CD4⁺CD25⁺ T Regulatory Cells Inhibit CD8⁺ IFN- γ Production During Acute and Chronic FIV Infection Utilizing a Membrane TGF- β -Dependent Mechanism

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Abstract

CD8⁺ lymphocytes are critical to the control and elimination of viral pathogens. Impaired CD8⁺ responses are well recognized in lentiviral infections; however, the mechanisms underlying CD8⁺ impairment remain elusive. Using the feline immunodeficiency virus (FIV) model for human AIDS, we reported previously that CD4⁺CD25⁺ Treg cells in both the acute and long-term, asymptomatic phase of infection are constitutively activated and suppress CD4⁺CD25⁻ T cell responses. In the current study, we have demonstrated that CD4⁺CD25⁺ Treg cells suppress CD8⁺ responses to immune stimulation during both the acute and chronic, asymptomatic phase of FIV infection and that the mechanism of suppression may be mediated by membrane-associated TGF- β (mTGF- β) on CD4⁺CD25⁺ lymphocytes. Depletion of CD4⁺CD25⁺ lymphocytes from lymph node suspensions significantly enhanced production of IFN- γ during the acute phase of infection and coculture of CD8⁺ lymphocytes with CD4⁺CD25⁺ lymphocytes resulted in suppression of CD8⁺ IFN- γ during both the acute and chronic stages of infection. FACS analysis indicated that there was TGF- β RII upregulation on CD8⁺ cells from FIV⁺ cats during the acute and chronic stage of infection. In addition, there was upregulation of mTGF- β on the CD4⁺CD25⁺ subset in chronically infected cats. In support of activation of the TGF- β signaling pathway, Western blotting showed Smad 2 phosphorylation in CD8⁺ targets following CD4⁺CD25⁺/CD8⁺ coculture. These results demonstrate the suppressive effect CD4⁺CD25⁺ Treg cells have on the CD8⁺ immune response during the acute and chronic stages of FIV infection and suggest that the mechanism of suppression may be mediated by mTGF- β .

Introduction

IN FELINE IMMUNODEFICIENCY VIRUS (FIV) and human immunodeficiency virus (HIV) INFECTIONS, progressive immune dysfunction is evident during the course of infection.^{1,2} Early immune dysfunction is characterized by reduced capacity to respond to FIV/HIV antigens, by poor response to recall antigens, and by the diminished capacity to mount a primary cell-mediated immune response to new infections.^{3,4} Later in the course of disease, immune dysfunction is characterized by a loss of mitogenic responses, a reduction in CD4⁺ T cell counts, and the development of opportunistic infections signaling the onset of AIDS.^{1,2,5}

Although AIDS lentivirus infections are typically associated with an early CD8⁺ lymphocytosis, recent data indicate that the quality of the CD8⁺ response, not the magnitude of

the response, appears to be most critical in controlling virus replication.^{6,7} Experiments utilizing AIDS lentivirus animal models have demonstrated that CD8⁺ depletion leads to increased virus production *in vitro* and to increased viremia and more severe disease *in vivo*.^{8,9} These combined results suggest that the CD8⁺ response plays a major role in the control of AIDS lentivirus replication.⁶⁻⁹ More importantly, these results indicate that even in the face of a robust CD8⁺ response there appear to be both viral and host factors that allow the virus to escape elimination and establish and maintain a chronic infection.

In acute and chronic viral infections, there are immunoregulatory mechanisms in place to control inflammatory responses that might prove detrimental to the host.^{1,10,11} A key component of the virus-host interaction is CD4⁺CD25⁺ T regulatory cells (Tregs) that temper the inflammatory

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response, but may also promote development of chronic infections. In a variety of different viral infections such as herpes simplex virus (HSV), hepatitis C virus (HCV), and Friend virus, CD4⁺CD25⁺ Tregs appear to play a pivotal role in immunopathogenesis.^{12–15} Suvas *et al.*¹² demonstrated that CD4⁺CD25⁺ depletion, prior to HSV infection, enhanced CD8⁺ responses to immunodominant peptides 3- to 4-fold. Boettler *et al.*¹⁵ demonstrated that Tregs inhibited CD8⁺ interferon (IFN)- γ production and proliferation in response to HCV antigen. Furthermore, they reported that chronic HCV infection led to *in vivo* CD4⁺CD25⁺ expansion and impaired CD8⁺ responses to other viral antigens such as influenza. The common theme of these models is that depletion of CD4⁺CD25⁺ Tregs leads to enhanced CD4⁺ and CD8⁺ effector responses, both against viral antigens and against unrelated recall antigens, and this heightened effector activity may lead to virus clearance. Conversely, depletion of CD4⁺CD25⁺ Tregs may also result in increased immunopathology associated with unchecked inflammation. For example, in a murine HSV keratitis model, Suvas and colleagues reported that CD4⁺CD25⁺ depletion prior to infection led to more severe corneal lesions associated with a vigorous T effector response.¹¹

The CD8⁺ immune response plays an important role in both the acute and chronic phases of lentiviral infection and the ability to escape elimination during the acute CD8⁺ response is one of the keys to establishing a chronic infection.^{16,17} Understandably, much attention has been focused on the mechanisms lentiviruses use to escape the initial CD8⁺ response and persistently evade elimination during the chronic phase of infection. Based on evidence from other models of virus infection, it is likely that Tregs play an important role in both the acute and chronic stages of HIV infection. Kinter *et al.*¹⁸ reported that CD4⁺CD25⁺ T cells in the majority of healthy, chronically infected HIV⁺ patients significantly suppressed proliferation and cytokine production by CD4⁺ and CD8⁺ T cells stimulated with HIV peptides *in vitro*. Interestingly, the level of Treg suppressor function correlated inversely with plasma viremia. More recently, Kinter and others¹⁹ demonstrated that Tregs from HIV-infected individuals suppressed CD8⁺ antigen-specific HIV gag responses, expression of IFN- γ and tumor necrosis factor- α (TNF- α), and antiviral chemokine secretion.

Using the FIV model we have previously shown that FIV infection phenotypically and functionally activates CD4⁺CD25⁺ Tregs during both the acute and chronic stages of infection and these cells are capable of inhibiting CD4⁺ effector responses.^{20,21} Activated feline Tregs from FIV⁺ cats upregulate CTLA4, B7.1 (CD80), and B7.2 (CD86), and suppress CD4⁺ proliferation and interleukin (IL)-2 production.^{20,21} Furthermore, we have demonstrated preferential *in vitro* and *in vivo* replication of FIV in the CD4⁺CD25⁺ subset of CD4⁺ T cells. We believe that due to FIV infection of CD4⁺CD25⁺ Tregs early during the course of infection as well as during the chronic stage of infection, Tregs become activated and capable of suppressing CD4⁺CD25⁺ T helper responses.^{21,22}

The mechanism(s) of CD4⁺CD25⁺ Treg-mediated suppression remains elusive. Different mechanisms of contact-mediated suppression have been proposed such as granzyme-dependent, CTLA-4-dependent, and tumor growth factor (TGF)- β -dependent suppression.^{23,24} Grossman

and others²⁵ reported that after CD3/CD46 stimulation, CD4⁺CD25⁺FOXP3⁺ lymphocytes isolated from human blood showed increased granularity caused by an accumulation of cytoplasmic granzyme A. In coculture experiments these stimulated Tregs were able to kill autologous CD4⁺, CD8⁺, and CD14⁺ target cells in a granzyme A-perforin-dependent fashion. However, granzyme-mediated killing of activated CD4⁺ and CD8⁺ targets is probably more relevant to autoimmune and transplantation models, because it appears that in many infectious disease models, effector cells are anergized and not destroyed following Treg interactions.^{24,25}

Tregs constitutively express CTLA-4 and some studies have suggested that CD80/CD86 ligation by CTLA-4 accounts for at least partial T effector suppression.^{23,26,27} However, Katoaka *et al.*²⁸ demonstrated that CTLA-4 was not required for *in vitro* Treg-mediated suppression. Therefore, it may be that the CTLA-4/CD80/86 pathway represents an alternative suppressive pathway in models of chronic viral infection such as FIV.^{29,30}

Several laboratories, including ours, have demonstrated surface bound TGF- β (mTGF- β) on activated Tregs.^{31–33} However, the role of TGF- β in Treg-mediated suppression is controversial. Nakamura *et al.*³¹ demonstrated that activated Tregs expressed mTGF- β and that their contact-dependent suppressor function was abrogated by treatment with anti-TGF- β neutralizing antibody. Others have demonstrated that suppressor function of both mouse and human CD4⁺CD25⁺ thymocytes is at least partially inhibited by neutralization of TGF- β .^{32,34} In contrast, Piccirillo *et al.*³⁵ reported that anti-TGF- β had no effect on *in vitro* Treg cell-mediated suppression. Additionally, studies have shown that Tregs from TGF- β -deficient mice were capable of suppression as well as those from normal mice.^{35,36}

We previously reported that activated CD4⁺CD25⁺ Tregs express mTGF- β and that these cells mediate suppressor function by engagement of TGF- β RII on the surface of activated CD4⁺ target cells.³⁷ Importantly, the TGF- β pathway is also essential to controlling CD8⁺ lymphocyte responses. Mice expressing a dominant negative TGF- β RII (dnTGF- β RII) specific to their T cells develop a profound CD8⁺ hyperproliferation resulting in massive expansion of their peripheral lymphoid tissue by 2–9 months of age.³⁸ Interestingly, CD8⁺ T cell development in the thymus was normal, suggesting that the homeostatic control occurs in the periphery. In addition, introduction of a dnTGF- β RII into antigen-specific CD8⁺ cells rendered them resistant to Treg cell-mediated suppression.³⁹ Further findings in this model demonstrated that TGF- β inhibits CD8⁺ antitumor effector function, although suppressed CD8⁺ lymphocytes maintain an activated phenotype.⁴⁰ Similarly, in an autoimmune diabetes model, delayed progression of diabetes was associated with an increased percentage of mTGF- β -positive, CD4⁺CD25⁺ lymphocytes within the peripheral nodes and pancreas.⁴¹

It is evident from this discussion that the role of the TGF- β /TGF- β RII signaling pathway in Treg cell-mediated suppression likely depends on the model system used. Using the FIV model, we have demonstrated transient upregulation of TGF- β on the surface of CD4⁺CD25⁺ cells during acute FIV infection and we have shown that blocking TGF- β signaling blocks the Treg-mediated conversion of CD4⁺CD25⁺ target

cells to suppressor cells.^{21,37} The experiments that follow support the hypothesis that Treg-mediated CD8⁺ suppression likely occurs via the interaction between Tregs displaying mTGF- β and TGF- β RII on target CD8⁺ cell surfaces.

Materials and Methods

Cats

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

Infection with FIV

The NCSU₁ isolate of FIV was originally obtained from a naturally infected cat at the North Carolina State University College of Veterinary Medicine and has been described in detail elsewhere.⁴² Virus inoculum was grown as a single tissue culture passage in an IL-2-dependent feline CD4⁺ cell line (FCD4-E cells) as previously described.⁴³ The cats were inoculated intravenously with 1×10^5 TCID₅₀ of cell-free virus culture and control cats were sham inoculated with equal volumes of sterile FCD4-E cell culture medium. Most of the cats used for the FIV acute phase infection studies were then utilized for the FIV chronic infection studies, and at the time of chronic phase experiments had been infected with FIV for approximately 1 year.

Sample collection

Blood was collected via jugular venipuncture into EDTA vacutainer tubes from FIV-infected and control cats at the indicated time points postinoculation. Two milliliters was retained for a complete blood cell count and lymphocyte subset analysis by multicolored flow cytometry. Plasma was separated from the remaining blood and frozen for analysis of viral load by reverse transcriptase polymerase chain reaction (RT-PCR). During acute phase studies cats were grouped such that popliteal lymph nodes (LNs) were biopsied at days 14, 21, 28, 35, 42, 56, and 84 from two to four FIV⁺ cats and four sham-inoculated cats were used as uninfected controls. LN biopsies were performed as previously described.^{21,44} Briefly, cats were anesthetized with intravenous ketamine and valium and anesthesia was maintained with isoflurane gas. Popliteal lymph nodes were excised through a small incision in the caudal aspect of the stifle and the incision was sutured with monofilament sutures. LNs were processed into a single cell suspension as previously described and used for phenotypic analysis by flow cytometry and for purification of lymphocyte subsets (CD8⁺, CD4⁺CD25⁺ cells). Two LNs were collected from each cat during the course of the acute phase study. For experiments during the chronic stage of infection, lymphocytes were harvested either by LN excision or following euthanasia.

Antibodies

Murine monoclonal antifeline CD4 [monoclonal antibody (mAb) 30A], CD8 (mAb 3.357), and CD25 (mAb 9F23) were

produced in our laboratory.⁴⁵ The antifeline CD25 (mAb 9F23) was originally provided by K. Ohno (University of Tokyo). The antibodies were conjugated to fluorescein isothiocyanate (FITC) (anti-CD8, anti-CD25), phycoerythrin (PE) (anti-CD4, anti-CD8), or biotin (anti-CD8) (developed with Streptavidin/PerCP). Antihuman TGF- β (mAb 240, R&D Systems), previously shown to bind feline TGF- β , was conjugated to antigen-presenting cells (APC).³⁷ Goat anti-TGF- β receptor II (AF 241 NA, R&D systems) was conjugated to PE. Mouse anti-phospho-SMAD2 (138D4) was purchased from Cell Signaling Technologies. The isotype control antibody for the TGF- β -APC was irrelevant mouse IgG1 purchased from Southern Biotec. The isotype control for the TGF- β receptor II-PE was irrelevant polyclonal goat antibody purchased from R&D systems and conjugated to PE.

Lymphocyte subset analysis

The phenotype of lymphocytes from blood and LNs was determined by multicolored flow cytometric analysis. Peripheral blood mononuclear cells (PBMCs) were prepared using an established whole blood lysis protocol.³ Membrane TGF- β expression was determined using APC-conjugated antihuman TGF- β in combination with anti-CD4-PE and anti-CD25-FITC. TGF- β RII expression was determined using PE-conjugated anti-TGF- β RII in combination with anti-CD8-FITC. For flow cytometric analysis, lymphocytes were gated based on forward vs. side scatter, and approximately 20,000 gated events were acquired and stored list-mode fashion for analysis using CellQuest software. Absolute numbers of lymphocytes were calculated using the percentage from FACS analysis, multiplied by the total lymphocyte count from either peripheral blood or LNs.

T regulatory cell depletion assay

LN cells were stained with anti-CD4-PE and anti-CD25-FITC and CD4⁺CD25⁺ dual positive cells were depleted by flow cytometric cell sorting (MoFlo, Beckman-Coulter). The resulting CD4⁺CD25⁺-depleted cells were cultured in the absence or presence of concanavalin A (Con A) (5 μ g/ml) for 18 h then analyzed for production of IFN- γ by ELISpot. Unfractionated (nondepleted) LN cells were also passed through the cell sorter to ensure similar processing conditions then cultured in the presence or absence of Con A and analyzed for IFN- γ production. The effect of depleting CD4⁺CD25⁺ cells on LN IFN- γ production was determined by dividing the number of spot-forming cells (SFCs) from depleted cultures by the number of SFCs from unfractionated cultures.

T regulatory cell/CD8⁺ suppression assay

LN cells were stained with anti-CD4-PE, anti-CD25-FITC, and anti-CD8-biotin, developed with Streptavidin-PerCP and sorted into CD4⁺CD25⁺ and CD8⁺ populations. The sorted populations were greater than 95% pure. The CD8⁺ cells were incubated for 18 h in the presence or absence of Con A (5 μ g/ml). Following incubation, 3×10^5 CD8⁺ cells per well were added to an IFN- γ ELISpot plate, and CD4⁺CD25⁺ cells added at a ratio of 0.5:1 (CD4⁺CD25⁺ to CD8⁺). The plates were analyzed for the presence of IFN- γ -producing cells as described below.

IFN- γ ELISpot

Following incubation without or with Con A, cells were plated into precoated (monoclonal antifeline IFN- γ , R&D systems) 96-well ELISpot plates at a concentration of 3×10^5 cells/well. The plates were incubated for 24 h, stained with detection antibody, and developed per the manufacturer's instructions. Once dry, each well was counted with an automated ELISpot reader for quantification of SFCs per number of cells plated in each well. Controls included recombinant feline IFN- γ (positive control), media only wells (to assess background staining), and wells treated without detection antibody (for nonspecific staining).

Plasma and cell-associated viremia

Evidence of infection was assessed on each plasma sample using a commercially available snap test ELISA (IDEXX laboratories) to detect antibodies against FIV. In addition, quantitative real-time PCR was used to determine viral gag-mRNA loads in each plasma sample. Briefly, 1 ml of plasma was used to extract viral RNA using the Qiagen QIAamp Ultrasense Virus Isolation kits. Then 10 μ l of the isolated viral RNA was reverse transcribed in a separate reaction using the Promega Reverse Transcription System with random primers. This reaction was followed by a real-time PCR step using specific primers for FIV-gag mRNA, universal Taqman PCR Mastermix (Applied Biosystems), and the FIV-specific probe in the relative concentrations specified by the manufacturer. Primers sequences were as follows: (491f-GATTAGGAGGT GAGGAAGTTCAGCT), (617r-CTTTCATCCAATATTCT TTATCTGCA), and (probe FIVNC555P-FAM/CATGGCCA CATTAAATAATGGCCGCA/36-TAMSp).

The reactions were run in duplicate in 96-well plates and incubated at 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min, before returning to 25°C. A standard curve was run in each reaction using serial dilutions of previously sequenced and quantified FIV-gag-mRNA. The standard curve was used to determine absolute viral mRNA copy numbers per milliliter of plasma. Sorted populations of $0.5\text{--}3 \times 10^6$ CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells from blood and LNs were maintained in RNA later and evaluated for relative quantities of FIV-gag mRNA using the $\Delta\Delta C_t$ method.⁴⁶ Equal amounts of RNA were used in each test. The calibrator sample consisted of a mixture of RNA samples obtained from all groups of cells. Cells from two to eight cats were evaluated individually at each time point.²¹

Reverse transcription real-time PCR for IFN- γ mRNA

CD8⁺ lymphocytes ($2\text{--}4 \times 10^6$) from FIV⁻ and FIV⁺ were untreated, Con A stimulated, or stimulated with Con A followed by either TGF- β (10 ng/ml) for 120 min or coculture with CD4⁺CD25⁺ cells for 120 min (CD4⁺CD25⁺ to CD8⁺ ratio = 1:1). RNA from cell cultures was isolated using the Qiagen RNeasy plus Mini Kit and reverse transcription was performed using the Promega Reverse Transcription System, following the manufacturer's instructions for both. This reaction was followed by a real-time PCR step using the universal Taqman PCR Mastermix (Applied Biosystems) and the Qiagen Quantitect Sybr Green PCR Kit (probe) using feline-specific IFN- γ primers. Primer sequences were as follows:

(141f-TGGTGGGTCGCTTTTCGTAG) and (225r-GAAGGA GACAATTTGGCTTTGAA). The reactions were run in duplicate in 96-well plates. The fold induction was calculated by using the $\Delta\Delta C_t$ value, where fold induction = $2^{-(\Delta\Delta C_t)}$, as described by Winer *et al.*⁴⁶ PBMCs from an FIV-negative cat and GAPDH as the internal control were used as the calibration sample value in the $\Delta\Delta C_t$ equation.

Smad 2 Western blots

FACS purified CD8⁺ and CD4⁺CD25⁺ lymphocytes were serum starved for 18 h. Then 4×10^6 CD8⁺ cells were incubated with rhTGF- β (10 ng/ml, R&D Systems) or cocultured with

FIG. 1. Peripheral blood and LN CD8⁺ lymphocyte counts during the acute phase of FIV infection. Peripheral blood (a) and lymph node (b) lymphocytes were collected at the time points post-FIV infection (shaded triangles) or sham infection (open circles) and stained with anti-CD8 and analyzed by flow cytometry. The CD8⁺ nadir for FIV⁺ cats occurs at day 14 in both the peripheral blood and LNs. A manual lymphocyte count multiplied by the percentage of CD8⁺ cells obtained via FACS analysis yielded the absolute count for the CD8⁺ population. Each time point represents the mean cell count (FIV⁺ cats $n = 4$, FIV⁻ cats $n = 2$).

CD4⁺CD25⁺ cells at a 1:1 ratio for 2 h. The CD8⁺ cells were then lysed with NP-40 and separated by SDS-PAGE. For the cocultured groups, CD4⁺CD25⁺ lymphocytes were depleted from CD8⁺ cultures using anti-PE microbeads and MACS separation columns (>90% CD8⁺ purity), both purchased from Miltenyi Biotec. The blots were analyzed using rabbit monoclonal anti-P-SMAD 2 (138D4, Cell Signaling Technology), followed by HRP-conjugated goat antirabbit IgG and detected by chemiluminescence. The blots were then stripped and reprobed with antiactin and HRP-conjugated goat antimouse antibody. For each treatment group, actin and P-SMAD 2 were evaluated by photodensitometry and normalized, with unstimulated CD8⁺ controls assigned a value of 1.

Statistical analysis

Statistical analysis was performed using a Student's *t* test to compare FIV⁻ and FIV⁺ groups and/or treatment groups as indicated in each of the figures. *p*-values less than 0.1 (Fig. 3) or 0.05 (Figs. 4–7 and 9) were considered significant.

Results

CD8⁺ lymphocyte dynamics and plasma viremia during acute FIV infection

The 28 SPF cats inoculated with FIV-NCSU₁ followed an infection course similar to that described previously for this isolate.^{21,43} All cats were antibody positive for FIV gag proteins (ELISA) by 5 weeks postinfection (pi). Blood and lymph node CD4⁺ cells as well as plasma were positive for FIV RNA by RT-PCR as early as 2 weeks postinfection (data not shown). Typical of FIV infection, there was an early T cell lymphopenia at day 14 pi in both the blood and lymph node followed by a gradual rebound in CD8⁺ cells (Fig. 1). At approximately week 5, all FIV-infected cats exhibited peripheral lymphadenopathy as has been previously described for acute FIV infection.^{5,47} Plasma viremia peaked at day 14 pi coinciding with the CD8⁺ nadir and then decreased as the CD8⁺ cell count increased (Fig. 2). FIV replication in the CD4⁺CD25⁺ subset paralleled the plasma viremia with FIV gag mRNA levels peaking at 14 days pi (Fig. 2).

FIG. 2. Lymph node CD8⁺ cell count with plasma viremia and FIV gag mRNA from CD4⁺CD25⁺ lymphocytes superimposed. Quantification of plasma viremia by real-time PCR for FIV-gag mRNA is depicted by the dashed line (circle), with the scale in copies per milliliter of plasma on the right-hand side (*n* = 8). Relative quantities of FIV gag mRNA from purified CD4⁺CD25⁺ lymphocytes were determined by the $\Delta\Delta C_t$ method. The fold change in FIV gag mRNA (square, relative scale not shown, *n* = 8) in CD4⁺CD25⁺ lymphocytes coincides with both the peak in plasma viremia and the CD8⁺ nadir. As a point of reference, the CD8⁺ lymphocyte count (triangle) as shown in Fig. 1 is duplicated here.

FIG. 3. IFN- γ production from FIV⁺ cats following CD4⁺CD25⁺ Treg depletion is enhanced at all time points after 21 days postinfection. Popliteal lymph nodes were surgically excised at the indicated time point and the lymphocytes were sorted into two populations: unfractionated LN cells and CD4⁺CD25⁺-depleted LN cells. Cells were stimulated for 18h with Con A then assayed for IFN- γ by ELISpot. The control group (C) was composed of sham-inoculated cats and the test group was represented by FIV⁺ cats sampled at the indicated time point pi. (a) The bars represent the absolute number of IFN- γ SFCs (mean $\pm$ SEM) as measured by ELISpot from unfractionated LNs (gray bars) and CD4⁺CD25⁺-depleted LNs (dark bars). (b) To compare control cats to FIV⁺ cats and to account for individual variation, the fold change following CD4⁺CD25⁺ depletion was calculated using the data shown in (a). The bars represent the mean $\pm$ SEM of fold increase in IFN- γ SFCs as measured by ELISpot, calculated by the following: SFCs from CD4⁺CD25⁺ depleted LN culture $\div$ SFCs from unfractionated LN culture. The fold change following CD4⁺CD25⁺ depletion from each FIV⁺ group (gray) was compared to the control group (diagonal line, $p < 0.1$, asterisks). Control cats $n = 4$, FIV⁺ cats $n = 2-5$ for each time point. (Note: all numbers greater than 1 reflect an increase in IFN- γ SFCs following CD4⁺CD25⁺ depletion.)

CD4⁺CD25⁺ lymphocytes inhibit CD8⁺ IFN- γ production during acute FIV infection

As shown in Fig. 2, acute FIV infection is associated with a rebound in CD8⁺ cell numbers, following the CD8⁺ nadir. However, despite this rapid rebound, there is persistent, low-

level plasma viremia. We recently reported that CD4⁺CD25⁺ Tregs are activated and suppress CD4⁺ T helper cells as early as 3 days post-FIV infection; we then asked whether Tregs also suppressed the CD8⁺ T cell response during acute FIV infection.²¹ CD8⁺ IFN- γ production as measured by ELISpot has been correlated with CD8⁺ CTL function and we used this method as an indicator of CD8⁺ antiviral potential.⁴⁸ Lymphocytes from popliteal LNs were sorted into unfractionated LN cells and CD4⁺CD25⁺-depleted LN cells and were assayed for IFN- γ SFCs. Figure 3a depicts the absolute number of SFCs (mean $\pm$ SEM) for unfractionated and CD4⁺CD25⁺-depleted LNs. To account for the amount of individual variation between cats, the number of SFCs from CD4⁺CD25⁺-depleted LNs was divided by the number of SFCs from unfractionated LNs to reflect the fold increase following CD4⁺CD25⁺ depletion. As shown in Fig. 3b, the number of IFN- γ SFCs following CD4⁺CD25⁺ depletion was enhanced at all time points after 21 post-FIV infection. Once the cats transitioned toward the chronic phase of infection (days 56 and 84 pi), a sustained 2-fold increase in IFN- γ SFCs was evident following CD4⁺CD25⁺ depletion. The results indicate that CD4⁺CD25⁺ depletion from LN suspensions during acute FIV infection enhanced IFN- γ production after 21 days pi.

To characterize the effect of CD4⁺CD25⁺ Tregs specifically on CD8⁺ IFN- γ production, we performed a direct Treg suppression assay using a subset of the FIV⁺ cats depicted in Fig. 3. Lymphocytes were sorted into CD8⁺ and CD4⁺CD25⁺

FIG. 4. CD4⁺CD25⁺-mediated suppression of CD8⁺ IFN- γ production in acute FIV infection occurs after 21 days pi. FACS-purified CD8⁺ cells from FIV⁺ cats were stimulated with Con A for 18h then cocultured with autologous CD4⁺CD25⁺ cells at a ratio of 0.5:1 (CD4⁺CD25⁺ to CD8⁺) and IFN- γ production was determined by ELISpot. The gray shaded bars represent CD8⁺ cells alone and the black bars represent CD8⁺/CD4⁺CD25⁺ cocultures with each pair of bars representing an individual cat. There is significant CD4⁺CD25⁺-mediated CD8⁺ suppression in six of seven cats at days 28–56 pi ($p < 0.05$). CD8⁺ suppression was not evident during the first 21 days of infection (not shown).

populations as described in the Materials and Methods section. After sorting, CD4⁺CD25⁺ lymphocytes were cocultured with CD8⁺ lymphocytes at a ratio of 0.5:1 (CD4⁺CD25⁺ to CD8⁺ targets) and assayed for IFN- γ SFCs. Consistent with the observations made in Fig. 3, during the first 21 days of infection, there was no evidence of CD4⁺CD25⁺-mediated suppression of CD8⁺ IFN- γ production (data not shown). However, as shown in Fig. 4, from days 28 to 56 pi, there is significant CD4⁺CD25⁺-mediated suppression of CD8⁺ IFN- γ SFCs, as measured by ELISpot.

CD8⁺ lymphocytes exhibit increased TGF- β RII expression during acute FIV infection

We reported previously that mTGF- β is transiently upregulated on CD4⁺CD25⁺ Treg cells from acutely FIV-infected cats, therefore lymphocytes targeted for T regulatory suppression should express TGF- β RII.²¹ As described in the introduction, TGF- β is important in controlling CD8⁺ responses. To assess expression of TGF- β RII on CD8⁺ cells during the acute stage of infection, peripheral blood CD8⁺ lymphocytes were examined by FACS for the percentage of TGF- β RII-positive cells. A representative dot plot from an FIV⁻ control and an FIV-infected cat at day 14 pi is shown in Fig. 5a. As depicted in Fig. 5b, the mean percentage of TGF- β RII⁺CD8⁺ lymphocytes was approximately three times greater in FIV⁺ cats (5%) at day 14 pi, when compared to age-matched FIV⁻ control cats (<2%). The mean percentage of TGF- β RII⁺CD8⁺ lymphocytes in FIV⁺ cats was also increased at days 42 and 98 pi (data not shown).

CD8⁺ lymphocytes upregulate TGF- β RII expression during the course of FIV infection

As demonstrated above, during acute FIV infection, TGF- β RII expression is increased in peripheral blood CD8⁺ cells. We then examined both peripheral blood and LN CD8⁺ lymphocytes from FIV chronically infected cats at 1 year pi. A representative dot plot from an FIV⁻ control and FIV-infected cat at 1 year pi is shown in Fig. 6a. Figure 6b shows that in chronically infected cats the mean percentage of TGF- β RII⁺CD8⁺ lymphocytes in both PBMCs and LNs was more than five times greater in FIV⁺ cats when compared to age-matched FIV⁻ control cats. More importantly, the mean expression of TGF- β RII on CD8⁺ lymphocytes was five times greater in FIV⁺ cats 1 year pi (22%) when compared to 14 days pi (5%). The results from Figs. 5 and 6 suggest TGF- β RII is progressively upregulated on CD8⁺ lymphocytes during the course of FIV infection.

CD4⁺CD25⁺ lymphocytes exhibit increased expression of mTGF- β in chronically infected FIV⁺ cats

We have shown that mTGF- β is transiently upregulated on CD4⁺CD25⁺ Tregs from acutely FIV-infected cats and recently we have demonstrated that CD4⁺CD25⁺ Tregs convert CD4⁺CD25⁻ T helper cells to T regulatory-like cells via a TGF- β -dependent mechanism.^{21,37} Data suggest this may also be the mechanism of T regulatory-mediated suppression.^{31,37,49} A representative dot plot from an FIV⁻ control and FIV-infected cat at 1 year pi is shown in Fig. 7a. As shown in Fig. 7b, during the chronic stage of infection, there was a 2-fold in-

crease in the percentage of lymph node mTGF- β ⁺CD4⁺CD25⁺ lymphocytes from FIV⁺ cats (56%) compared to FIV⁻ cats (27%). The mean percentage of mTGF- β ⁺CD4⁺CD25⁺ cells from peripheral blood of FIV⁺ cats was somewhat higher than FIV⁻ cats, but did not reach significance ($p = 0.24$).

CD4⁺CD25⁺ lymphocytes from chronically infected FIV⁺ cats activate the TGF- β signaling pathway and inhibit IFN- γ production in CD8⁺ target cells

The data presented above suggest that mTGF- β may be a mechanism of T regulatory-mediated CD8⁺ suppression during FIV lentiviral infection. We asked if treatment of CD8⁺ cells with soluble TGF- β or coculture with CD4⁺CD25⁺ Tregs induced intracellular Smad 2 phosphorylation, a marker specific to the TGF- β signaling pathway.⁵⁰ Synchronization of Smad 2 phosphorylation for visualization by Western blotting requires coordinated effector cell to target cell interaction. Therefore, we selected a CD4⁺CD25⁺ effector to CD8⁺ target ratio of 1:1. Figure 8 shows that after a 2-h coculture with CD4⁺CD25⁺ lymphocytes, CD8⁺ lymphocytes exhibit Smad 2 phosphorylation exceeding that of TGF- β -treated CD8⁺ cells.

To further characterize the role of TGF- β in Treg cell-mediated suppression of CD8⁺ cells, lymphocytes from chronically infected FIV⁺ cats and FIV⁻ control cats were sorted into CD8⁺ and CD4⁺CD25⁺ populations. Following treatment of CD8⁺ cells with TGF- β or following CD4⁺CD25⁺ coculture, IFN- γ mRNA from CD8⁺ lymphocytes was assessed by real-time RT-PCR. Figure 9 shows a modest reduction in CD8⁺ IFN- γ mRNA following treatment with soluble TGF- β for 120 min. More importantly, in FIV⁺ cats there was marked suppression in CD8⁺ IFN- γ mRNA following a 120-min CD8⁺/CD4⁺CD25⁺ coculture. These results indicate that CD4⁺CD25⁺ Tregs from chronically infected FIV cats exhibit potent CD8⁺ immunosuppressive capability. The induction of Smad 2 phosphorylation combined with IFN- γ mRNA suppression in CD8⁺ target cells suggests that CD4⁺CD25⁺ Tregs utilize the TGF- β /TGF- β RII signaling pathway to inhibit CD8⁺ lymphocyte responses.

Discussion

During acute lentivirus infection, a CD8⁺ T cell lymphocytosis is noted several weeks pi.^{8,9,51-54} Investigations of acute HIV infection have shown that the initial control of plasma viremia parallels HIV-specific CTL activity.^{54,55} Using the FIV lentivirus model, Bucci *et al.*⁹ reported that cats with poor CD8⁺ anti-FIV activity did not have a subsequent reduction in cell-associated viremia. Conversely, cats with robust CD8⁺ anti-FIV activity demonstrated a progressive reduction in cell-associated virus, with several cats appearing to eliminate the virus entirely. In the experiments shown here, we have not observed any cats that appeared to clear their acute FIV infection. In a second study, Bucci *et al.*⁵³ reported that CD8⁺ lymphocytes from FIV⁺ cats displayed an activated phenotype and that *in vitro* depletion of CD8⁺ lymphocytes led to increased FIV replication in PBMC cultures from FIV chronically infected cats. Similarly, Schmitz and others⁵⁶ showed that *in vivo* depletion of CD8⁺ lymphocytes during chronic SIV infection led to increased viremia,

aTGF β RII Surface Expression on CD8⁺ cells

FIG. 6. CD8⁺ lymphocytes exhibit increased expression of TGF- β RII in FIV⁺ cats when compared to FIV⁻ control cats during chronic FIV infection. Peripheral blood and lymph node cells were stained with anti-CD8 and anti-TGF- β RII and then analyzed by FACS. (a) Dot plots (upper row) demonstrating the percentage of TGF- β RII⁺ cells within the CD8⁺ population from the blood of an uninfected control cat and an FIV⁺ cat 1 year pi (4% and 22%, respectively). The lower row of dot plots represents irrelevant isotype control antibodies for anti-TGF- β RII surface staining. (b) The graph depicts the mean percent TGF- β RII⁺ cells within the CD8⁺ population (horizontal line) and dots represent individual cats. The mean percent TGF- β RII⁺ was higher in FIV⁺ cats in both the blood FIV⁺ (22%, $n = 15$), FIV⁻ (5%, $n = 7$) and LN FIV⁺ (15%, $n = 15$), FIV⁻ (2%, $n = 6$).

which resolved with the reappearance of virus-specific CD8⁺ lymphocytes.

More recent investigations have examined the interaction between CD4⁺CD25⁺ Tregs and CD8⁺ targets in chronic HIV infection. Kinter *et al.*¹⁸ reported that CD4⁺CD25⁺ T cells in

the majority of healthy, chronically infected HIV⁺ patients significantly suppressed proliferation and cytokine production by CD4⁺ and CD8⁺ T cells stimulated with HIV peptides *in vitro*. More recently, Kinter and others¹⁹ demonstrated that Tregs from HIV-infected individuals suppressed CD8⁺

FIG. 5. CD8⁺ lymphocytes exhibit increased expression of TGF- β RII in FIV⁺ cats when compared to FIV⁻ control cats during acute FIV infection. Peripheral blood was stained with anti-CD8 and anti-TGF- β RII then analyzed by FACS. (a) Dot plots (upper row) demonstrating the percentage of TGF- β RII⁺ cells within the CD8⁺ population from the blood of an uninfected control cat and an FIV⁺ cat 14 days pi (1% and 5%, respectively). The lower row of dot plots represents irrelevant isotype control antibodies for anti-TGF- β RII surface staining. (b) The graph depicts the mean percent TGF- β RII⁺ cells within the CD8⁺ population (horizontal line) and dots represent individual cats. The mean percent TGF- β RII⁺ from the blood of FIV⁺ (5%, $n = 6$) cats 14 days pi is higher than that of FIV⁻ (1%, $n = 5$) controls.

FIG. 7. LN CD4⁺CD25⁺ lymphocytes exhibit increased expression of mTGF- β during chronic FIV infection. Peripheral blood and lymph node cells were stained with anti-CD4, anti-CD25, and anti-mTGF- β and analyzed by FACS. CD4⁺CD25⁺ cells were gated and then analyzed for percent mTGF- β ⁺ cells. (a) Dot plots (upper row) demonstrating the percentage of mTGF- β -positive cells within the CD4⁺CD25⁺ population from the LNs of an uninfected and chronically infected FIV⁺ cat. The upper right quadrant represents the percent mTGF- β ⁺ cells (24% and 46%, respectively). The lower row of dot plots represents irrelevant isotype control antibodies for anti-mTGF- β surface staining. (b) The graph depicts the mean percent mTGF- β ⁺ of the CD4⁺CD25⁺ population (horizontal line) and dots represent individual cats. There was no difference between the FIV⁻ (6%, $n = 7$) and FIV⁺ (8%, $n = 15$) blood. However, the mean mTGF- β ⁺ was considerably higher in the FIV⁺ LNs (50%, $n = 15$) when compared to the FIV⁻ LNs (27%, $n = 6$).

We recently reported that acute FIV infection leads to increased intranuclear FOXP3 expression in CD4⁺CD25⁺ lymphocytes as early as day 21 pi.²¹ A short lag time between the increase in Treg cell FOXP3 expression and CD8⁺ suppression would be expected. These data suggest that the overall CD8⁺ response during acute FIV is diminished by CD4⁺CD25⁺ Tregs, and that the magnitude of CD8⁺ inhibition follows the course of FOXP3 upregulation in the CD4⁺CD25⁺ subset.

The exact mechanism of Treg-mediated suppression is not completely understood and it is likely that Tregs are capable of suppression by different means and that the mechanisms of suppression vary between the experimental models used. In 2001, Nakamura and colleagues³¹ reported that CD4⁺CD25⁺-mediated suppression of CD4⁺CD25⁻ proliferation was inhibited by the presence of anti-TGF- β antibody. More importantly, they showed that CD4⁺CD25⁺ regulatory T cells stimulated with anti-CD3 and IL-2 expressed high levels of

FIG. 7. (Continued)

membrane TGF- β , as assessed by FACS and western blot. In a follow-up study, Nakamura *et al.*⁴⁹ reported that treatment of CD4⁺CD25⁺ T cells with recombinant latency-associated peptide of TGF- β (rLAP) also inhibited CD4⁺CD25⁺-mediated suppression. They also described experiments in which CD4⁺CD25⁺ cells from TGF- β -deficient mice were capable of suppressing CD4⁺CD25⁻ proliferation *in vitro*, but did not protect recipient mice from the development of colitis in an *in vivo* SCID transfer model.

In concert with the findings from Nakamura *et al.*, we have reported that CD4⁺CD25⁺ Tregs from FIV-infected cats exhibit increased expression of mTGF- β .^{21,61} We have recently reported that mTGF- β is transiently upregulated on lymph node CD4⁺CD25⁺ lymphocytes during acute FIV infection.²¹ Similar to the findings from acute FIV infection, Fig. 7 shows a significantly increased percentage of mTGF- β ⁺CD4⁺CD25⁺ lymphocytes within the lymph node of FIV⁺ cats, approximately 1 year postinfection. An increased percentage of mTGF- β ⁺CD4⁺CD25⁺ lymphocytes was not evident in the blood during acute or chronic FIV infection. Taken together these observations suggest that mTGF- β is upregulated on lymph node CD4⁺CD25⁺ T regulatory cells during the progression from acute to chronic FIV infection.

The inhibitory action of TGF- β is important in regulating CD8⁺ immune responses. For example, mice expressing a dnTGF- β RII specific to their T cells develop a profound CD8⁺ hyperproliferation and adoptive transfer experiments utilizing dnTGF- β RII CD8⁺ lymphocytes have demonstrated the development of different autoimmune diseases.^{38,41,62,63}

In a human melanoma vaccine study, TGF- β was shown to inhibit CD8⁺ effector function *in vitro*, whereas the suppressed CD8⁺ lymphocytes maintained an activated phenotype.⁴⁰ In an infectious disease model, blocking TGF- β restored *in vitro* CD8⁺ T cell IFN- γ production, and more importantly restored protective immunity against *Plasmodium yoelli* *in vivo*.⁶⁴

There is little information regarding the role of TGF- β in controlling CD8⁺ immune responses during lentiviral infections, but there is ample evidence for CD8⁺ immune dysfunction during the course of AIDS lentiviral infections.^{1,2,5,65,66} In considering possible mechanisms for CD8⁺ dysfunction, we hypothesized that FIV infection may cause increased TGF- β RII expression on CD8⁺ lymphocytes, thereby making them much more sensitive to TGF- β inhibition. Figures 5 and 6 demonstrate that CD8⁺ lymphocytes from FIV⁺ cats have increased surface expression of TGF- β RII when compared to FIV⁻ cats and these findings suggest that there is progressive upregulation of TGF- β RII during the course of FIV infection. Similar to the human melanoma vaccine study referenced above, activation of the TGF- β pathway in CD8⁺ lymphocytes may help to explain one of the apparent paradoxes of chronic lentiviral infections, in that CD8⁺ T cells display an activated phenotype but exhibit reduced effector function.^{1,53}

In CD8⁺ lymphocytes, TGF- β ligation of TGF- β RII leads to TGF- β RI/TGF- β RII dimerization followed by Smad 2/3 phosphorylation for intracellular propagation of the TGF- β signal. The phosphorylated Smad complex translocates to the nucleus and binds transcription factors, which promote

FIG. 8. Smad 2 phosphorylation is induced in CD8⁺ lymphocytes by TGF- β and following CD4⁺CD25⁺/CD8⁺ cocultures. In all, 4×10^6 FACS-purified CD8⁺ lymphocytes were serum starved overnight and were either untreated, treated with TGF- β (10 ng/ml) for 120 min, or cocultured with 4×10^6 FACS-purified CD4⁺CD25⁺ Tregs for 120 min at a ratio of 1:1, then lysed, and the amount of p-Smad 2 was determined by Western blotting. In the case of the CD4⁺CD25⁺/CD8⁺ cocultures, CD4⁺CD25⁺ cells were depleted by magnetic bead separation prior to lysis. (a) The upper and lower Western blots are from an FIV⁺ and FIV⁻ cat, respectively. Only a trace amount of Smad 2 phosphorylation is visible in untreated CD8⁺ cells (Lane 3). Treatment of CD8⁺ lymphocytes with soluble TGF- β results in Smad 2 phosphorylation (Lane 4). Following coculture with CD4⁺CD25⁺ Tregs, CD8⁺ targets exhibit robust Smad 2 phosphorylation (Lane 5). The lower row represents the actin (A) control and Lane 6 represents the kDa marker (M, phosphorylated Smad 2 is found between 50 and 60 kDa). (b) Smad 2 phosphorylation in CD8⁺ targets following coculture with CD4⁺CD25⁺ cells exceeds that of TGF- β -treated targets. The bar graph represents the mean of Smad 2 phosphorylation from eight FIV⁺ cats and two FIV⁻ control cats that were treated as described above. Untreated groups were given a value of 1, and the fold Smad 2 phosphorylation was then calculated for each treatment group.

cell cycle arrest and inhibit cytolytic gene products, leading to CD8⁺ anergy and reduction in CD8⁺ function.^{67,68} Studies of CD8⁺ antitumor activity have emphasized the importance of the TGF- β signaling pathway as a mechanism for tumor escape. Using the murine EL-4 thymoma model, Tomas and Massague⁶⁷ showed in a series of experiments that TGF- β inhibits the expression of CD8⁺ IFN- γ through Smad-ATF1-mediated binding of the IFN- γ promoter.

In accordance with these findings, Fig. 8b shows that coculture of CD8⁺ lymphocytes with CD4⁺CD25⁺ Tregs induces Smad 2 phosphorylation exceeding that of soluble TGF- β . Utilization of an mTGF- β -dependent mechanism relies on effector and target cell contact and in feline CD8⁺ lymphocytes Smad 2 phosphorylation occurs within 2–4 h of TGF- β RII ligation.³¹ Therefore, a CD4⁺CD25⁺ to CD8⁺ ratio of 1:1 was selected to maximize effector to target cell contact and to coordinate Smad 2 phosphorylation. Vahlenkamp *et al.*²⁰ have previously reported that Tregs from FIV⁺ cats inhibit IL-2 production in CD4⁺CD25⁻ targets at relatively low CD4⁺CD25⁺ to CD4⁺CD25⁻ ratios. However, at effector to target ratios of 1:1, they showed that Tregs from FIV⁻ cats also suppressed IL-2 production in CD4⁺ targets. Figure 7b shows that approximately 24% of the CD4⁺CD25⁺ cells from the FIV⁻ control cats are also mTGF- β ⁺. Given the high CD4⁺CD25⁺ to CD8⁺ ratio and the results of Fig. 7b, it is not surprising that Fig. 8b demonstrates that CD4⁺CD25⁺ cells from FIV⁻ cats can also induce Smad 2 phosphorylation. However, despite the induction of Smad 2 phosphorylation in FIV⁻ cats, Fig. 9 shows that only activated Tregs from FIV⁺ but not FIV⁻ control cats are capable of suppressing IFN- γ production in stimulated CD8⁺ target cells. Figure 9 also demonstrates that coculture of CD8⁺ cells with activated CD4⁺CD25⁺ Tregs from FIV⁺ cats results in more potent inhibition of CD8⁺ IFN- γ production than treatment with soluble TGF- β .

In conclusion, during the course of FIV infection, mTGF- β is upregulated on CD4⁺CD25⁺ Tregs, with these cells being found in LNs during the acute and chronic stage of infection. CD8⁺ lymphocytes exhibit an increased percentage of TGF- β RII in both the blood and LN when compared to FIV⁻ cats. It is likely that once in the LN CD8⁺ lymphocytes interact with CD4⁺CD25⁺ lymphocytes in a TGF- β -dependent fashion. In support of this, TGF- β -dependent Smad 2 phosphorylation in CD8⁺ lymphocytes was evident following CD4⁺CD25⁺ coculture. We have also shown that CD8⁺ IFN- γ production is inhibited following CD4⁺CD25⁺ coculture in FIV⁺ cats but not in FIV⁻ controls. Taken together, these results indicate that in FIV lentiviral infection, CD4⁺CD25⁺ Tregs suppress CD8⁺ responses during both the acute and chronic stages of infection and that CD4⁺CD25⁺ Tregs utilize a membrane TGF- β -dependent mechanism to suppress IFN- γ in CD8⁺ lymphocytes.

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FIG. 9. CD8⁺ IFN- γ mRNA is reduced in FIV⁺ cats by TGF- β treatment and following coculture with CD4⁺CD25⁺ Tregs. In all, 2–4 × 10⁶ purified CD8⁺ lymphocytes from FIV⁻ (gray bars) or FIV⁺ (dark bars) cats were serum starved overnight and were either untreated, treated with TGF- β , or cocultured with CD4⁺CD25⁺ Tregs, and RNA from CD8⁺ cells was then harvested for determination of IFN- γ mRNA by RT-PCR. For the CD4⁺CD25⁺/CD8⁺ cocultures, CD4⁺CD25⁺ cells were depleted by magnetic bead separation prior to RNA collection. The CD8⁺ cells were either untreated, Con A (5 μ g/ml) stimulated for 30 min followed by TGF- β (10 ng/ml) for 120 min, or treated with Con A for 30 min followed by coculture with CD4⁺CD25⁺ cells (1:1 ratio) for 120 min. In FIV⁺ cats, there was a significant reduction in IFN- γ mRNA following coculture with CD4⁺CD25⁺ Tregs ($p < 0.05$, asterisks). Each bar represents the mean $\pm$ SEM from five experiments.

Author Disclosure Statement

No competing financial interests exist.

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