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***In vivo* depletion of CD4⁺CD25⁺ regulatory T cells in cats**

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Abstract

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

1. Introduction

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

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CD4⁺CD25⁺ Tregs were recently described in the cat and were demonstrated to be chronically activated in feline immunodeficiency virus (FIV)-positive cats (Vahlenkamp et al., 2004). Analysis of these cells from both normal and FIV-infected cats demonstrated that they have the salient characteristics of CD4⁺ Tregs in humans and rodents, as they constitute about 5-10% of the peripheral T cell population, are arrested in the G0/G1 stage of the cell cycle, do not proliferate in response to mitogen, and are relatively resistant to activation-induced programmed cell death. When activated *in vitro* with LPS, CD4⁺CD25⁺ T cells from uninfected cats are able to suppress the proliferative response of Con A-stimulated CD4⁺CD25⁻ T cells. Interestingly, freshly isolated, unstimulated CD4⁺CD25⁺ T cells from FIV-infected cats significantly inhibit proliferation of Con A-stimulated CD4⁺CD25⁻ T cells, suggesting that these cells are activated *in vivo* as a result of the chronic FIV infection. As activated Tregs are non-antigen specific in their suppressive function, it is possible that these cells could in turn suppress or anergize CD4⁺ T helper cell responses to a variety of antigens including FIV antigen and thereby contribute to the acquired immunodeficiency syndrome (AIDS) that is characteristic of this infection. Similar observations have recently been described in HIV-1 infected people (Aandahl et al., 2004; Weiss et al., 2004). Currently, it is unknown whether Treg-mediated immunosuppression undermines a successful anti-viral T cell response or beneficially limits a destructive cycle of inflammation and viral replication. This question cannot be addressed in human subjects but rather requires a well-characterized animal model such as FIV and a method for depletion of CD4⁺CD25⁺ Tregs.

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

2. Materials and Methods

2.1. Animals

To determine the effect of 9F23 on circulating CD25⁺ T cells and the most effective route of monoclonal antibody (mAb) administration, twenty specific-pathogen-free (SPF) cats purchased from Liberty Labs (Liberty, NY) were divided into four groups of five cats each. At the time of euthanasia cats were about nineteen months old. To determine the extent of CD25⁺ cell depletion in the tissues, eight cats were divided into two groups of four cats each. Data from a separate study conducted by K. Howard provided comparative normal values for lymphoid compartment cell subsets in five additional untreated SPF cats. Cats were housed in the Laboratory Animal Resource Facility at the College of Veterinary Medicine, North Carolina State University in conditions approved of by the Institutional Animal Care and Use Committee. Animals were anesthetized with Telazol[®] administered i.v. and/or i.m. (Fort Dodge Animal Health, Overland, KS) during sample collection and euthanized with sodium pentobarbital administered i.v. (Vortech Pharmaceuticals, Dearborn, MI). As indicated, some cats were immunized two or three times i.p. with 200 µg FIV p24-GST fusion protein (Reid et al., 1991) and 0.5 ml MPL[®] + TDM adjuvant (Sigma-Aldrich, St. Louis, MO) per dose.

2.2. CD25⁺ cell depletion strategy

The hybridoma clone 9F23 produces a mouse mAb against feline CD25 and was generously provided by M. Honda (National Institutes of Health, Tokyo, Japan) (Ohno et al., 1992). Mouse anti-yellow fever antigen (YFA) mAb hybridoma (CRL-1689, ATCC, Manassas, VA) was also obtained to produce an IgG2a kappa light chain antibody as an isotype control. Antibody-

producing hybridomas were grown in serum-free medium (Gibco, Grand Island, NY) and antibodies were protein A purified using endotoxin-free buffers. For some studies, purified antibodies were produced and certified endotoxin-free and mycoplasma-free by Leinco, Inc. (St. Louis, MO). To determine the best dosing strategy, two groups of cats received 3 mg/kg of mouse anti-feline CD25 or the anti-YFA mAb i.v. on days 0, 3, and 7. The third group of cats received 9 mg/kg of anti-CD25 mAb i.p. in one dose on day 0. Sham-treated cats in the fourth group received PBS i.v. on days 0, 3, and 7. Thereafter all cats received 9 mg/kg of anti-CD25 mAb i.p. in one dose on day 0.

2.3. Sample collection and processing

Whole blood was collected via jugular venipuncture into Vacutainer™ tubes (Becton-Dickinson, Franklin Lakes, NJ) containing ACD on days 0, 3, 7, 9, 11, 14, 21, 28, and 35 after treatment. Centrifugation of blood over Histopaque (Sigma-Aldrich, St. Louis, MO) density gradient at 400xg for 30 min at 25°C yielded isolated PBMCs. Cells were washed once in 1x PBS and once in PBMC medium (RPMI 1640 medium supplemented with 15% FBS, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 55 µM β-mercaptoethanol) before resuspension in PBMC medium for cell counting by trypan blue dye exclusion. Serum for enzyme-linked immunosorbent assays (ELISA) was collected in Vacutainer™ tubes without additive, centrifuged at 1300xg for 10 min at 25°C and aliquots were frozen at -80°C. Plasma for complete blood counts and leukocyte differentials was collected in Vacutainer™ tubes containing EDTA. Complete blood counts were performed using a VetScan HMT hematology system (Abaxis, Union City, CA). Peripheral LN biopsies were performed on anesthetized cats and one popliteal LN was surgically excised per time point. Sectioned LN were disassociated over a #40 wire mesh screen and cell suspensions were centrifuged at 400xg for 10 min at 25°C. Pellets were washed twice in PBMC medium and filtered through 150 µm nylon mesh (Sefar, Freibach, Austria) before counting single-cell suspensions. Viability was routinely >90%. At necropsy, mesenteric, retropharyngeal, and medial iliac lymph nodes as well as spleen, thymus, and distal small intestine were harvested and processed as previously described (Dean and Pedersen, 1998; Howard et al., 2005). Cell aliquots were frozen in RLT buffer (Qiagen, Valencia, CA) supplemented with 1% β-mercaptoethanol for subsequent RNA isolation with the RNeasy Mini Kit (Qiagen).

2.4. Antibodies

Mouse anti-feline pan T cell mAb (1.572) (Tompkins et al., 1990) and mouse anti-feline CD25 (9F23) were conjugated to FITC using standard protocols. Mouse anti-feline CD4 (30A) and mouse anti-feline CD8α (3.357) (Tompkins et al., 1990) were conjugated to FITC using standard protocols, to PE using Alexa Fluor® 647 mAb labeling kit (Invitrogen, Eugene, OR), or to biotin using Molecular Probes™ Mini-biotin-XX labeling kit (Invitrogen). Streptavidin-PerCP (BD Pharmingen, San Diego, CA) or Streptavidin-APC (Southern Biotech, Birmingham, AL) were used as secondary fluorochromes. Mouse anti-feline CD4-PE (3-4F4, Southern Biotech, Birmingham, AL), mouse anti-canine CD21 (CA2-1D6, Serotec, Raleigh, NC), and goat anti-mouse IgG2a-FITC (R19-15, BD Pharmingen, San Diego, CA) were also used in flow cytometric analysis.

2.5. Flow cytometric staining and analysis

At least 5×10^5 freshly isolated cells were stained for phenotypic analysis in 1x PBS and incubated with directly conjugated antibodies for 15 min at 4°C. Cells were washed 2 times with cold PBS and centrifuged at 600xg for 5 min at 10°C. This procedure was repeated as necessary for complete staining with streptavidin-APC or streptavidin-PerCP. To determine whether CD25+ cells were bound by 9F23 in vivo, PBMC were incubated with goat anti-mouse IgG2a-FITC only. To determine the total number of CD25+ cells, cells were first incubated

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

2.6. FOXP3 real-time RT-PCR

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

2.7. ELISAs

For the FIV p24 antibody ELISA, Immulon-2HB plates (Dynex Technologies, Chantilly, VA) were coated with 1.0 μ g/ml p24-GST fusion protein and the assay was performed as previously described (Staats et al., 1996). For the mouse antibody ELISA, Maxisorp plates (Nunc, Rochester, NY) were coated with 5 μ g/ml mouse anti-feline CD25 in bicarbonate coating buffer (0.5 M NaHCO₃, pH 8.6) at 4°C overnight. Plates were blocked with 200 μ l/well blocking buffer (0.5 M NaHCO₃, 5% nonfat dry milk, pH 8.6) for two hours at room temperature. Serum samples were diluted in ELISA sample diluent (5% nonfat dry milk, 5% goat serum, 0.05% Tween 20, and 0.1% Kathon in 1x PBS) and added to blocked plates at 100 μ l/well. Plates were incubated for two hours at 37°C and then washed four times with wash buffer (0.5% Tween 20 in 1x PBS). Antibody was identified with goat anti-cat IgG-horseradish peroxidase (HRP) (Bethyl Labs, Montgomery, TX) diluted 1:20,000 and incubated for one hour at room temperature. Plates were washed four times with a 5 min incubation between the third and fourth wash before the addition of tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD) for 10 min. The reaction was stopped with 1 N H₂SO₄ before reading the optical density at 450 nm on a Tecan Sunrise absorbance reader (Zurich, Switzerland). A threefold higher optical density of tested sample over preinoculation samples determined positive antibody titers. Differences in post-treatment endpoint titers were determined by t-test using a 0.05 level of significance (SAS, Cary, NC).

2.8. Anti-FIV p24-GST T cell proliferation

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

2.9. IFA for Anti-nuclear antibody (ANA)

Day 21 serum samples from five cats treated by i.p. anti-CD25 administration were evaluated for anti-nuclear antibody activity. Positive control serum was obtained from the NCSU-CVM clinical immunology laboratory. The control and experimental serum samples were stored at -80°C. Ten-fold serial dilutions (1:20 to 1:200) of control serum and each sample were prepared using PBS as a diluent. Ten µl of 1:20 dilution from the control and from each sample was placed on ANA Hep-2 substrate slides® (Immuno Concepts, Sacramento, CA). Slides were incubated at 37°C for fifteen minutes. The slides were then washed three times with PBS. FITC conjugated anti-feline IgG (Cappel, West Chester, PA) was then applied to each sample and incubated at 37°C for fifteen minutes. The slides were again washed three times with PBS. Slides were mounted with a coverslip and evaluated on a Zeiss IM 35 fluorescence microscope (Jena, Germany). All five samples were negative (less than 1:20) for ANA activity.

2.10. Coculture suppression assay

Mesenteric and retropharyngeal LN from cats were stained with anti-feline CD4-PE (3-4F4) and anti-feline CD25-FITC (9F23). CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were purified on a MoFlo Cell Sorter (Dako, Fort Collins, CO). Target CD4⁺CD25⁻ cells were washed in 1xPBS and stained with 1.25 µM CFSE for 5 min in the dark. Target cells were added to a 96-well round-bottom plate at 5×10⁴ cells/well and were incubated alone or with suppressor cells. Autologous CD4⁺CD25⁺ cells were added to CD4⁺CD25⁻ cells to yield suppressor:target cell ratios from 0.016:1 to 0.5:1, or were incubated alone. Cells were incubated for 4 days at 37°C with or without 2 µg/ml ConA, then washed in 1xPBS containing 10% FBS and analyzed on an LSR II flow cytometer with FACSDiva software. Suppression of proliferation was calculated as (% proliferation of CD4⁺CD25⁻ cells alone - % proliferation of CD4⁺CD25⁻ cells in coculture) / % proliferation of CD4⁺CD25⁻ cells alone.

3. Results

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

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

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

In addition to high expression levels on regulatory T cells, CD25 is also expressed on activated T and B cells. Therefore, we evaluated the effect of CD25⁺ cell depletion on CD8⁺CD25⁺ and

CD21⁺CD25⁺ cells as well as CD4⁺CD25⁺ cells. Indeed, all three populations were depleted in peripheral blood by treatment with 9F23. Figure 2 shows the kinetics of depletion and rebound of CD4⁺, CD8⁺, and B cell populations expressing CD25 in cats treated intraperitoneally. Just as depletion of the CD4⁺CD25⁺ population was mirrored by a corresponding decrease in total CD4⁺ T cells, total CD8⁺ and CD21⁺ absolute cell number was reduced surrounding the nadir of depletion in the group receiving 9F23 intraperitoneally (Fig. 1C and 1D). B cells and CD8⁺ T cells rebounded to pre-treatment levels by day 14 and exceed pre-treatment numbers at day 21. This is presumably due to the antigenicity of the mouse antibody and the ensuing activation of responding B cells and CD8⁺ T cells.

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

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

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

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

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

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

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

CD25⁺ cells were depleted (Table 2). IgG titers to FIV p24-GST were also monitored and showed minimal fluctuation after Treg depletion as compared to titers before treatment with antibody (Fig. 6).

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

In order to characterize the rebound of CD4⁺CD25⁺ T cells in secondary lymphoid organs by day 35 after depletion, CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated from mesenteric and retropharyngeal LN by FACS and cocultured in a functional suppression assay. Because of the small number of CD4⁺CD25⁺ T cells in lymph nodes on day 11 after depletion, no attempt was made to isolate this cell subset at this time point. CD4⁺CD25⁺ Tregs were cultured in a two-fold series of 1:2 to 1:64 ratios with CFSE-stained CD4⁺CD25⁻ cells. CD4⁺CD25⁺ cells remained stained with low levels of residual anti-CD25-FITC from FACS isolation and were not included in the proliferating population (Fig. 7A). CD4⁺CD25⁺ cells displayed a Treg functional phenotype by inhibiting CD4⁺CD25⁻ ConA-induced proliferation at a suppressor:target cell ratio as low as 0.31:1 (Fig. 7B).

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

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

4. Discussion

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

Administration of a single dose of anti-CD25 monoclonal antibody (9F23) by the intraperitoneal route consistently resulted in the depletion of CD4⁺CD25⁺ Tregs in blood and tissues. Clone 9F23 is of the IgG2a isotype, and can potentially support antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. We believe that depletion of CD4⁺CD25⁺ cells occurred after binding by 9F23 since CD4⁺ absolute cell numbers declined concurrent with the nadir of CD4⁺CD25⁺ cell numbers (Fig. 1A and 1B). Furthermore, relative FOXP3 mRNA levels also declined in tissues as CD4⁺CD25⁺ cells were depleted (Fig. 5). The reduction in circulating CD4⁺CD25⁺ cells was most pronounced at day 11 post-depletion (82%) in the blood and remained significantly low until day 28. CD4⁺CD25⁺ cells in lymphoid inductive sites and the thymus were also significantly diminished by day 11, later rebounding by day 35. In lymphoid effector sites CD4⁺CD25⁺ cells were minimally reduced on day 11 but significantly decreased at day 35. The reasons for the unique depletion kinetics in this compartment are not clear. Perhaps this is related to differential distribution of the anti-CD25 antibody to tissues initially and the subsequent trafficking of Tregs thereafter. Although poorly understood, it is clear that there are tissue-specific mechanisms for Treg migration and retention (Annacker et al., 2005; Wei et al., 2006).

There are two concerns regarding the use of anti-CD25 monoclonal antibodies to deplete Tregs. First, not all Tregs are CD25⁺, however, we have shown in the cat that, similar to human and mouse, the majority of FOXP3⁺ cells (>70%) are also CD25⁺ (unpublished observations). Currently, FOXP3 expression is the most specific marker for Tregs (Banham et al., 2006).

However, there are no means to selectively deplete FOXP3⁺ cells due to the intranuclear localization of this transcription factor. Given the limitations of in vivo antibody-depletion strategies, CD25 remains the best target available to eliminate Tregs.

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

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

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

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

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

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Abbreviations

Treg	regulatory T cell
FIV	feline immunodeficiency virus
ADCC	antibody-dependent cellular cytotoxicity
CDC	complement-dependent cytotoxicity
SPF	specific-pathogen-free
mAb	monoclonal antibody
YFA	yellow fever antigen
CFSE	carboxyfluorescein succinimidyl ester
ANA	anti-nuclear antibody
ConA	concanavalin A
PBMC	peripheral blood mononuclear cell
LN	lymph node

IEL

intraepithelial lymphocytes

LPL

lamina propria lymphocytes

Fig. 1.

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

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

Fig. 3. Anti-mouse antibody response precedes rebound in CD25⁺ cells. The absolute number of CD25⁺ cells (closed squares) and 9F23-coated cells (closed triangles) are shown relative to the primary Y-axis for cats receiving 9F23 by i.p. route. The log₂ anti-mouse antibody titer (closed diamonds) is shown relative to the secondary Y-axis. Bars are SEM.

Fig. 4.

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

Fig. 5.

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

Fig. 6. Maintenance of anti-FIV p24-GST IgG titers after administration of 9F23. Cats were immunized against FIV p24-GST and treated with 9F23 i.p. at day 0. FIV p24-GST feline IgG ELISAs were performed on serum samples. * indicates $P < 0.05$ by t-test compared to Day 0. Bars are SEM.

Fig. 7. Suppression of CD25⁻ T cell proliferation by CD25⁺ peripheral lymph node T cells. FACS purified CD4⁺CD25⁻ T cells from the mesenteric and retropharyngeal lymph nodes of four cats sacrificed 35 days after 9F23 mAb i.p. administration were labeled with CFSE and cocultured with FACS purified autologous CD4⁺CD25⁺ T cells in the presence of ConA (2 µg/ml) for four days. CD4⁺CD25⁺ T cells were also labeled with CFSE and incubated alone in the presence of ConA for four days. Histograms of T cell proliferation from representative cocultures of one mesenteric lymph node are shown (A). The top left and top middle histograms show proliferation of ConA-stimulated CD4⁺CD25⁻ and CD4⁺CD25⁺ cells, respectively. The top right histogram demonstrates residual FITC present on FACS purified CD4⁺CD25⁺ cells.

The middle and bottom rows of histograms show the level of proliferation of ConA-stimulated CD4⁺CD25⁻ cells cocultured with decreasing numbers of CD4⁺CD25⁺ cells (Treg:Teff). The percent of cells that proliferated are indicated on each histogram. Suppression of proliferation was calculated as (% proliferation of CD4⁺CD25⁻ cells alone - % proliferation of CD4⁺CD25⁻ cells in coculture) / % proliferation of CD4⁺CD25⁻ cells alone (B). Bars are SEM.

Table 1

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

	Forward	Reverse	Probe
FOXP3	GCCTGCCACCTGGAATCAAC ^a	GTGTGCTGGGGCTTGGGA	CAGTGCTGGCTCCCTGGACACCCA ^b
HPRT	TTACGCTGAGGATTTGGAAAAGGT	GCCACCCATCTCCTTCAACAC	CTCGGGCAAGACGCTCGGTCCTGTC

^a All sequences listed 5'- to -3'.^b Probes labeled at 5'- with 6-FAM™ and -3' with Black Hole Quencher™

Table 2

Maintenance of anti-FIV p24-GST T cell proliferation after administration of 9F23. Cats were immunized against FIV p24-GST and treated with 9F23 i.p. at day 0. PBMCs stained with CFSE were incubated for four days with FIV p24-GST. Subsequent flow cytometric evaluation of the dilution of CFSE in replicating cells provided percentage dividing cells out of total lymphocyte population. Positive responders were defined as >3% p24-GST-specific proliferating cells over background proliferation as determined by media controls.

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