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Feline Immunodeficiency Virus Infection Phenotypically and Functionally Activates Immunosuppressive CD4$^+$CD25$^+$ T Regulatory Cells[1]

Thomas W. Vahlenkamp,[2] Mary B. Tompkins,[3] and Wayne A. F. Tompkins

Disease progression of feline immunodeficiency virus (FIV) infection is characterized by up-regulation of B7.1 and B7.2 costimulatory molecules and their ligand CTLA4 on CD4$^+$ and CD8$^+$ T cells. The CD4$^+$CTLA4$^+$B7$^+$ phenotype described in FIV$^+$ cats is reminiscent of CD4$^+$CD25$^+$CTLA4$^+$ cells, a phenotype described for immunosuppressive T regulatory (Treg) cells. In the present study, we describe the phenotypic and functional characteristics of CD4$^+$CD25$^+$ T cells in PBMC and lymph nodes (LN) of FIV$^+$ and control cats. Similar to Treg cells, feline CD4$^+$CD25$^+$ but not CD4$^+$CD25$^-$ T cells directly isolated from LN of FIV$^+$ cats do not produce IL-2 and fail to proliferate in response to mitogen stimulation. Unstimulated CD4$^+$CD25$^+$ T cells from FIV$^+$ cats significantly suppress the proliferative response and the IL-2 production of Con A-stimulated autologous CD4$^+$CD25$^-$ T cells compared with unstimulated CD4$^+$CD25$^+$ T cells from FIV$^-$ cats. Flow-cytometric analysis confirmed the apparent activation phenotype of the CD4$^+$CD25$^+$ cells in LN of chronically FIV$^+$ cats, because these cells showed significant up-regulation of expression of costimulatory molecules B7.1, B7.2, and CTLA4. These FIV-activated, anergic, immunosuppressive CD25$^+$CTLA4$^+$B7$^+$CD4$^+$ Treg-like cells may contribute to the progressive loss of T cell immune function that is characteristic of FIV infection. *The Journal of Immunology,* 2004, 172: 4752–4761.

T he hallmark of HIV infection is a progressive decline of CD4$^+$ cells and loss of immune functions, leading to the development of AIDS. Disease progression is accompanied by progressive immune activation (1–4) and the inability of T cells to produce IL-2 and proliferate in vitro in response to recall Ags and mitogen during the asymptomatic stage of the infection (5–7). These immunological abnormalities correlate with a high degree of T cell apoptosis in lymph nodes (LN)[4] of infected individuals (3, 8–10). Apoptosis does not correlate with the number of virus-infected cells, level of viremia, or stage of disease, but rather to the state of T cell activation.

In feline immunodeficiency virus (FIV)-infected cats, a natural infection model for HIV, CD4$^+$ T cell depletion, decreased responsiveness to T cell mitogens, and LN T cell apoptosis are also common features (11–13). We recently reported that, with time after FIV infection, LN and, to a lesser extent, the blood become increasingly populated with CD4$^+$ and CD8$^+$ T cells expressing the B7.1 and B7.2 costimulatory molecules, as well as their anergy signaling ligand CTLA4 (13). A similar increase in CD4$^+$ cells expressing B7.1, B7.2, and CTLA4 in PBMC of HIV$^+$ individuals

has also been reported (14, 15). Three-color flow cytometry revealed that B7.1$^+$B7.2$^+$CTLA4$^+$ CD4$^+$ and CD8$^+$ activation phenotypes in FIV$^+$ cats have a higher frequency of apoptosis as opposed to the B7.1$^-$B7.2$^-$CTLA4$^-$ CD4$^+$ and CD8$^+$ phenotypes (13). Although we do not know whether there is a cause-and-effect relationship between these activated T cells and the T cell immunopathology associated with these infections, the surface phenotype and function of the FIV-induced B7$^+$CTLA4$^+$CD4$^+$ T cells are reminiscent of CD25$^+$CTLA4$^+$CD4$^+$ T regulatory (Treg) cells that are also noted for their interaction with other activated CD4$^+$ or CD8$^+$ cells in a manner to induce T cell anergy and apoptosis.

Studies in a number of experimental models have firmly established the existence of CD4$^+$CD25$^+$ Treg cells that perform an important anti-autoimmunity function by inhibiting the activation of autoreactive T cells and maintaining peripheral self-tolerance (16–19). More recently, it has been reported that CD4$^+$CD25$^+$ Treg cells are activated in animals by a number of infectious agents, and that these Ag-specific Treg cells are responsible for regulating protective immune responses to the pathogens (20–23). CD4$^+$CD25$^+$ Treg cells are a distinct, naturally occurring T cell lineage comprising 5–10% of the total CD4$^+$ population in the blood of normal rodents and humans (24–27). CD4$^+$CD25$^+$ Treg cells are a partially activated anergic phenotype capable, when stimulated via their TCR, of inhibiting the proliferative response of Ag- or mitogen-stimulated CD4$^+$ and CD8$^+$ T cells (28, 29). Suppression is mediated by cell contact-dependent transcriptional down-regulation of IL-2, a mechanism that is not Ag specific (30, 31). Because suppression is observed only upon activation of the CD4$^+$CD25$^+$ T cells, and is cell contact dependent, it is likely that inducible cell surface protein(s) are involved in immune suppression (32, 33). B7.1 and B7.2 costimulatory molecules on APCs perform such a regulatory function by binding receptors on Ag-activated T cells (34). Interestingly, it has been shown that B7 molecules, usually restricted to APC, are up-regulated on a fraction of T cells upon activation in vitro and in vivo in situations of

chronic antigenic stimulation, such as autoimmune disease (35–38) and lentivirus infection (13, 39–41). Nakamura et al. (33) and Caramalho et al. (42) have shown that B7.1 and B7.2 are also up-regulated on the surface of $CD4^+CD25^+$ Treg cells following in vitro activation with anti-CD3 or LPS.

The present study was designed to investigate the phenotypic and functional characteristics of $CD4^+CD25^+$ T cells in cats and to explore their potential role in the immunodeficiency associated with FIV infection. Our studies demonstrate that $CD4^+CD25^+$ T cells in normal and FIV^+ cats display the salient characteristics of $CD4^+$ Treg cells in humans and rodents, because they constitute ~5–10% of the peripheral T cell population, are characterized by an IL-2-deficient anergic phenotype, and are responsive to LPS. Compared with $CD4^+CD25^+$ T cells from control animals, $CD4^+CD25^+$ T cells directly isolated from LN of FIV^+ cats significantly suppress the proliferative response and the IL-2 production of Con A-stimulated autologous $CD4^+CD25^-$ T cells in a dose-dependent manner, suggesting that they are activated in vivo. Flow-cytometric analysis of PBMC and LN cells from asymptomatic FIV^+ cats confirmed this apparent in vivo activation state of $CD4^+CD25^+$ Treg cells in chronically infected animals, because the expression of B7.1 and B7.2 costimulatory molecules and their ligand CTLA4 is significantly up-regulated.

Materials and Methods

Cats and virus

Specific pathogen-free cats were obtained from Liberty Labs (Liberty Corners, NJ) or Cedar River Laboratory (Mason City, IA) and housed at the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University. Cats were inoculated with the $NCSU_1$ isolate of FIV as described by Bucci et al. (43). The $NCSU_1$ isolate of FIV is a pathogenic clade A virus, first described by English et al. (44). Infected cats were positive for FIV infection as confirmed by Ab immunoblot analysis and provirus detection by PCR using primers specific for the FIV p24 encoding sequence. All infected cats had inverted $CD4^+$: $CD8^+$ ratios in the blood. At the time samples were taken, cats had been infected with FIV for 3–5 years and did not show any clinical signs. Uninfected control cats ranged in age from 4 to 6 years and were housed separately from FIV^+ cats.

Blood and LN cell collection

Whole blood was collected by jugular venipuncture into EDTA Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ). PBMC were isolated by Percoll (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation as previously described (45). LN cells were obtained by peripheral LN biopsies as described by Levy et al. (46). Briefly, cats were anesthetized with i.v. ketamine and diazepam and maintained with inhalant isoflurane. One popliteal LN was excised, and butorphanol tartrate was administered to control postoperative discomfort. Single-cell suspensions of LN cells were prepared by gently passing the tissue through a steel mesh screen. After isolation, cells counts and viability were determined by trypan blue dye exclusion. Viability was routinely >90%.

Purification of T cells

To investigate single lymphocyte subsets, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells derived from PBMC were enriched by biomagnetic bead depletion using goat anti-mouse IgG-coated beads (Dynabeads M-450; Dynal, Great Neck, NY) as described by Bucci et al. (43). PBMC were depleted of B cells with anti-CD21 mAb (P. Moore (University of California, Davis, CA))-coated beads. Monocytes and macrophages were depleted with anti-CD14 mAb (TUK_4; DAKO, Carpinteria, CA)-coated beads, and $CD8^+$ cells were depleted with mAb 3.357 (47)-coated beads. The CD25-positive $CD4^+$ cell population was then enriched by positive selection using anti-feline CD25 mAb (9F23; kindly provided by K. Ohno (University of Tokyo, Tokyo, Japan)). The purity of the enriched $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells was verified by flow-cytometric analysis and ranged from 90 to 95%. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells derived from LN of the cats were purified using a high-speed FACS (MoFlo; DakoCytomation, Fort Collins, CO). The purity of the $CD4^+CD25^+$ and $CD4^+CD25^-$ LN cell populations was ≥97%. In all cases, viability of the purified $CD4^+$

subsets was determined by trypan blue dye exclusion, and all assays were based on viable cell numbers. Viability of sorted cells was always >90%.

Flow cytometry analysis

At least 5×10^5 PBMC or purified cell populations were stained for surface expression of various markers using FITC-conjugated anti-CD4 (mAb 30A) (47), PE-conjugated anti-CD8 (mAb 3.357), and allophycocyanin-conjugated anti-CD25 mAb (mAb 9F23). Expression of costimulatory molecules was performed as described by Tompkins et al. (13) using polyclonal rabbit anti-feline B7.1, B7.2, or CTLA4 followed by incubation with a PE-labeled donkey anti-rabbit IgG F(ab′)$_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA). The production and specificity of the polyclonal anti-B7 and anti-CTLA4 Abs have been described previously (13). At least 20,000 cells were analyzed by flow cytometry (FACSCalibur; CellQuest software; BD Biosciences, Mountain View, CA). Cells undergoing apoptosis were differentiated from nonapoptotic cells by staining for the presence of phosphatidyl-serine on their surface using annexin V staining (Roche Laboratories, Basel, Switzerland). Necrosis vs apoptosis was determined by propidium iodide and annexin V staining according to the manufacturer's instructions followed by two-color flow-cytometric analysis.

T cell stimulation assays

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, and 1 mM HEPES buffer. For Con A stimulation, 2×10^4 viable cells/well were cultured in a round-bottom 96-well plate in the presence or absence of 5 μg/ml Con A or 100 U/ml recombinant human IL-2 (rhIL-2) (National Institutes of Health AIDS Research and Reagent Program, Rockville, MD). For LPS stimulation, 2×10^5 viable cells/well were cultured in a round-bottom 96-well plate in the presence or absence of 10 μg/ml LPS (*E. coli* 055:B5; Sigma-Aldrich) and/or 100 U/ml rhIL-2. All assays were run in triplicate. After 2 days of incubation (Con A-stimulated cells) or 4 days of incubation (LPS-stimulated cells), cells were pulsed with 1 μCi of [^{3}H]TdR/well and harvested 16 h later using a Filtermake Harvester (Packard Bioscience, Meriden, CT). [^{3}H]TdR uptake as a measure of proliferation was determined in cpm using a Top Count NXT Microplate scintillation counter (Packard Bioscience).

Coculture suppression assay

Purified $CD4^+CD25^-$ target cells (10^6 cells/ml) were stimulated for 3 h with 5 μg/ml Con A, washed twice in RPMI 1640, and plated at 5×10^4 viable cells/well in 96-well plates. Varying numbers of autologous $CD4^+CD25^+$ effector cells were then added to the wells to yield E:T ratios ranging from 0.006:1 to 1:1. The autologous $CD4^+CD25^+$ effector cells were added either as freshly isolated, untreated cells, or were treated in 24-well plates at 10^6 cells/ml for 4 days with 10 μg/ml LPS or 100 U/ml rhIL-2, or for 2 days with LPS followed by 2 days with rhIL-2. The cells were then washed, counted, and added to the target cells. Depending on the number of effector cells, assays were run in either duplicate or triplicate. Effector and target cells were cocultured for 3 days, then pulsed with 1 μCi of [^{3}H]TdR/well, and harvested 16 h later as described above. Percent inhibition of proliferation was determined based on proliferation of $CD4^+CD25^-$ target cells alone and calculated as follows: percent inhibition = (cpm of target cells alone − cpm of coculture)/cpm of target cells alone.

Measurement of IL-2 production

The IL-2-dependent CTLL cell line was used to determine the presence of IL-2 in culture supernatants of feline $CD4^+$ subsets as described (45). CTLL cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin, 1% sodium bicarbonate, 1% sodium pyruvate, 1% L-glutamine, and 1 mM HEPES buffer. rhIL-2 (10^3 U/ml) was added daily, and the culture analyzed for cell viability by trypan blue staining. Exponentially growing cultures were centrifuged twice, and the rhIL-2-containing medium was removed. CTLL cells (2×10^3) were cultured in 100 μl of RPMI 1640 medium without rhIL-2 in a flat-bottom 96-well plate, and 100 μl of the feline cell culture supernatant to be tested was added. After 36 h of incubation, the cultures were pulsed with 1 μCi of [^{3}H]TdR/well and harvested 16 h later as described above.

Statistical analysis

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

Results

Prevalence of CD4⁺CD25⁺ T cells in feline peripheral blood and LN

Two-color flow-cytometric analysis of feline PBMC and LN cells revealed that the percentage of $CD4^+CD25^+$ T cells in the peripheral blood ranged between 5 and 10% of the total $CD4^+$ T cells (Fig. 1A). Interestingly, the percentage of $CD4^+CD25^+$ cells was significantly ($p < 0.0001$) higher in the LN, comprising 20–30% of the total $CD4^+$ T cells. Also, the mean fluorescence intensity (MFI) of CD25 expression on $CD4^+$ T cells was significantly higher in the LN than PBMC of both FIV^+ ($p = 0.037$) and FIV^- cats ($p = 0.014$) (Fig. 1B). There is no difference in the percentage of $CD4^+CD25^+$ T cells or MFI of CD25 expression in chronically FIV^+ cats compared with age-matched control animals. These data suggest that neither the percentage of $CD4^+$ cells expressing CD25 nor the surface density of CD25 on existing $CD4^+CD25^+$ T cells is affected by FIV infection.

Freshly isolated feline CD4⁺CD25⁺ LN cells are unresponsive to Con A stimulation

One characteristic of Treg cells is that they are unresponsive to antigenic or mitogenic stimulation (19, 27). To determine whether
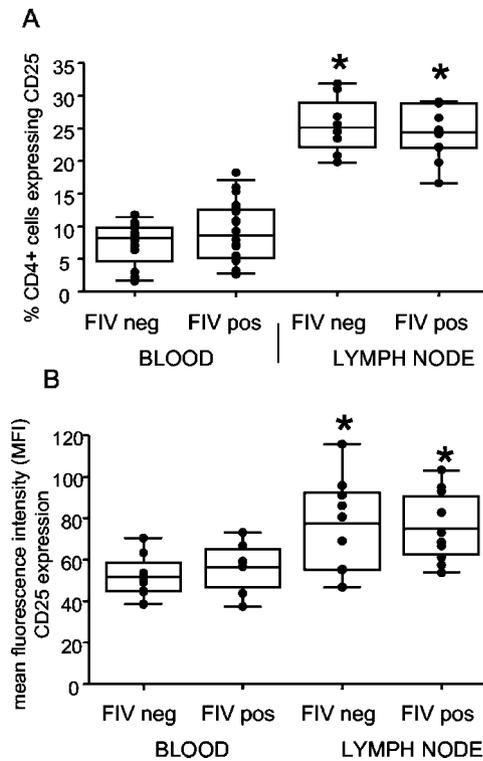
this is the case for feline $CD4^+CD25^+$ cells, FACS-purified $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from the LN of FIV^+ and control cats were stimulated with Con A and analyzed for cell proliferation by [³H]TdR uptake. Freshly isolated $CD4^+CD25^-$ T cells from FIV^+ and control cats were able to proliferate in response to Con A stimulation (Fig. 2A). In contrast, the $CD4^+CD25^+$ cell population from either FIV^+ or control cats failed to proliferate (Fig. 2A). However, addition of rhIL-2 and Con A to the cultures resulted in a strong proliferative response by both $CD4^+CD25^-$ and $CD4^+CD25^+$ cell populations (Fig. 2A). The presence of rhIL-2 alone in the culture medium failed to induce a significant proliferative response in $CD4^+CD25^-$ or $CD4^+CD25^+$ T cells. The unresponsiveness of $CD4^+CD25^+$ T cells to polyclonal mitogen activation was not due to high levels of apoptosis in this cell population, because annexin V analysis of 36-h cultured cells with or without rhIL-2 or Con A was <30% and not significantly different in the $CD4^+CD25^+$ and $CD4^+CD25^-$ cell cultures in both FIV^+ and FIV^- cats (data not shown).

Stimulated CD4⁺CD25⁺ T cells are deficient in IL-2 production

The inability of $CD4^+CD25^+$ Treg cells to proliferate in response to stimulation has been attributed to the lack of IL-2 production by these cells (30). To address the possibility that $CD4^+CD25^+$ T cells are deficient in IL-2 production, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were stimulated with Con A for 3 days, and

FIGURE 1. Expression of CD25 on $CD4^+$ cells in peripheral blood and LN from FIV^+ cats and age-matched control animals. Two-color analysis was performed using cat-specific anti-CD4 and anti-CD25 mAb. Box-whisker plots representing 5th and 95th percentiles (whisker), 25th and 75th percentiles (box), and median. A, The percentage of $CD4^+$ cells in the peripheral blood and LN that express CD25. Values represent the percentage of $CD4^+CD25^+$ cells of total $CD4^+$ T cells. Symbols indicate values for individual cats. The percentage of $CD4^+$ cells expressing CD25 was significantly higher in the LN compared with the peripheral blood both in control and FIV^+ animals ($p < 0.0001$). B, The MFI of CD25 expressed on $CD4^+$ cells in the peripheral blood and LN. The MFI of CD25 expression on $CD4^+$ cells was significantly higher in the LN of control ($p = 0.014$) and FIV^+ cats ($p = 0.037$) compared with the PBMC. There was no difference in the percentage of $CD4^+$ cells expressing CD25 and the MFI of CD25 expression in the blood and LN between control and FIV^+ cats. Symbols indicate values for individual cats.
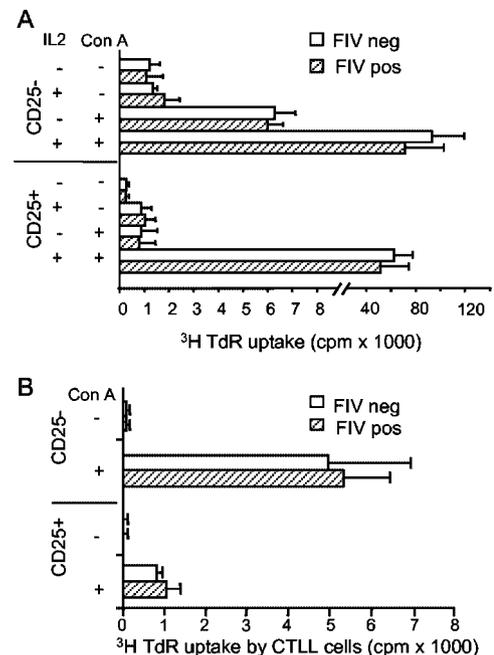

FIGURE 2. Response of LN $CD4^+$ cells from uninfected and FIV^+ cats to Con A and/or rhIL-2 stimulation. A, Proliferative response of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells. FACS-purified cells (2×10^4) were incubated in the presence or absence of Con A (5 μg/ml) and/or IL-2 (100 U/ml) for 2 days, pulsed with [³H]TdR, and harvested 16 h later. Bars represent the mean ± SEM of four cats. B, Proliferation of the IL-2-dependent murine cell line CTLL in the presence of supernatant from $CD4^+CD25^+$ or $CD4^+CD25^-$ LN T cell cultures. FACS-purified cells (2×10^4) were incubated in the presence or absence of Con A (5 μg/ml) for 3 days. The culture supernatants (100 μl) were then collected and added to CTLL cultures. Following 36 h of incubation, 1 μCi of [³H]TdR/well was added, and cells were harvested 16 h later. Bars represent the mean ± SEM of three cats.

the culture supernatants were assayed for IL-2 by the CTLL proliferation bioassay. As shown in Fig. 2B, the CTLL cells proliferated in the presence of supernatant from Con A-stimulated CD4$^+$CD25$^-$ cells, indicating that the supernatant contained IL-2. In contrast, supernatant from Con A-stimulated CD4$^+$CD25$^+$ cell cultures did not support the proliferation of CTLL cells. These data suggest that the unresponsiveness of feline CD4$^+$CD25$^+$ T cells to mitogenic stimulation is due to a deficiency in IL-2 production, which can be overridden by the addition of exogenous IL-2.

CD4$^+$CD25$^+$ T cells become activated and proliferate in response to LPS

Recent studies have demonstrated that CD4$^+$CD25$^+$ Treg cells can be phenotypically and functionally activated, as defined by increased expression of costimulatory molecules and proliferation in response to stimulation of the Toll-like receptor (TLR)-4 with LPS (42). We therefore tested the response of purified CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells from normal cats to LPS stimulation. As shown in Fig. 3A, incubation for 4 days in the presence of 10 μg/ml LPS increased the expression of the activation markers B7.1, B7.2, and CTLA4 on CD4$^+$CD25$^+$ T cells, whereas there was no change in expression of these molecules on CD4$^+$CD25$^-$ T cells or CD4$^-$ cells. In addition to phenotypic activation, LPS stimulation induced proliferation of the CD4$^+$CD25$^+$ cells compared with untreated CD4$^+$CD25$^+$ cells ($p = 0.05$) and with untreated or LPS-treated CD4$^+$CD25$^-$ cells (Fig. 3B). As has been reported for murine Treg cells (42), there was an even greater proliferative response by the CD4$^+$CD25$^+$ cells in the presence of rhIL-2 plus LPS. Con A stimulation of these cell populations gave results similar to that shown in Fig. 2A, i.e., only the CD4$^+$CD25$^-$ cells proliferated (data not shown).

In vitro-activated CD4$^+$CD25$^+$ T cells suppress proliferation of CD4$^+$CD25$^-$ T cells

The hallmark of Treg cells is their ability, when activated in vivo or in vitro, to suppress proliferative responses of other activated T

cells in a contact-dependent manner. Caramalho et al. (42) demonstrated that in vitro activation of Treg cells by LPS, LPS plus IL-2, or IL-2 alone induces suppressor activity as measured by their ability to inhibit proliferation of CD4$^+$CD25$^-$ cells. As phenotypic and functional data presented to this point suggest that the feline CD4$^+$CD25$^+$ T cells could be Treg cells, we tested the suppressor function of LPS-treated CD4$^+$CD25$^+$ cells from normal cats. Purified CD4$^+$CD25$^+$ cells were treated for 4 days with 10 μg/ml LPS, or treated for 2 days with LPS followed by the addition of 100 U/ml rhIL-2 for 2 days, or treated with rhIL-2 alone for 4 days. Varying numbers of the treated cells were then cocultured for 3 days with a constant number of autologous Con A-stimulated CD4$^+$CD25$^-$ cells, and proliferation was measured by [^{3}H]TdR incorporation. Fig. 4 shows the results from two different cats. In both cases, stimulation by LPS alone induced only a low level of suppression. However, addition of rhIL-2 to LPS-treated cells, or rhIL-2 alone induced strong suppressive activity by CD4$^+$CD25$^+$ cells. To verify that cell-cell contact is required for this suppressor activity, similar assays were done in a Transwell system, and no suppression of CD4$^+$CD25$^-$ cell proliferation occurred (data not shown). Similar treatment of CD4$^+$CD25$^-$ cells with LPS and/or rhIL2 did not induce any suppressor activity (data not shown). These data suggest that the feline CD4$^+$CD25$^+$ T cells are Treg cells.

CD4$^+$CD25$^+$ T cells from FIV$^+$ cats have an activated phenotype characterized by up-regulation of costimulatory molecules B7.1, B7.2, and their ligand CTLA4

Recent studies have demonstrated that a number of microbial pathogens can activate CD4$^+$CD25$^+$ Treg cells in vivo (20–23). We therefore asked whether chronic FIV infection results in activation of Treg cells. Because activated CD4$^+$CD25$^+$ Treg cells up-regulate B7 costimulatory molecule expression on their surface, CD4$^+$CD25$^+$ T cells from FIV$^+$ and FIV$^-$ cats were analyzed for surface expression of B7.1 and B7.2. The percentage of CD4$^+$CD25$^+$ T cells expressing B7.1 (Fig. 5A) and B7.2 (C) was
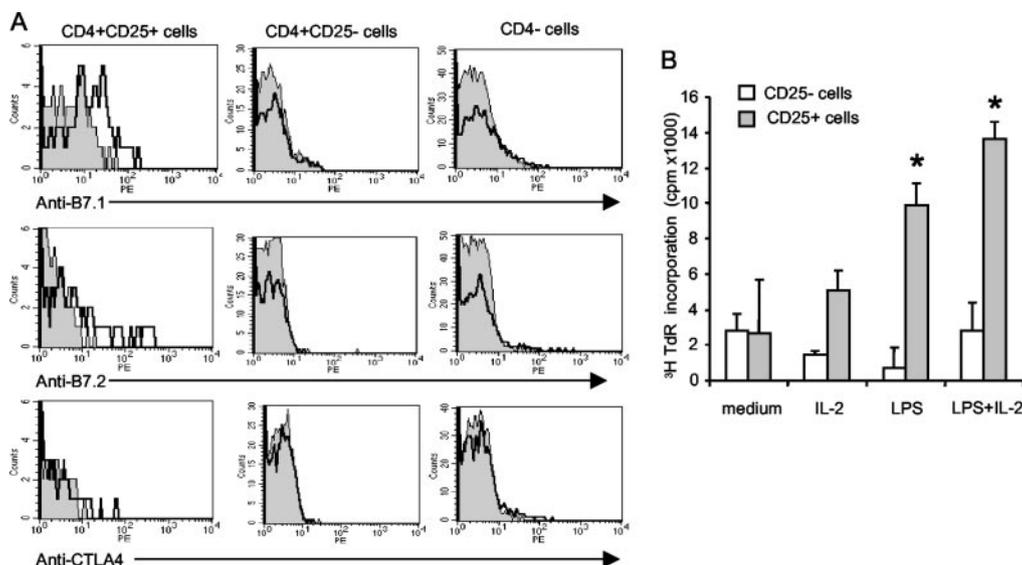
FIGURE 3. Activation of CD4$^+$CD25$^+$ T cells by stimulation with LPS. A, Costimulatory molecule expression on PBMC from normal cats following 4 days of culture in the presence (bold line) or absence (thin line) of LPS (10 μg/ml). Three-color analysis was performed for CD4, CD25, and either B7.1, B7.2, or CTLA4. A representative result of three independent experiments is shown. B, Proliferative response of CD4$^+$CD25$^+$ T cells to stimulation by LPS alone, rhIL-2 alone, or LPS plus rhIL-2. Magnetic bead-purified CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells (2 × 10^5) were incubated for 3 days in medium supplemented with IL-2 (100 U/ml), LPS (10 μg/ml), or LPS plus rhIL-2, pulsed with [^{3}H]TdR, and harvested 16 h later. Bars represent the mean ± SEM of four cats. Proliferation of CD4$^+$CD25$^+$ T cells in the presence of LPS and LPS plus rhIL2 was significant ($p = 0.05$) compared with the same cells in medium alone.
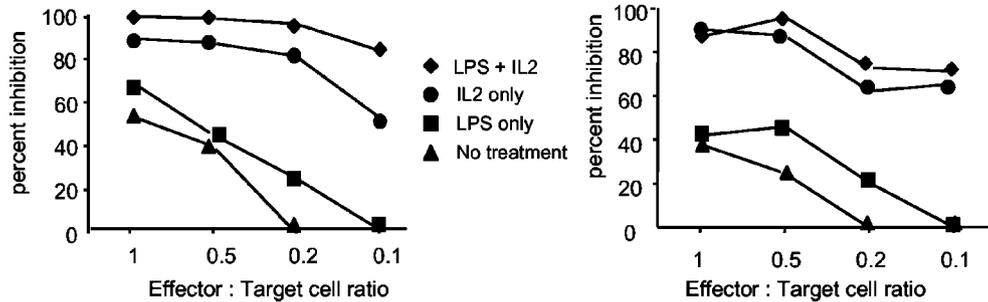
FIGURE 4. Suppressor activity of LPS-activated CD4$^+$CD25$^+$ T cells from two normal cats. Magnetic bead-purified CD4$^+$CD25$^+$ cells were treated either for 4 days with LPS (10 μg/ml) or IL-2 (100 U/ml), or 2 days with LPS followed by 2 days with IL-2, then cocultured for 3 days with Con A (5 μg/ml)-stimulated autologous CD4$^+$CD25$^-$ target cells. Cocultures were pulsed with [^{3}H]TdR and harvested 16 h later. A constant number of CD4$^+$CD25$^-$ target cells (5 × 10^4/well) were cultured with varying numbers of CD4$^+$CD25$^+$ effector cells. Percent inhibition of proliferation was calculated based on proliferation of CD4$^+$CD25$^-$ target cells alone ((cpm of target cells alone $-$ cpm of coculture)/cpm of target cells alone).

significantly higher in PBMC from both FIV$^-$ and FIV$^+$ cats compared with the percentage of CD4$^+$CD25$^-$ T cells expressing these molecules. In the LN, B7.1 (Fig. 5B), but not B7.2 (D) expression was significantly increased on CD4$^+$CD25$^+$ T cells from FIV$^-$ cats, whereas both B7.1 and B7.2 expression was increased in FIV$^+$ cats. Importantly, in both blood and LN, the percentage of CD4$^+$CD25$^+$ T cells expressing B7.1 and B7.2 from FIV$^+$ cats was significantly greater than in FIV$^-$ cats.

Because expression of the B7 ligand CTLA4 has been shown to be associated with Treg cells, and because we have previously reported that FIV infection induces up-regulation of CTLA4 on the surface of CD4$^+$ cells, we examined the CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ cell subsets for differential expression of CTLA4. In the peripheral blood, there was no significant difference between FIV$^-$ and FIV$^+$ cats in the percentage of cells expressing CTLA4. However, in the LN, there was a significantly greater percentage of CD4$^+$CD25$^+$ T cells expressing CTLA4 compared with CD4$^+$CD25$^-$ T cells in FIV$^+$ cats (Fig. 5F), but not in negative cats. CTLA4 expression on CD4$^+$CD25$^+$ cells in the LN of FIV$^+$ cats was also significantly greater than in LN of negative cats. Thus, the CD4$^+$CD25$^+$ cells in FIV$^+$ cats express an activated phenotype as determined by costimulatory molecule expression.

Proliferation of CD4$^+$CD25$^-$ T cells is inhibited by coculture with unstimulated CD4$^+$CD25$^+$ T cells from FIV$^+$ cats

Because the phenotype of freshly isolated CD4$^+$CD25$^+$ cells from FIV$^+$ cats suggested they were activated, we tested the ability of this cell population to suppress the proliferation of Con A-stimulated autologous CD4$^+$CD25$^-$ T cells. As shown in Fig. 6A, unstimulated CD4$^+$CD25$^+$ T cells from FIV$^+$ cats but not FIV$^-$ cats markedly suppressed the proliferation of autologous CD4$^+$CD25$^-$ cells. CD4$^+$CD25$^+$ effector cell titration assays, using a constant number of CD4$^+$CD25$^-$ target cells and varying numbers of CD4$^+$CD25$^+$ effector cells, revealed that CD4$^+$CD25$^+$ T cells from FIV$^+$ cats suppressed the proliferation of mitogen-stimulated T cells in a dose-dependent manner (Fig. 6B).

Activated Treg cells suppress the proliferation of other T cells through down-regulation of IL-2 in the target cells (30). To verify this in the autologous coculture suppressor assay system, we measured the cytokine production in the culture supernatant using the IL-2-dependent CTLL murine cell line. Culture supernatants from nonstimulated CD4$^+$CD25$^+$ T cells cocultured with Con A-stimulated autologous CD4$^+$CD25$^-$ T cells from FIV$^+$ cats did not support the proliferation of CTLL cells (Fig. 6C), whereas supernatants from similar cocultures of negative cat CD4$^+$CD25$^+$ T cells did. These results suggest that the CD4$^+$CD25$^+$ T cells in chronically FIV-infected cats behave as activated Treg cells by

interfering with the induction of IL-2, suppressing cell proliferation of Con A-stimulated CD4$^+$CD25$^-$ T cells.

Discussion

We previously reported a time-related progressive expansion of B7.1$^+$B7.2$^+$CTLA4$^+$ CD4$^+$ and CD8$^+$ T cells in the LN of FIV$^+$ cats (13). These cells correlate with a high frequency of spontaneous T cell apoptosis leading to the hypothesis that T cells coexpressing B7 and CTLA4 are capable of T-T cell interactions that mediate anergy and apoptosis. The activated phenotype and the proposed anergy signaling function of the FIV-induced B7$^+$CTLA4$^+$CD4$^+$ T cells closely resembles that of CD25$^+$CTLA4$^+$B7$^+$CD4$^+$ Treg cells that have been recently described in different species, including humans and rodents, and shown to suppress the proliferative responses of T cells in the periphery (16–19, 24–27). In this study, we provide evidence that feline CD4$^+$CD25$^+$ T cells have characteristics of Treg cells. Furthermore, we demonstrate that, in FIV$^+$ animals, these cells are activated such that they are able to suppress mitogen-induced responses of CD4$^+$CD25$^-$ Th cells.

Analysis of feline PBMC from uninfected and chronically FIV$^+$ cats revealed that 5–10% of the CD4$^+$ cells express CD25, which is similar to the number of CD4$^+$CD25$^+$ Treg cells described in the peripheral blood of humans and rodents (24–27). There was no significant difference in the percentage of CD4$^+$ cells expressing CD25 between control and FIV$^+$ cats. Expression of CD25 on CD4$^+$ cells in the peripheral blood of HIV$^+$ patients has been reported to be decreased compared with healthy individuals (48). The percentage of CD4$^+$ T cells expressing CD25 was significantly higher in the LN compared with the blood in both FIV$^+$ and control animals and was paralleled by increased CD25 fluorescence intensity. Whether these CD4$^+$CD25$^+$ T cells in the LN are all Treg cells or some are activated Th cells is not known, because CD25 can be expressed on activated CD4$^+$ Th cells (49). However, it is possible that there are functional differences between blood and LN CD4$^+$CD25$^+$ Treg cells, because it is the LN where we would expect Treg cells to be activated and perform their function. This conjecture is supported by the observation that a significantly higher fraction of LN CD4$^+$CD25$^+$ express CTLA4 on their surface than those in the blood of FIV$^+$ cats. Unfortunately, we were unable to further phenotypically characterize CD4$^+$CD25$^+$ cells due to the limited availability of reagents in the feline model. We are in the process of doing this by assessing the expression of TGF-β, Foxp3, and other markers for CD4$^+$CD25$^+$ Treg cells.

CD4$^+$CD25$^+$ Treg cells are distinguished from CD4$^+$CD25$^-$ Th cells in that they are deficient in IL-2 production and anergic to
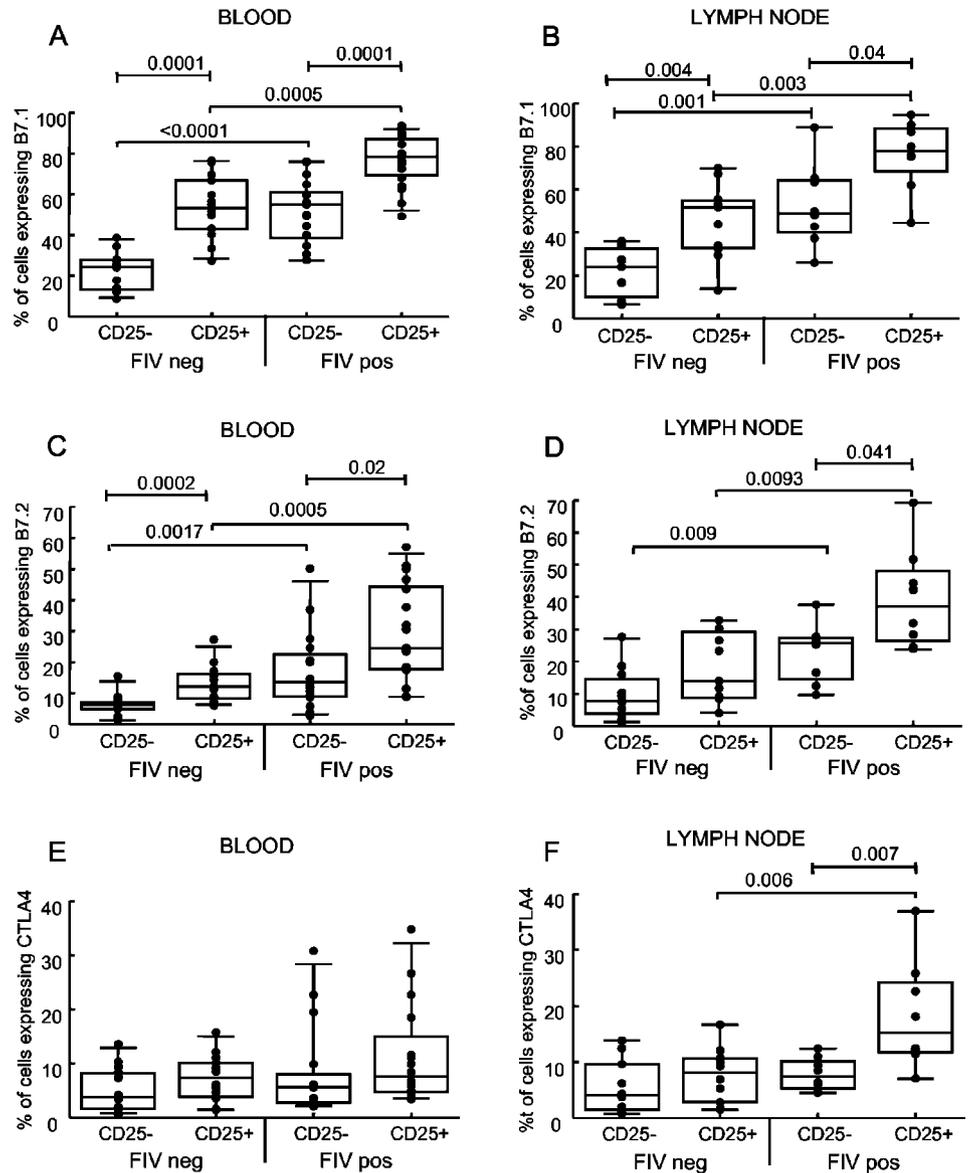
FIGURE 5. Up-regulation of B7.1/B7.2 costimulatory molecules and their ligand CTLA4 on CD4$^+$ T cell subsets from FIV$^+$ cats. Three-color analysis was performed on PBMC and LN cells from FIV$^-$ and FIV$^+$ cats for CD4, CD25, and either B7.1 (*A* and *B*), B7.2 (*C* and *D*), or CTLA4 (*E* and *F*). Box-whisker plots are as described in Fig. 1. Symbols indicate values for individual cats. Numbers represent the percentage of B7.1-, B7.2-, or CTLA4-positive cells of total CD4$^+$CD25$^+$ or CD4$^+$CD25$^-$ T cells.

antigenic or mitogenic stimulation (19, 27). The studies described herein show that LN CD4$^+$CD25$^+$ cells from both FIV$^+$ and FIV$^-$ cats possess the major characteristics of CD4$^+$CD25$^+$ Treg cells. Consistent with an anergic Treg phenotype, CD4$^+$CD25$^+$ T cells from FIV$^+$ or FIV$^-$ cats failed to proliferate when stimulated with Con A, whereas CD4$^+$CD25$^-$ T cells in the absence of CD4$^+$CD25$^+$ Treg cells proliferated normally. These data suggest that presumably naive CD4$^+$CD25$^-$ LN or PBMC Th cells in FIV$^+$ cats are immune to immunosuppression by activated CD4$^+$CD25$^+$ Treg cells. Thus, the suppressor activity of feline CD4$^+$CD25$^+$ cells against CD4$^+$CD25$^-$ Th cells seems to dependent upon activation of the target cell population, as is the case for the immunosuppressive activity of human and rodent Treg cells (28, 30). Thus, naive CD4$^+$CD25$^-$ cells from FIV$^-$ or FIV$^+$ cats in the absence of CD4$^+$CD25$^+$ cells would be responsive to Con A but would be suppressed when stimulated in the presence of CD4$^+$CD25$^+$ T cells that would also be activated by Con A. In support of this, Joshi et al. (50) demonstrated that unfractionated PBMC from FIV$^+$ cats were unresponsive to Con A stimulation, whereas CD25$^+$-depleted cultures proliferated normally. Interestingly, as has been reported for CD4$^+$CD25$^+$ Treg cells (27, 28), the unresponsive state of feline CD4$^+$CD25$^+$ cells to mitogenic

stimulation could be overridden by addition of IL-2. In agreement with this observation, Con A-stimulated CD4$^+$CD25$^+$ T cell cultures were deficient in biologically active IL-2 production as compared with CD4$^+$CD25$^-$ cells, as has been reported for human and rodent Treg cells (28, 32). These data suggest that the failure of feline CD4$^+$CD25$^+$ to proliferate in response to Con A stimulation can be attributed to a poor IL-2 response. These data collectively suggest that CD4$^+$CD25$^+$ T cells from FIV$^+$ and FIV$^-$ cats possess major characteristics of Treg cells, because they are deficient in IL-2 production and do not proliferate in response to mitogenic stimulation, and that this unresponsive state can be overridden with exogenous IL-2.

The hallmark characteristic of CD4$^+$CD25$^+$ Treg cells is their ability to inhibit the proliferation of other activated T cells when stimulated by their cognate Ag (30). More recently, Caramalho et al. (42) reported that CD4$^+$CD25$^+$ Treg cells express a number of TLRs including TLR-4, and that exposure of CD4$^+$CD25$^+$ T cells to the TLR-4 ligand LPS up-regulated several activation markers, including B7.1, and increased their proliferative response and immunosuppressive activity in vitro and in vivo. Addition of IL-2 augmented these CD4$^+$CD25$^+$ T cell responses to LPS. We also found that LPS and LPS plus IL-2 stimulation of CD4$^+$CD25$^+$ but
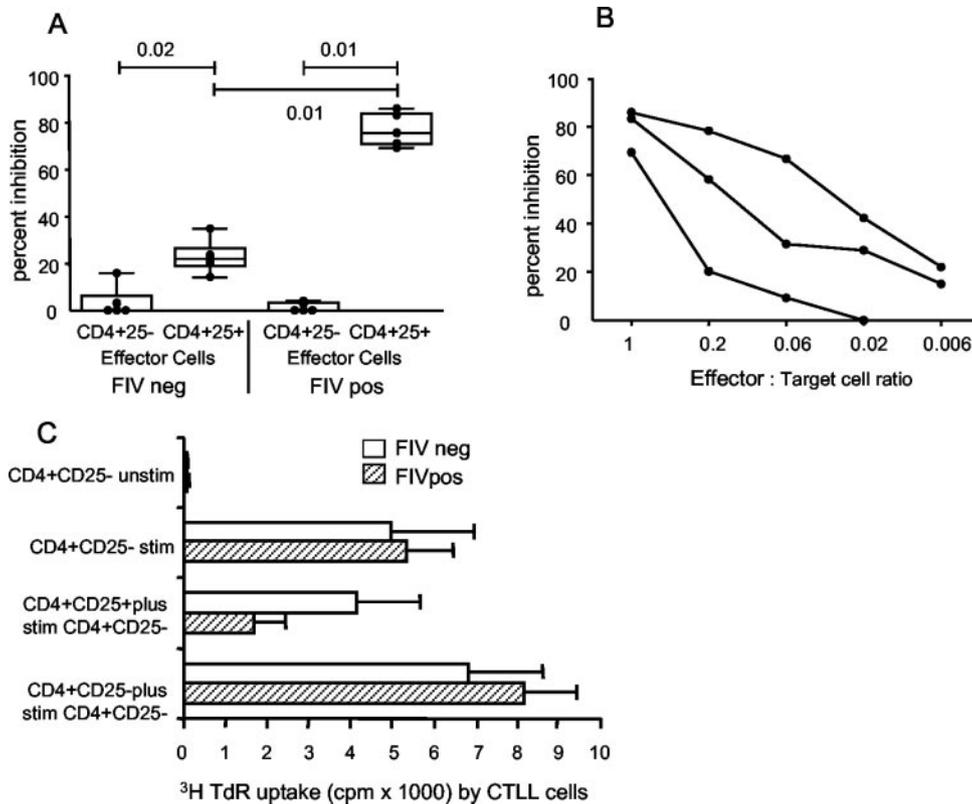
FIGURE 6. Suppressor activity of unstimulated CD4$^+$CD25$^+$ T cells from FIV$^+$ cats. *A*, Con A (5 μg/ml)-stimulated CD4$^+$CD25$^-$ T cells (5 $\times$ 10^4) were cocultured with equal numbers of freshly isolated autologous, unstimulated CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ effector cells from FIV$^-$ or FIV$^+$ cats. T cell proliferation was measured after 3 days of incubation and an additional 16-h pulse with [^{3}H]TdR. Percent inhibition of proliferation was calculated as described in Fig. 4. Box-whisker plots are as described in Fig. 1. The results from five control and FIV$^+$ cats are shown. *B*, CD4$^+$CD25$^+$ T cells inhibit T cell proliferation in a dose-dependent manner. Con A-stimulated CD4$^+$CD25$^-$ T cells (5 $\times$ 10^4) were cocultured in the presence of different numbers of freshly isolated autologous, unstimulated CD4$^+$CD25$^+$ T cells from FIV$^+$ cats. Proliferation and percent inhibition were determined as described in Fig. 4. Each line represents a single FIV$^+$ cat. *C*, CD4$^+$CD25$^+$ T cells from FIV$^+$ cats mediate their suppression through down-regulation of IL-2. Culture supernatants from 3-day cocultures as described in *A* were added to IL-2-dependent CTLL cells, and proliferation was determined by [^{3}H]TdR incorporation as described in Fig. 2*B*. Bars represent the mean $\pm$ SEM of three cats.

not CD4$^+$CD25$^-$ T cells up-regulated B7.1 expression, as well as B7.2 and CTLA4. We also observed that LPS treatment of CD4$^+$CD25$^+$ T cells from FIV$^-$ cats induced a proliferative response and activated their suppressor function against Con A-stimulated autologous CD4$^+$CD25$^-$ Th cells. As reported for LPS-stimulated CD4$^+$CD25$^+$ Treg cells, IL-2 enhanced these responses and could to a large extent mimic the LPS response. These data suggest that CD4$^+$CD25$^+$ T cells in the cat have the phenotypic and functional characteristics of classic Treg cells described in humans and rodents.

Because there are reports of Treg cell activation in vivo under conditions of chronic immune stimulation (20–23), we asked whether these cells might be activated in chronically FIV-infected cats. Interestingly, freshly isolated, unstimulated CD4$^+$CD25$^+$ T cells from FIV$^+$ cats significantly inhibited the proliferative response of autologous Con A-stimulated CD4$^+$CD25$^-$ T cells when compared with CD4$^+$CD25$^+$ T cells from FIV$^-$ cats, although CD4$^+$CD25$^+$ T cells from FIV$^-$ cats also showed some suppressor function, similar to what has been reported in humans and mice (27, 42). Because TCR signaling is thought to be a prerequisite for CD4$^+$CD25$^+$ T cell-mediated suppressor function (27, 30), the results indicate that the CD4$^+$CD25$^+$ T cell population derived from FIV$^+$ cats have been activated in vivo in response to viral Ag. The suppressive effect of the CD4$^+$CD25$^+$ T cells from FIV$^+$ cats correlated with the down-regulation of target cell IL-2, which provides additional evidence that the

CD4$^+$CD25$^+$ T cells in chronically FIV$^+$ cats behave as activated Treg cells that suppress T cell proliferation via down-regulation of IL-2 in target cells (30). The antigenic specificity of the activated CD4$^+$CD25$^+$ Treg cells in FIV$^+$ cats remains an unanswered question, because these cells can be activated by less specific stimuli, such as ligands for TLR, in the absence of TCR engagement.

Phenotypic analysis of the CD4$^+$ T cell subsets demonstrated that FIV infection also up-regulated activation markers on CD4$^+$ T cells, including up-regulation of B7.1 and B7.2 expression on both CD4$^+$CD25$^+$ and CD4$^+$CD25$^-$ T cells in the blood and LN. However, the highest expression of B7.1 and B7.2 was detected on CD4$^+$CD25$^+$ T cells derived from the LN of FIV$^+$ cats. Whether these costimulatory molecules play a role in CD4$^+$CD25$^+$ immunosuppressive function is not known. It is anatomically significant that the expansion of anergic CD4$^+$CD25$^+$B7$^+$ cells occurs predominantly in the LN as opposed to the blood of FIV$^+$ animals, because it is the LN where we would expect immune responses to Ag to occur, and where we would expect Treg cells to exert their immunoregulatory function. In support of this, we demonstrated a significant up-regulation of surface CTLA4 expression on CD4$^+$CD25$^+$ T cell only in the LN of FIV$^+$ cats. We also detected an increased expression of CTLA4 on the surface of CD4$^+$CD25$^+$ cells as opposed to CD4$^+$CD25$^-$ cells in the blood and LN of FIV$^-$ cats. However, this increase was not significant. These results support our previous report that CTLA4 is up-regulated on the surface of CD4$^+$ LN cells in cats with long-term FIV infection

(13). Because of its rapid turnover on the cell surface, CTLA4 is usually detected by intracellular staining, and studies have demonstrated that increased levels of intracellular CTLA4 is a characteristic of $CD4^+CD25^+$ Treg cells (24). We were unable to measure intracellular CTLA4 in feline Treg cells in control and FIV^+ cats, because the feline-specific CTLA4 Ab is a polyclonal and did not lend itself well to intracellular staining. We are in process of developing a mAb to feline CTLA4 to address this issue. However, in support of our data, constitutive expression of CTLA4 on the surface of $CD4^+CD25^+$ cells as opposed to $CD4^+CD25^-$ cells has been reported in mice and humans (51–53). Increased surface expression of CTLA4 on $CD4^+CD25^+$ LN Treg cells plus the fact that the MFI of CD25 expression was significantly higher in the LN than in the blood suggest a possible functional difference of this phenotype in the two tissue compartments. Several studies reported that the $CD4^+CD25^{high}$ population predominantly harbors cells with regulatory suppressor function (54, 55). The role of CTLA4 and other surface molecules with respect to the suppressive properties of $CD4^+CD25^+$ Treg cells is currently controversial. Most investigators have found, similar to our experience, that the suppressive properties of $CD4^+CD25^+$ Treg cells in vitro are strictly contact dependent (56). Nevertheless, the suppressive properties of Treg cells in vivo might be more complex and based on diverse mechanisms including mediators such as IL-10 and TGF-β. Further studies are in progress to address these questions in the FIV infection model.

A number of recent studies have revealed that a variety of microbial pathogens including bacteria, viruses, fungi, and intracellular parasites are capable of activating $CD4^+CD25^+$ Treg cells in vivo (20–23). Belkaid et al. (20) reported that $CD4^+CD25^+$ Treg cells specific for *Leishmania* Ag accumulate at the site of infection and suppress the ability of the immune system to completely eliminate the parasite. Removal of the $CD4^+CD25^+$ T cells enhanced the immune response, resulting in complete eradication of the parasite. Similar studies have demonstrated that $CD4^+CD25^+$ Treg cells regulate the magnitude of the Th immune response to *Pneumocystis carini* and *Candida albicans*, which determines the course of infection and development of memory (57). Consistent with our observations, Iwashiro et al. (21) reported that chronic Friend leukemia virus infection increased the number and activity of Treg cells, and these mice lose their ability to reject certain tumor transplants. Moreover, similar to our observations in chronic FIV^+ cats, Treg from chronically Friend leukemia virus-infected mice were immunosuppressive in vitro, capable of inhibiting T cell proliferative response to antigenic stimulation. These two studies add strong support for an activated Treg mechanism to explain retrovirus-induced immunosuppression in the absence of T cell depletion.

As with many studies of this nature, it is difficult to distinguish the relative contributions of loss of $CD4^+$ T cell numbers from loss of $CD4^+$ T cell function to the progressive immunodeficiency that is characteristic of AIDS lentiviruses. Similar to HIV infection, FIV infection is marked by an inverted $CD4^+$:$CD8^+$ ratio that is the result of an early and long-lasting $CD8^+$ lymphocytosis and a progressive $CD4^+$ cell decline. In the case of FIV infection, it is not known whether $CD4^+CD25^+$ Treg cells also decline with disease progression and what effect this might have on Th cell immunopathology. However, cats do not suffer a dramatic loss of $CD4^+$ cells until late in infection (44). Our data show that the immunosuppressive function of $CD4^+CD25^+$ cells from FIV^+ cats is >10-fold that of FIV^- cats, based on E:T ratios. Similarly, LPS/IL-2 stimulation increases the immunosuppressive function of $CD4^+CD25^+$ Treg cells from FIV^- cats by >10-fold. Therefore,

any loss in $CD4^+$ cell numbers would be more than offset by activation of suppressor function in FIV^+ cats.

The activation status of immunosuppressive $CD4^+CD25^+$ in the LN and PBMC of cats infected with the $NCSU_1$ isolate of FIV is consistent with the well-documented acquired immunodeficiency associated with this isolate that occurs before a significant loss of $CD4^+$ T cells. English et al. (44) reported that PBMC from FIV $NCSU_1$-infected cats with asymptomatic or clinical infections are hyporesponsive to Con A stimulation. In addition, Joshi et al. (50) demonstrated that unfractionated PBMC but not $CD4^+CD25^+$-depleted PBMC were unresponsive to Con A stimulation, suggesting that the reported unresponsiveness of PBMC to mitogen stimulation in FIV $NCSU_1$-infected cats could be attributed to activated $CD4^+CD25^+$ Treg cells. Additional evidence of immune dysfunction in FIV $NCSU_1$-infected cats comes from studies with a *Toxoplasma gondii*-FIV coinfection model. Normal cats develop transient chorioretinitis after *T. gondii* challenge that resolves after 3–4 wk. However, as early as 16 wk after infection with FIV $NCSU_1$, FIV^+ cats challenged with *T. gondii* developed severe interstitial pneumonia resulting in 50–75% mortality (58, 59). This suggests that severe $CD4^+$ cell-dependent immune dysfunction develops early after FIV infection, and that factors other than reduction in $CD4^+$ cells may be involved in the immunosuppression, including activated $CD4^+CD25^+$ Treg cells. Similarly, in the case of HIV infection, numerous studies indicate that a $CD4^+$ Th-dependent immune dysfunction develops before a significant decrease in $CD4^+$ cell numbers occurs (12, 44, 60, 61) that may also be attributed to activated Treg cells.

In addition to their potential role in immunosuppression in HIV and FIV infection, $CD4^+CD25^+$ Treg cells may contribute to the long-term virus persistence in these infections in vitro and in vivo. In vitro studies have shown that activated $CD4^+$ cells expressing CD25 can be productively infected with HIV in vitro, whereas highly purified resting $CD4^+CD25^-$ cells were resistant to HIV infection (62). However, the $CD4^+CD25^+$ cells reported to support HIV replication were not identified as Treg cells. Recent studies in our laboratory using the FIV infection model demonstrated that both $CD4^+CD25^+$ and $CD4^+CD25^-$ cells are susceptible to FIV infection. In contrast to $CD4^+CD25^-$ cells, which become latently infected, $CD4^+CD25^+$ cells isolated from FIV^+ cats or infected in vitro harbor a productive FIV infection, yet have functional characteristics of $CD4^+CD25^+$ Treg cells. They are arrested in the G_0/G_1 stage of the cell cycle, do not respond to mitogen stimulation, and are relatively resistant to activation-induced programmed cell death (50). Thus, $CD4^+CD25^+$ Treg cells could potentially provide a long-term, stable reservoir for FIV, as well as HIV, replication. Additional experiments will address the role of cellular transcription factors known to regulate FIV replication in the activation state and function of $CD4^+CD25^-$ and anergic $CD4^+CD25^+$ cells.

To the best of our knowledge, the potential role of immunosuppressive $CD4^+CD25^+$ Treg cells in the pathogenesis of persistent infections associated with severe T cell immunodeficiency has not been investigated. Our data support the speculation that chronically activated $CD4^+CD25^+$ Treg cells could suppress $CD4^+$ Th responses to FIV Ags and contribute to the AIDS that is characteristic of this infection.

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