axis from which TSP+ percentages are determined. Representative FIV- and FIV+ dot plots with indicated quadrant percentages. (D) Percentage of CD8+ TSP+ lymphocytes from the total live lymphocyte population isolated from peripheral blood of control (n = 11) and FIV+ cats (n = 6). There is no significant difference between FIV- and FIV+ populations ( $P > 0.05$ ), individual sample points are shown.

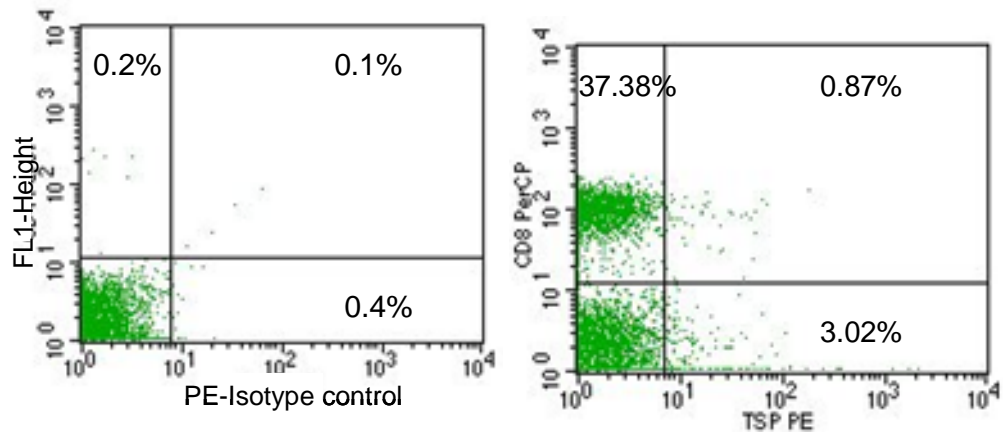
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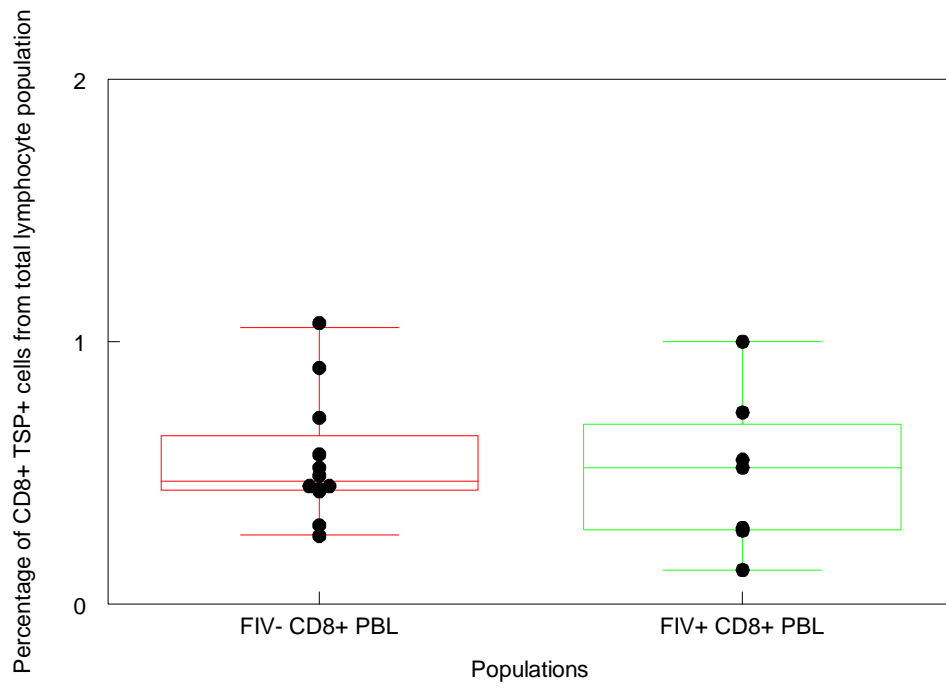
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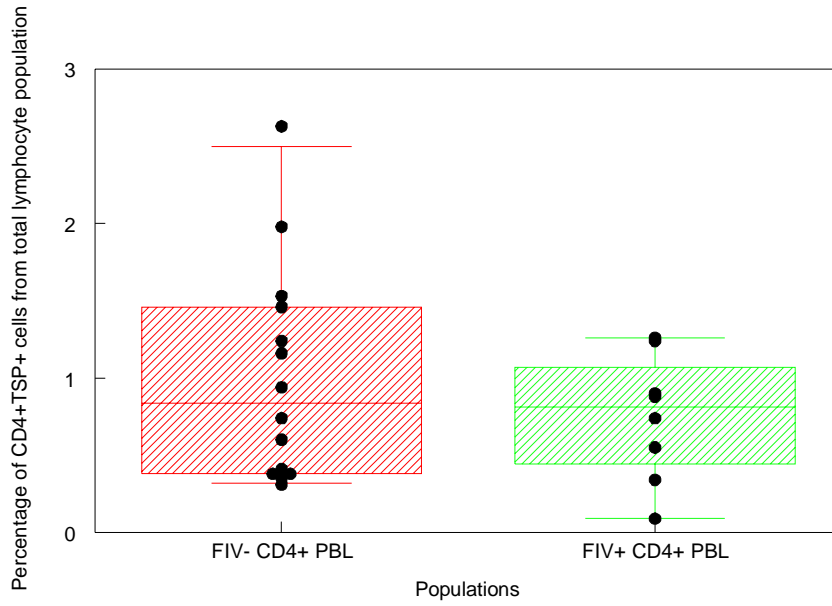
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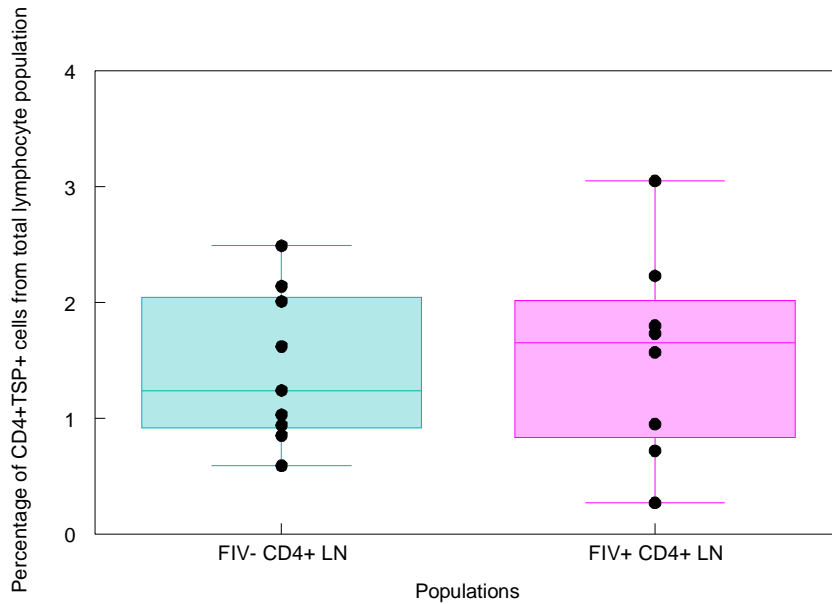
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**A**

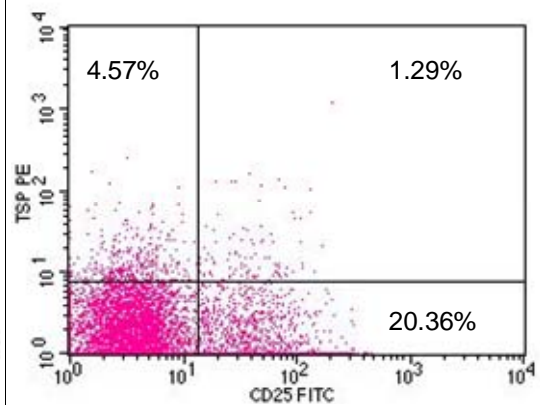
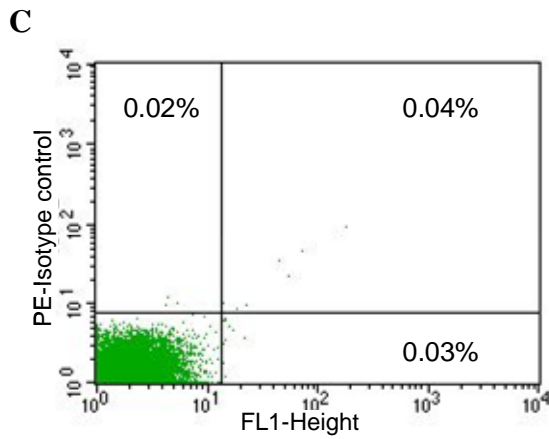
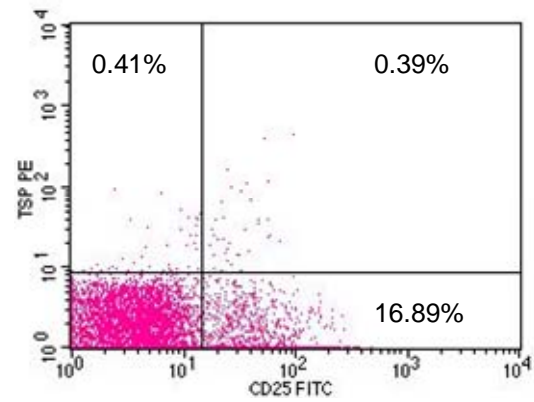
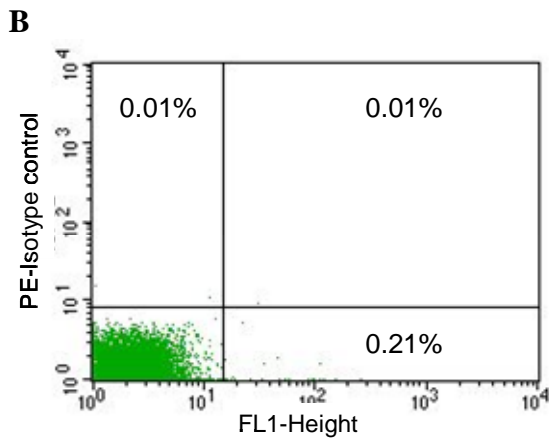
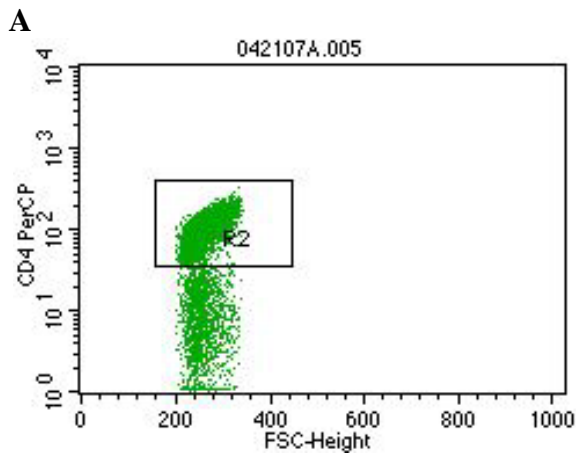


**B**

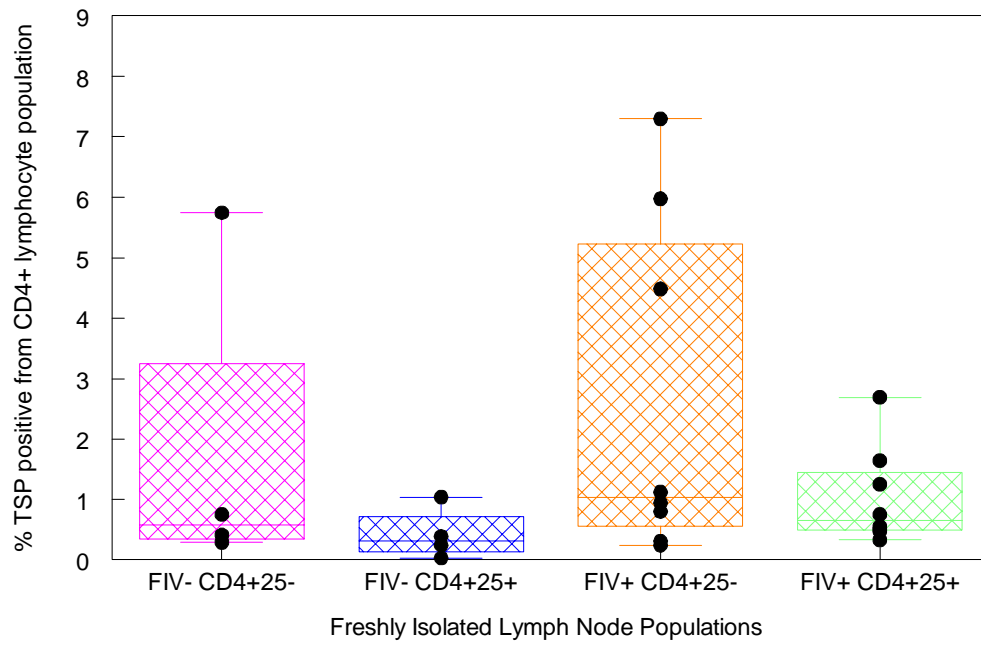


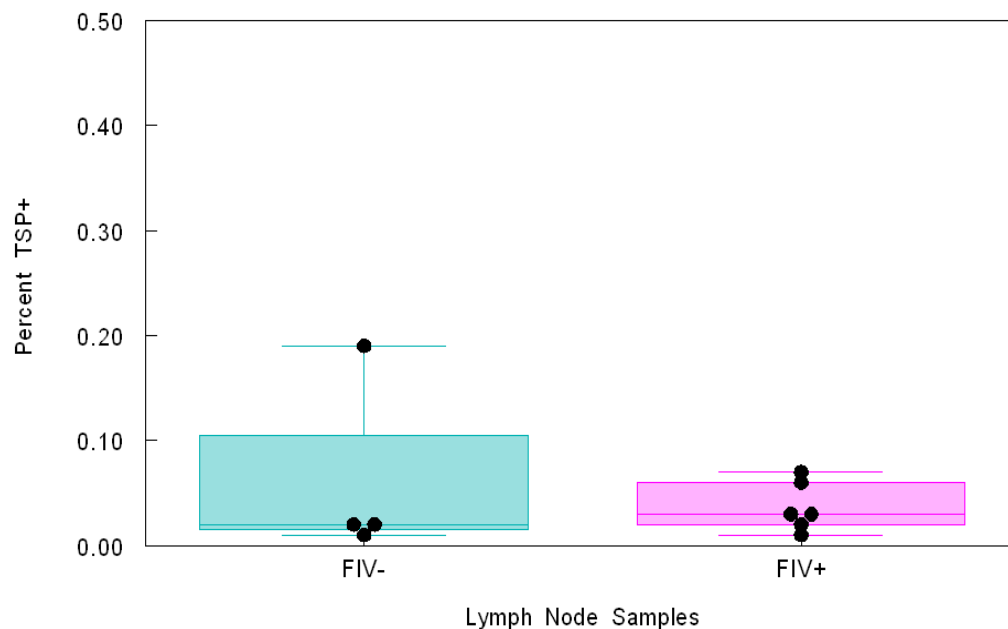
**Figure 4. Percent CD4+ TSP+ lymphocytes from peripheral blood or peripheral lymph nodes. (A) Peripheral blood populations, individual samples shown. (B) Lymph node populations, individual samples shown.**

**Figure 5. Percent CD4+ TSP+ lymphocytes with or without CD25 expression from peripheral lymph nodes.** Freshly isolated lymphocytes from peripheral lymph nodes of FIV negative and FIV infected cats were stained with anti-CD4, CD25 and TSP fluorescently labeled monoclonal antibodies. **(A)** These cells were gated on the live lymphocyte population (R1, see Fig. 3A) then gated for positive CD4 expression (R2). **(B and C)** Gated cells were then analyzed for TSP and CD25 expression. The left dot plots illustrate the anti-TSP PE-isotype control and the right dot plots are representative FIV- and FIV+ samples with indicated quadrant percentages. **(D)** Percent of CD4+ and CD25 + or CD25- populations expressing TSP from freshly isolated peripheral lymph nodes obtained from FIV+ (n = 8) and control cats (n = 6). There is no significant difference between any of the populations ( $p > 0.05$ ), individual samples are shown.

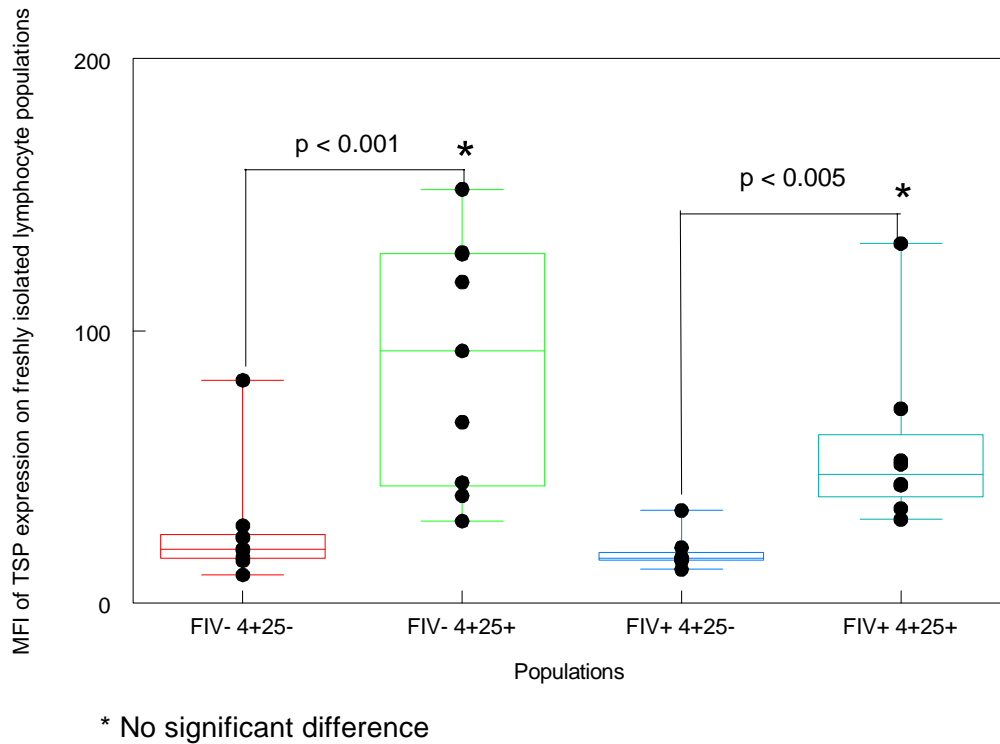


**D**





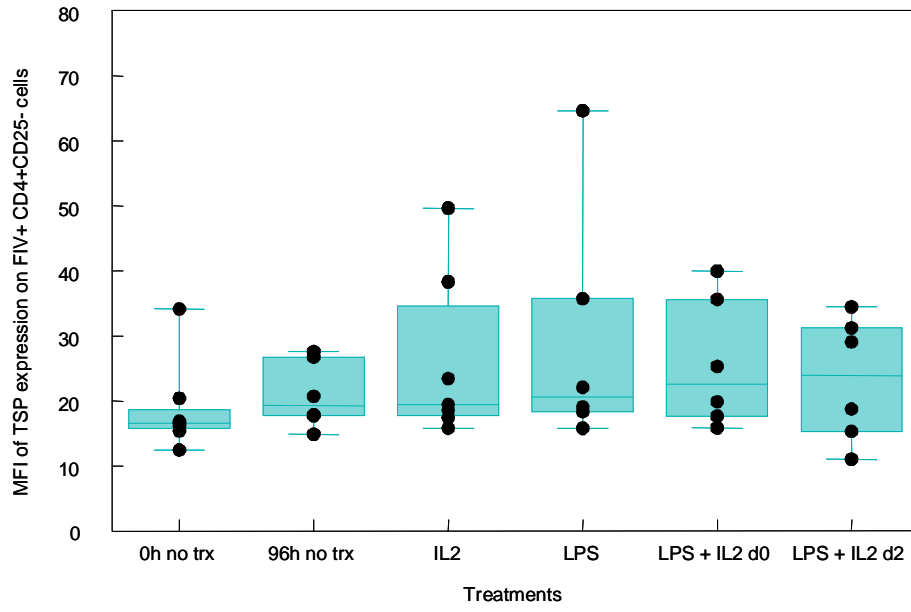
**Figure 6. Percentage of CD4+CD25+ population expressing TSP on the cell surface.** Lymphocytes were isolated from peripheral lymph nodes of FIV infected and uninfected cats then stained with monoclonal antibodies for CD4, CD25 and TSP and analyzed by flow cytometry directly after isolation. Percentage of TSP+ cells is calculated out of the CD4+CD25+ population only, representing a population of enriched T regulatory cells. There is no significant difference in the percentage of Tregs expressing TSP on their surface between FIV+ and FIV- samples ( $p > 0.05$ ). N = 4 samples were used from FIV negative cats and n = 6 samples were used from FIV positive cats.



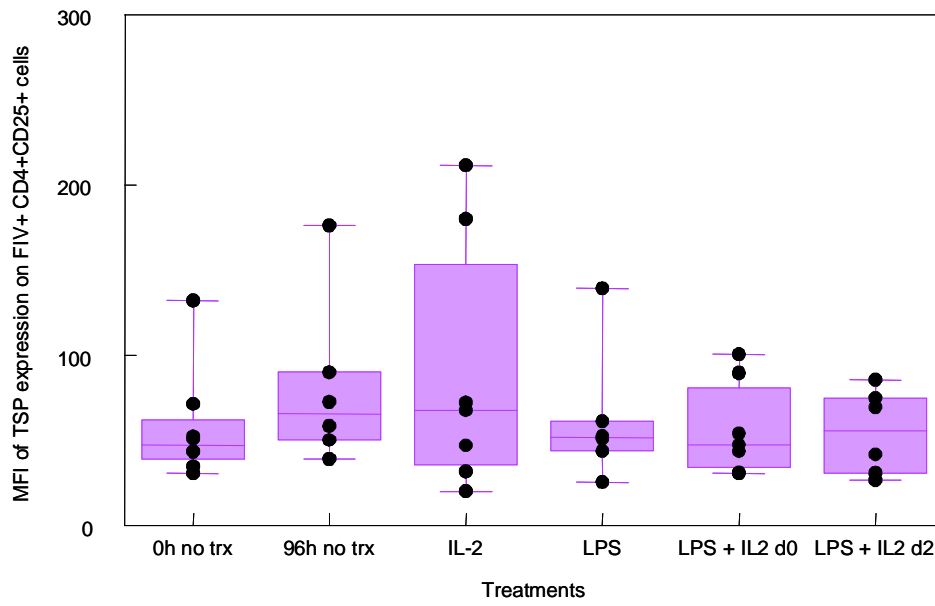
**Figure 7. Mean fluorescence intensity of thrombospondin expression on T cell populations from lymph nodes.** Lymphocytes were isolated from peripheral lymph nodes of experimental and control cats then stained with anti-CD4, CD25 and TSP fluorescently tagged monoclonal antibodies. Gated live lymphocytes on CD4+ expression then determined the intensity of TSP staining on CD25 +/- populations. No significant difference indicates  $p > 0.05$ . Individual samples shown.

**Figure 8. Mean fluorescence intensity of thrombospondin expression on stimulated T lymphocytes from peripheral lymph nodes of FIV+ cats.** Bulk unsorted lymphocytes from peripheral lymph nodes of FIV+ cats were stimulated with 100U/ml IL2 and/or 10µg/ml LPS. LPS + IL2 d0 indicates that LPS and IL2 were both added at day 0. LPS + IL2 d2 indicates LPS added at day 0 with IL2 added at 2 of culture. After 96h incubation the cells were stained with fluorescently tagged monoclonal antibodies against CD4, CD25 and TSP. Their CD4 CD25 phenotype as well as TSP expression was characterized after stimulation. **(A)** CD4+CD25- T cells as characterized after 96h in culture. **(B)** CD4+CD25+ T cells as phenotyped after 96h in culture. No significant difference was observed between the freshly isolated 0h no treatment control and any experimental treatment population,  $p > 0.05$ . Individual samples are shown.

**A**

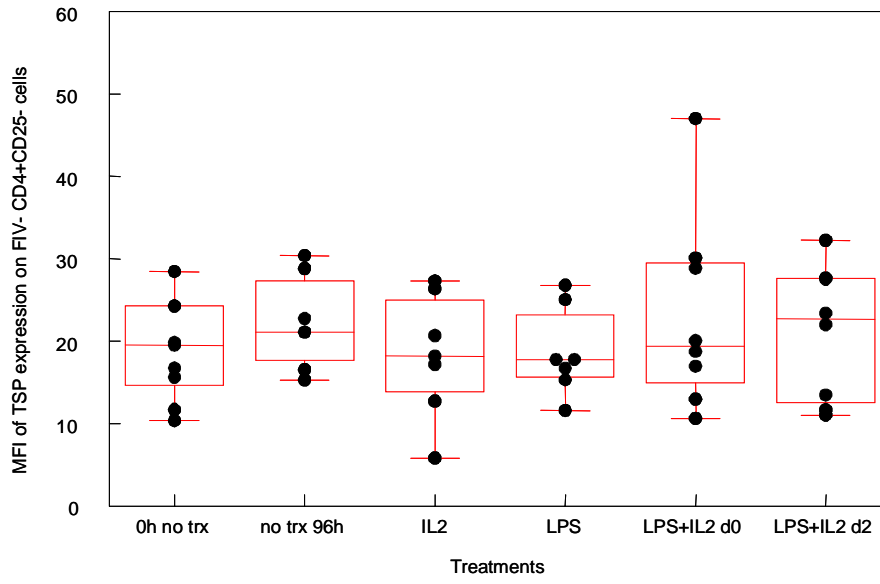


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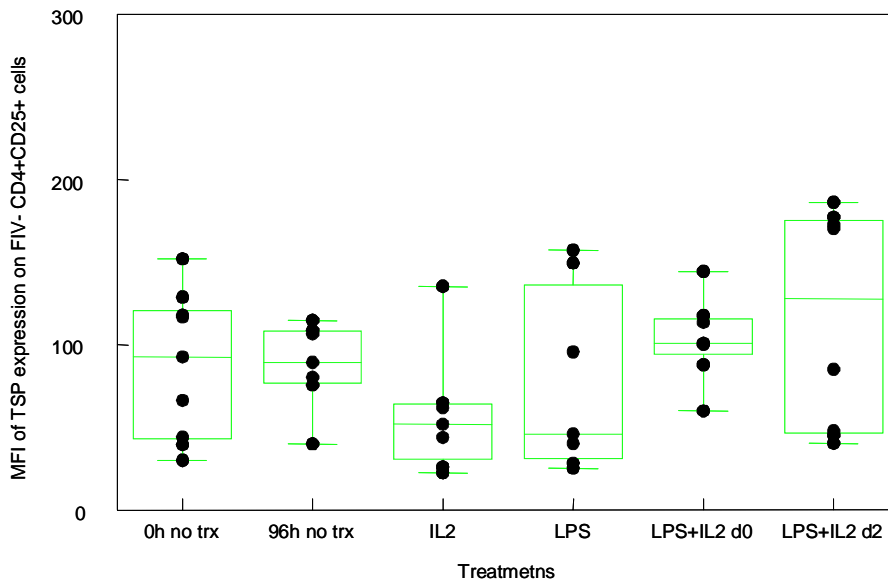


**Figure 9. Mean fluorescence intensity of thrombospondin expression on stimulated T lymphocytes from peripheral lymph nodes of FIV negative cats.** Bulk unsorted lymphocytes from peripheral lymph nodes of FIV negative cats were stimulated as indicated then stained with fluorescently tagged monoclonal antibodies against CD4, CD25 and TSP. Their CD4 CD25 phenotype as well as TSP expression was characterized after stimulation. **(A)** CD4+CD25- T cells as characterized after 96h in culture. **(B)** CD4+CD25+ T cells as phenotyped after 96h in culture. No significant difference was observed between the freshly isolated 0h no treatment control and any experimental treatment population,  $p > 0.05$ . Individual samples are shown.

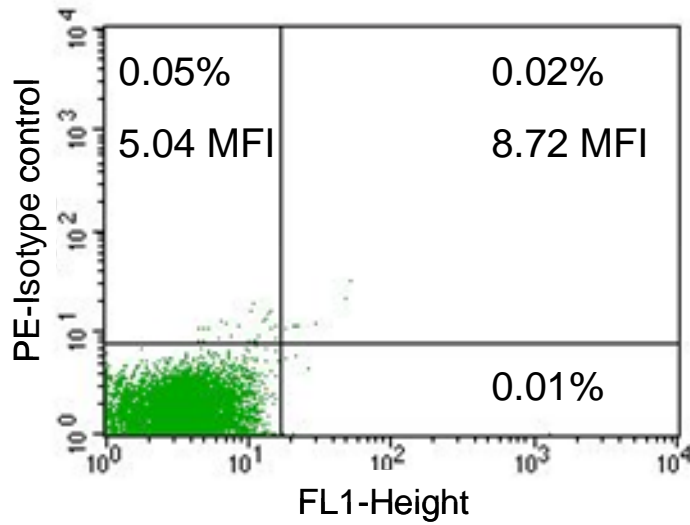
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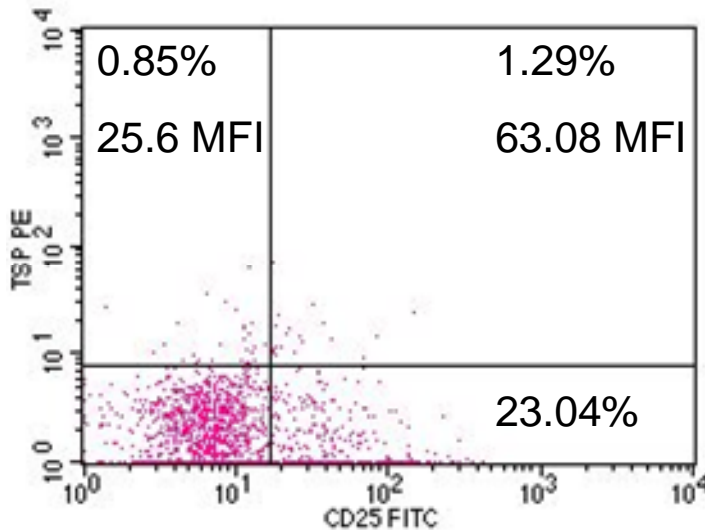
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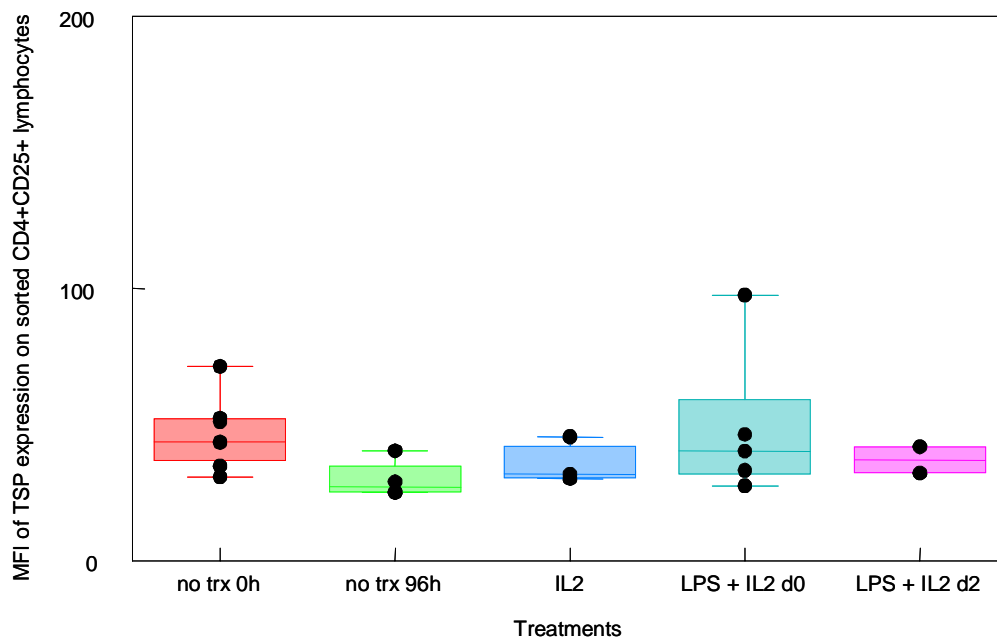
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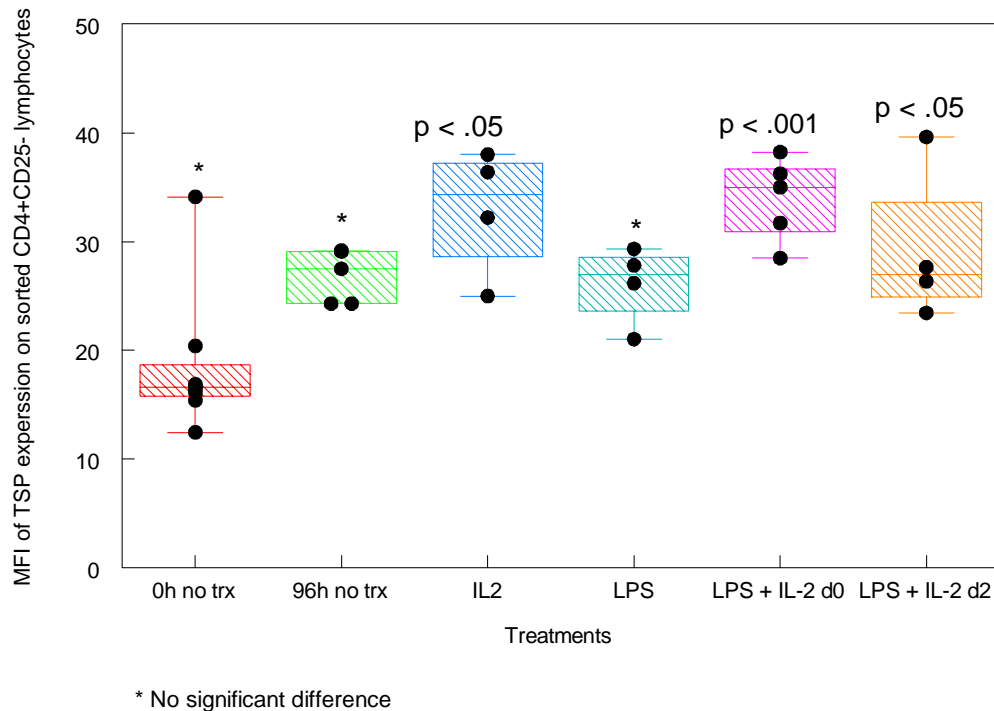
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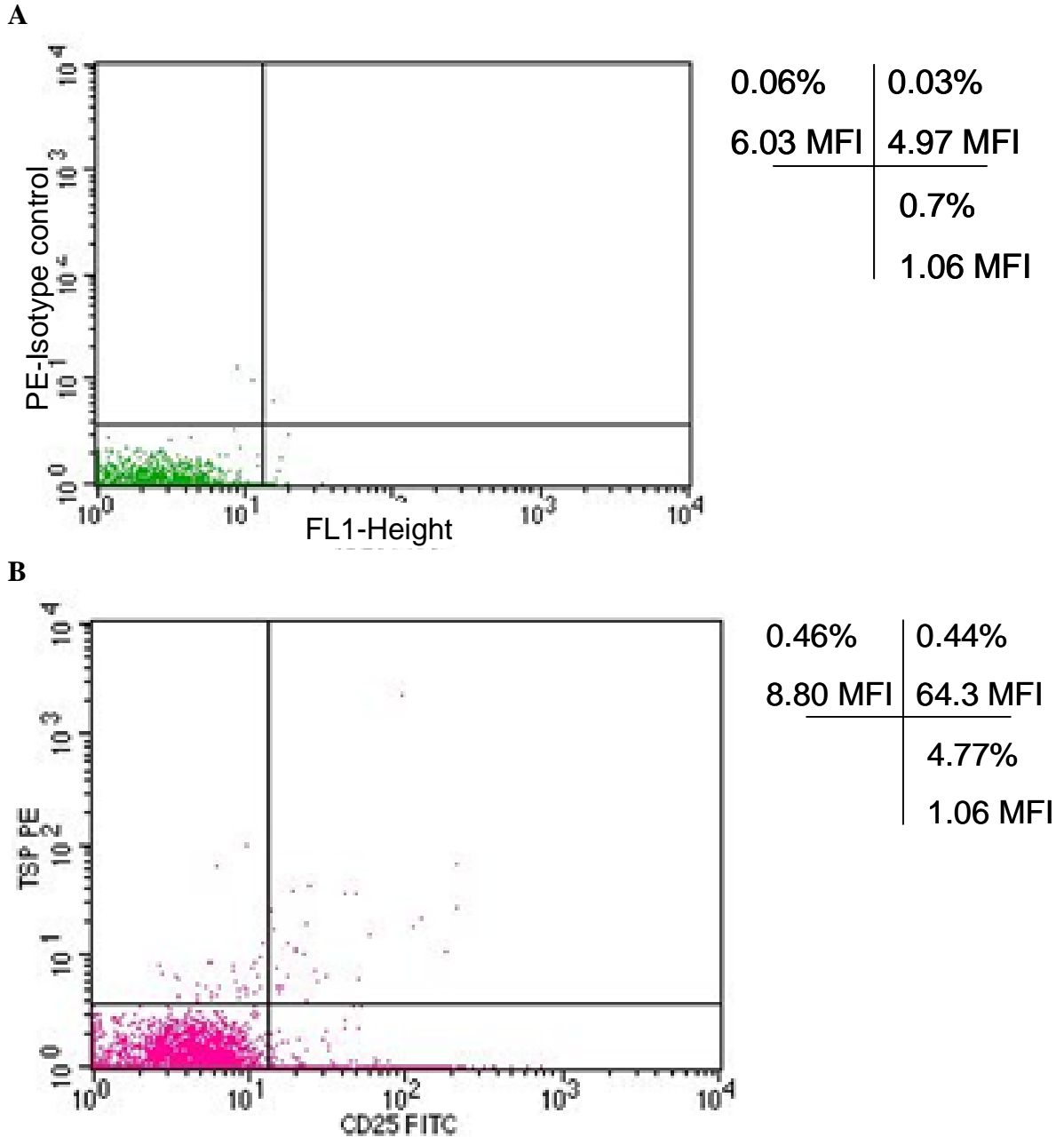
**Figure 10. Representative dot plot of unsorted lymphocytes from FIV+ peripheral lymph nodes stimulated with LPS and IL2 for 96h in serum-free medium.** (A) Unsorted FIV+ lymphocytes from peripheral lymph nodes were cultured for 96h in the presence of 10µg/ml LPS and 100U/ml of recombinant human IL2, washed, then stained with PE-conjugated mouse IgG1 (TSP isotype control). (B) Unsorted FIV+ lymphocytes from peripheral lymph nodes were cultured for 96h in the presence of 10µg/ml LPS and 100U/ml of recombinant human IL2, washed, then stained with biotinylated anti-CD4, PE-conjugated anti-TSP and FITC labeled anti-CD25 and analyzed by flow cytometry after gated on the live lymphocyte population and CD4+ cells. Quadrant percentages and mean fluorescence intensities are indicated.



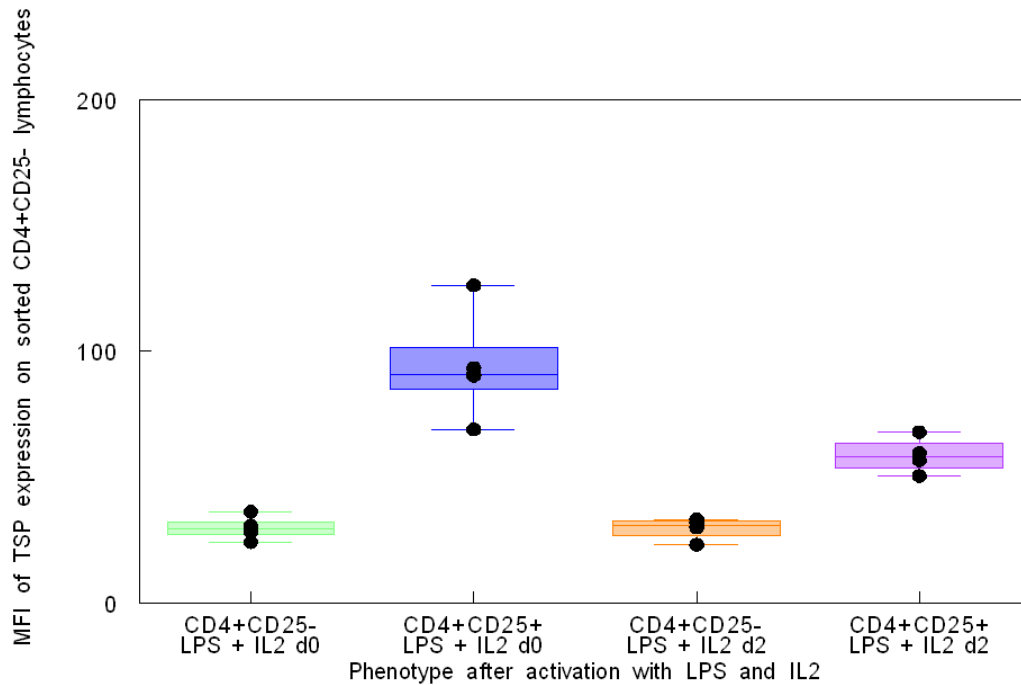
**Figure 11. Mean fluorescence intensity of thrombospondin expression on sorted CD4+CD25+ lymphocytes from FIV+ cats.** Lymphocytes were stained with fluorescently tagged monoclonal antibodies against CD4 and CD25 for MoFlo cell sorting or stained with anti-CD4, CD25 and TSP for analysis directly after isolation. Sorted CD4+CD25+ lymphocytes were stimulated, as indicated, for 96h, or stained for flow analysis directly after isolation. After stimulation, cells were stained with fluorescently tagged monoclonal antibodies for CD4, CD25 and TSP and gated on CD4+ expression. This graph indicates the mean fluorescence intensity of TSP expression on CD4+CD25+ lymphocytes as determined after 96h in culture. No significant difference,  $p > 0.05$ , was observed between the 0h no treatment control and any of the treatments. Individual samples are shown.



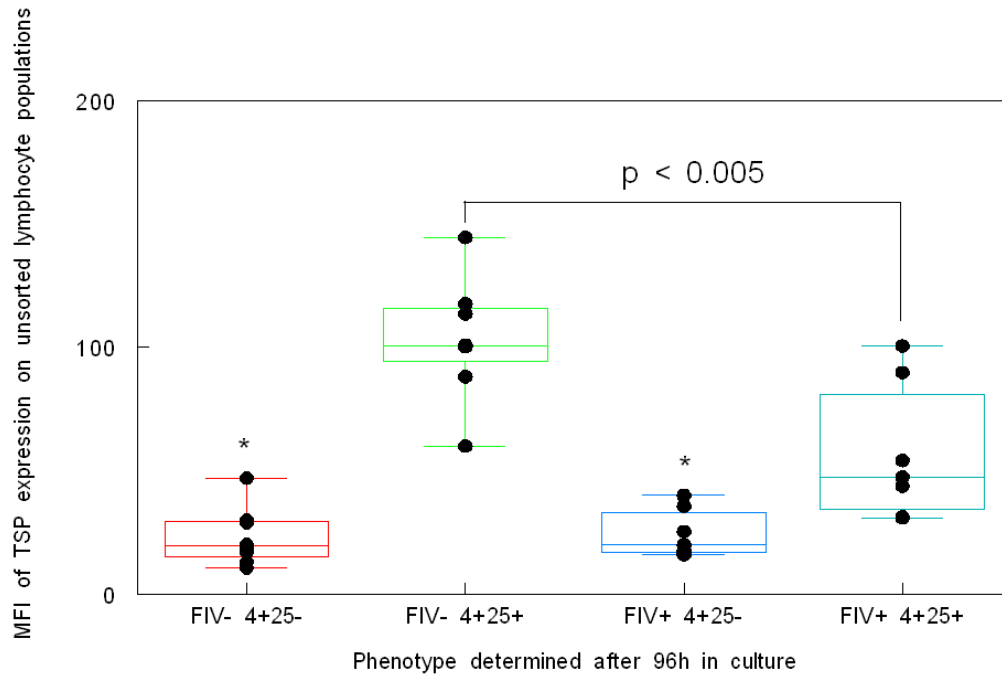
**Figure 12. Mean fluorescence intensity of thrombospondin expression on sorted CD4+CD25- T lymphocytes from FIV+ cats.** Lymphocytes were stained with fluorescently tagged monoclonal antibodies against CD4 and CD25 for cell sorting or stained with anti-CD4, CD25 and TSP for analysis directly after isolation. Sorted CD4+CD25- cells were stimulated for 96h as indicated then stained again with anti-CD4, CD25 and TSP for analysis. No treatment for 96 hours and LPS stimulation showed no significant difference,  $p > 0.05$ , in TSP expression compared with freshly isolated no treatment control. IL2 treatment and combinations of LPS and IL2 showed significantly increased expression of TSP compared with the 0h no treatment control group (p-values indicated). Individual samples are shown.



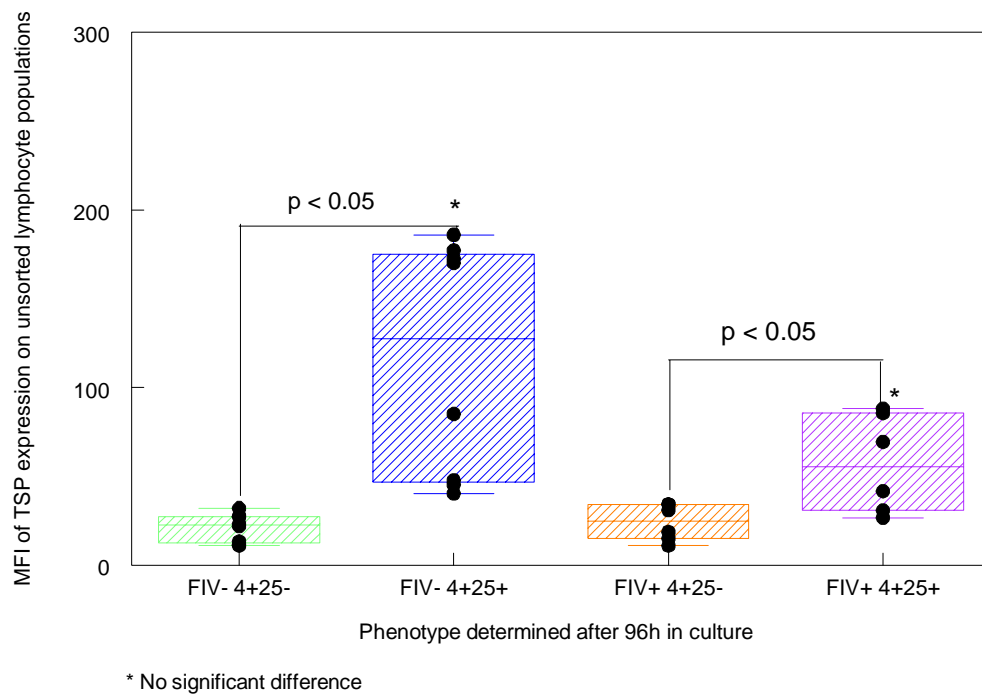
**Figure 13. Flow analysis of sorted CD4+CD25- lymphocytes from peripheral lymph nodes of an FIV+ cat after LPS and IL2 stimulation.** Mean fluorescence intensity of TSP staining and percent TSP positive of a representative FIV+ sample. Freshly isolated lymphocytes from the peripheral lymph nodes of an FIV infected cat were stained with anti-CD4 and anti-CD25 antibodies and sorted into CD4+CD25- and CD4+CD25+ populations. Sorted cells were incubated with 10 $\mu$ g/ml LPS and 100U/ml IL2 added to the cells at day 0 then stained with anti-CD4, anti-CD25 and anti-TSP with fluorescently tagged monoclonal antibodies. **(A)** PE-isotype control used to set axis. **(B)** Representative FIV+ sample. Respective quadrant percentages and mean fluorescence intensities are indicated for both dot plots.



**Figure 14. Mean fluorescence intensity of thrombospondin expression on sorted CD4+CD25- T cells from peripheral lymph nodes of FIV+ cats after activation with LPS and IL2.** Lymphocytes from FIV+ cats were collected from peripheral lymph nodes and stained with antibodies specific for CD4 and CD25. Lymphocytes were sorted into a greater than 98% pure CD4+CD25- population. Between  $5 \times 10^5$  and  $1 \times 10^6$  sorted lymphocytes were incubated for 96h in the presence of  $10\mu\text{g/ml}$  LPS with  $100\text{U/ml}$  added at day 0 (d0) or at day 2 (d2) of culture. After 96h hours, cells were washed and stained with antibodies against CD4, CD25 and TSP for flow analysis. The sorted lymphocytes expressing CD25 after incubation significantly ( $p < 0.05$ ) upregulated TSP expression compared with CD25- cells in both treatment groups. The cells receiving LPS + IL2 at day 0 expressing CD25 after 96h in culture upregulated TSP expression significantly more than the CD25+ cells in the LPS + IL2 d2 treatment group ( $p < 0.05$ ). N = 5 samples were used for LPS + IL2 d0 treatments and n = 4 samples were used for LPS + IL2 d2 treatments.



**Figure 15. Mean fluorescence intensity of thrombospondin expression on unsorted CD4+CD25- and CD4+CD25+ lymph node populations after 96h in culture with LPS and IL2 added at day 0.** There is a significant difference,  $p < 0.05$ , between the CD4+CD25- and CD4+CD25+ populations in both FIV+ and control groups. No significant difference,  $p > 0.05$ , exists between the CD4+CD25- populations in experimental and control groups. The CD4+CD25+ cells isolated from the control cats were able to significantly upregulate the intensity of thrombospondin staining compared with CD4+CD25+ cells isolated from FIV+ models, p-value indicated. Individual samples are shown.



**Figure 16. Mean fluorescence intensity of thrombospondin staining on unsorted CD4+CD25- and CD4+CD25+ lymph node populations after 96h in culture with LPS added at day 0 and IL2 added at day 2.** There is a significant difference,  $p < 0.05$ , between the CD4+CD25- and CD4+CD25+ populations in both FIV+ and control groups. No significant difference,  $p > 0.05$ , exists between the CD4+CD25+ populations between FIV+ and control models. Individual samples are shown

## DISCUSSION

CD4+CD25+ T regulatory cell function and expression of membrane bound TGF- $\beta$  on T regulatory cells in an FIV model has been extensively studied in our laboratory (Vahlenkamp, 2004; Petty, 2006; Emani, 2005). Other reports suggesting the presence of LAP on the surface of CD4+CD25+ Tregs led us to consider how TGF- $\beta$  was capable of mediating Treg suppressor function if it was bound in a biologically inactive state (**Figure 1**). The overwhelming evidence supporting a role for thrombospondin in activating LAP bound TGF- $\beta$ , and the data indicating that T lymphocytes were able to express TSP on their surface led us to consider a possible association between T lymphocyte TSP expression and TGF- $\beta$ -mediated Treg suppressor function.

Consistent with the findings reported by Li et al., (2006) we were able to demonstrate expression of thrombospondin on the surface of CD4+ and CD8+ lymphocytes isolated from peripheral lymph node tissue and peripheral blood of FIV+ and control felines. This group also reported that thrombospondin expression on the surface of T cells underwent rapid turnover on resting T lymphocytes, but activated T cells exhibited a greater stability in TSP expression (Li, 2006). Our results neither confirm nor refute these findings, as we only able analyzed two time points (0 and 96 hours). Further studies investigating the kinetics of TSP surface expression in combination with intracellular staining or RT-PCR analysis of TSP mRNA levels would better explain the manner and degree to which TSP expression can be induced after stimulation of feline T lymphocytes.

An earlier report published from our laboratory demonstrated that treating Tregs isolated from FIV negative control cats with a combination of LPS and IL2 increased their ability to suppress the proliferation of activated CD4+CD25- T<sub>h</sub> cells in a cell-cell contact dependent manner (Vahlenkamp, 2004). More recent studies conducted in our laboratory confirmed these findings, and those of Nakamura, demonstrating that TGF- $\beta$  on the surface of Tregs is responsible for mediating T<sub>h</sub> cell suppression (Nakamura, 2001). Blocking antibodies against TGF- $\beta$  and/or TGF- $\beta$ RII abrogated Treg suppressor function (Nakamura, 2001; Petty, 2006). As published data suggests that TSP mediates the biological activity of mTGF- $\beta$ , we hypothesized that the treatments employed by Vahlenkamp et al., (2004) would increase TSP expression on the surface of either CD4+CD25+ T regulatory cells or CD4+CD25- T helper cells so as to release surface bound TGF- $\beta$  from latency in order to mediate Treg suppress function.

T regulatory cells were originally defined as expressing both CD4 and CD25 (Sakaguchi, 1995; Suri-Payer, 1998). While this population is enriched with Tregs, the phenotype is not exclusive to the Treg population. Activated CD4+ T helper cells can also upregulate CD25 on their surface, thus complicating the distinction between CD4+ Tregs and CD4+ T helper cells. The initial experiments we performed, utilizing bulk unsorted lymphocytes that were stimulated for 96 hours and then analyzed by flow cytometry for CD25 expression, do not adequately distinguish between these populations. The results of these experiments (**Figures 8 and 9**) are not comparing Tregs to T<sub>h</sub> cells, but instead give us further information about TSP expression on activated T helper cells. We found

the results of these experiments particularly interesting when comparing FIV positive and FIV negative control cat samples (**Figures 15 and 16**).

The cell populations discussed in **Figures 15 and 16** were characterized post-stimulation and therefore the CD25+ populations most likely represent a mixture of T regulatory cells and activated T helper cells. From these results we can clearly conclude that a difference exists between the CD4+CD25+ populations isolated from FIV+ and control cats with respect to surface expression of thrombospondin. Lymphocytes isolated from the peripheral lymph nodes of control cats that express CD25 after culture with LPS and IL2 both added at day 0 are able to upregulate surface thrombospondin expression to a significantly higher level than CD25+ cells isolated from FIV+ samples, indicating a difference in the ability of these T cell populations to respond to stimuli.

Several articles detail a state of immune hyperactivation associated with HIV/FIV lentivirus infection (Kochli, 1999; Gebhard, 1999; Tompkins, 2002). We believe this hyperactivation renders the CD4+CD25+ lymphocytes isolated from FIV positive cats unable to upregulate surface expression of TSP to the same levels as CD4+CD25+ T lymphocytes from control cats after stimulation with LPS and IL2 added together on day 0 (**Figure 15**). The CD4+CD25+ cells isolated from FIV+ cats do not express significantly different levels of TSP directly upon isolation compared with FIV negative samples (**Figure 7**). We only observed a divergence in intensity of TSP expression when the lymphocytes were cultured under stimulating conditions (**Figure 15**). **Figure 8b** further supports the idea that T cells isolated from FIV+ cats are already activated by

showing that no significant change occurs in TSP expression between freshly isolated CD4+CD25+ T cells from FIV+ cats and those stimulated with combinations of LPS and IL2 after 96 hours in culture. Taken together, these data support our conclusion that the state of immune hyperactivation in the FIV+ samples caused no appreciable change in T cell TSP expression to occur upon stimulation.

The data on TSP expression on the surface of Tregs collected on freshly isolated lymphocytes gives us the most insight into what the levels of thrombospondin expression are on regulatory T cells (**Figures 5 and 6**). **Figure 5** illustrates that a greater proportion of CD4+CD25- T cells from peripheral lymph nodes express TSP on their surface compared with CD4+CD25+ T cells. This information is not surprising given that the CD4+CD25+ T cell population only constitutes 5-10% of the CD4+ T cell population in normal samples (Powrie, 2003; Vahlenkamp, 2004). The percentages used in this figure are taken out of the total lymphocyte population therefore placing the CD25+ population at a numeric disadvantage. Even when considering the regulatory T cell population alone, very few of these cells express thrombospondin on their surface (**Figure 6**). The data on intensity of TSP expression however, shows the CD4+CD25+ population having much greater levels of surface TSP expression compared to CD4+CD25- T cells in both FIV+ and control cats (**Figure 7**). The CD4+CD25+ populations represented here are primarily T regulatory cells, especially those isolated from FIV negative cats as these cats should not have undergone any immune activation that would cause T helper cells to activate.

To better test our original hypothesis, we sorted the lymphocyte populations based on CD4+CD25+ and CD4+CD25- directly upon isolation and then stimulated these populations separately, thus giving us a better picture of what was occurring in the Treg and T<sub>h</sub> cell subsets after incubation. Due to the limited number of Tregs present in our model, we were only able to accumulate enough cell numbers to perform these experiments by collecting multiple lymph nodes from sacrificed cats, or by pooling multiple lymph nodes collected from different cats. We decided against pooling cells from multiple sources since we observed marked variation in results between cats in our initial experiments. For these reasons we were only able to perform experiments on sorted cell populations collected from sacrificed FIV positive cats.

As expected, sorted CD4+CD25+ from FIV+ models did not significantly upregulate expression of TSP after stimulation with combinations LPS and IL2 or LPS and IL2 alone. These results led us to believe that either the Treg population in FIV positive cats is already activated and therefore not induced to upregulate TSP by these treatments or that this upregulation is ,perhaps, occurring before the 96h time point. Again, we based this stimulation protocol off of a prior publication indicating an increase in Treg activity at 96 hours (Vahlenkamp, 2004). If our original hypothesis was true, that TSP on the surface of Tregs must release latent TGF- $\beta$  from LAP before suppression can occur, this event is probably taking place prior to 96h. Further analysis into the expression on TSP on Tregs at earlier time points is needed to address this hypothesis. Flow cytometry analysis of surface TGF- $\beta$  expression with respect to TSP expression is also of great

interest to better understand a potential connection between thrombospondin and TGF- $\beta$ -mediated T regulatory cell immune suppressor function.

In parallel with our experiments using sorted CD4+CD25+ cells, we performed the same stimulations on sorted CD4+CD25- cells, again isolated from FIV+ models. We observed that CD4+CD25- did upregulate TSP expression with LPS and IL2 stimulation for 96hours (**Figure 12**). Upon further analysis of the flow cytometry data, we observed that some of the sorted CD4+CD25- T cells had upregulated CD25 on their surface and it was this population that expressed the highest levels of surface TSP. In this scenario, the CD25+ cells are most likely activated T helper cells as Chen et al. reported that CD4+CD25- T cells are not converted to CD25+ Tregs unless cultured under stimulating conditions with exogenous TGF- $\beta$  (Chen, 2003). Given our limited number of cells to repeat these experiments, further analysis must be done on sorted cell populations, especially from FIV negative control cats to confirm these findings.

The majority of these experiments were performed in medium containing fetal bovine serum (FBS), as this greatly increased the viability of our cells after 96h in culture. Experiments were also conducted, following the same procedures, using serum-free medium to confirm that the TSP detected in flow cytometry analysis was not from an external source. Indeed, these experiments produced comparable, and often greater, levels of surface TSP staining (**Figure 10**).

Functional studies regarding the potential for TSP function in activating surface bound TGF- $\beta$  present on Tregs are of further interest to our laboratory. Little work has been done regarding the effect TSP has on T cells outside of influencing cell migratory patterns. A recent report suggested a role for TSP in generating a new subset of regulatory T cells named T anergic/suppressor cells (Tas) (Grimbert, 2006). This report focused on exogenous TSP and its interaction with CD47 on the surface of CD4+CD25- T cells from human peripheral blood. They concluded that Tas suppressor function was contact-dependent and TGF- $\beta$ -independent, although more analysis using a blocking antibody against TGF- $\beta$  must be done to fully support this conclusion.

Studies demonstrating the presence of LAP on the surface of functional Tregs lend further credence to our hypothesis that TGF- $\beta$  on the surface of Tregs is in a latent state and must be activated to exert suppressor function (Ochi, 2006; Oida, 2003). We have acquired the LAP antibody utilized in these reports with the intention of repeating these experiments in a feline model. Preliminary data (not shown) indicate LAP expression on feline CD4+ T lymphocytes. The previously discussed reports on LAP described a method of incubating Tregs with the LAP antibody to block TGF- $\beta$  activation prior to coculturing with T helper cells in a suppressor assay. These results demonstrated that Tregs blocked with anti-LAP had decreased suppressor function compared with untreated Tregs. Repeating these studies in the feline model would contribute further to testing the theory that surface bound TGF- $\beta$  is responsible for Treg function and also help to decipher a potential role for TSP in Treg suppressor function. Further studies to test whether TSP contributes to Treg suppressor function could include incubating Tregs or

targets with anti-TSP at various time points during stimulation before plating them in a co-culture suppressor assay to determine if TSP has any effect on Treg function.

Continued research into the mechanism of CD4+CD25+ T regulatory cell immunosuppression is necessary to better understand the role these cells play in a lentivirus infection. Studies attempting to deplete the Treg population *in vivo* have attempted to answer this question but many complications obviously arise from such a model. By better understanding the mechanism of Treg function, we can design better models to explore the effects of this important immune cell subset in FIV and HIV infections.

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