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Use of FoxP3 expression to identify regulatory T cells in healthy dogs and dogs with cancer

B.J. Biller^a, R.E. Elmslie^c, R.C. Burnett^a, A.C. Avery^a, S.W. Dow^{a,b,*}

^a Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, United States

^b Department of Clinical Sciences, Colorado State University, Fort Collins, CO 80523, United States

^c 3550 S Jason Street, Englewood, CO 80110, United States

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Abstract

Regulatory T cells (Treg) are a distinct group of T lymphocytes with immunosuppressive properties that serve normally to prevent harmful autoimmune responses. However, Tregs can also interfere with beneficial immune responses such as anti-tumor and anti-viral immunity in humans and rodents. Given the overall importance of Tregs, it is likely that they play an important role in diseases of dogs as well. However, at present reagents required for identification of Tregs in dogs are not available. Therefore, we investigated whether expression of FoxP3, a transcription factor that is highly expressed in Tregs in humans and rodents could also be used to identify Tregs in dogs. We found that a cross-reactive FoxP3 antibody identified a subset of CD4⁺ T cells in blood and lymph nodes of dogs. By flow cytometry the mean percentage of FoxP3⁺CD4⁺ T cells in normal dogs was 4.3% in blood and 9.8% in the lymph nodes. In dogs with cancer, there was a significant increase in numbers of Treg in blood (7.5%) and tumor-draining lymph nodes (17.1%) compared to age-matched healthy control dogs. We also found that FoxP3⁺CD4⁺ T cells in dogs could be significantly expanded *in vitro* by TCR activation together with addition of TGF- β and IL-2. Treated cells also significantly increased expression of TGF- β and IL-10 mRNA. We conclude from these studies that a cross-reactive FoxP3 antibody can be used to identify Tregs in dogs and that this reagent may serve as a useful tool for investigating the role of Treg in a variety of diseases of dogs.

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1. Introduction

Regulatory T cells are a distinct lineage of T lymphocytes that comprise approximately 5–10% of all CD4⁺ T cells in rodents, cats and humans (Itoh et al., 1999; Shevach, 2002; Vahlenkamp et al., 2004). Several subsets of Treg have recently been described, including

naturally occurring, thymic-derived Treg and those induced by exposure to antigen and activated in the periphery (Beyer and Schultze, 2006; Levings et al., 2001). When stimulated through their TCR, endogenous CD4⁺ Treg function to directly suppress the proliferation of harmful self-reactive T cells in a cell-contact dependent, cytokine-independent manner (Sakaguchi, 2000; Shevach, 2000). In contrast, antigen-induced Treg typically regulate immune homeostasis via production of cytokines such as IL-10 and TGF- β (Hara et al., 2001; Nakamura et al., 2001). Despite functional and phenotypic differences between Treg subsets, the Treg population as a whole plays a

* Corresponding author at: Department of Clinical Sciences, Colorado State University, Ft. Collins, CO 80523, United States. Tel.: +1 970 221 4535; fax: +1 970 297 1275.

E-mail address: sdow@colostate.edu (S.W. Dow).

critical role in the prevention of autoimmune disease and maintenance of peripheral tolerance.

In mice and humans Treg can be tentatively identified based on surface expression of high levels of the IL-2-receptor- α chain (CD25). Other cell surface molecules such as glucocorticoid-induced TNF-receptor (GITR), CD103, and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have also been shown to identify a population of Treg (Dieckmann et al., 2001; Jonuleit et al., 2001). In humans, CD4⁺ T cells expressing low levels of CD127 have also been found recently to be highly enriched for functional Treg (Liu et al., 2006). However, it is often difficult to distinguish true Treg from activated CD4⁺ non-regulatory T cells using CD25 and other cell surface molecule expression, inasmuch as expression of these markers is also upregulated upon activation (Baecher-Allan and Anderson, 2006).

Recently, however, intracellular detection of the transcription factor FoxP3 has been shown to uniquely identify a highly enriched Treg population in rodents (Fontenot et al., 2003; Fontenot and Rudensky, 2005; Ramsdell, 2003). Although FoxP3 expression is also highly enriched within the human CD4⁺CD25^{high} population and is currently the most specific Treg marker known, several investigators have demonstrated induction of FoxP3 expression in activated CD4⁺ T cells that do not possess suppressive activity (Allan et al., 2005; Morgan et al., 2005; Walker et al., 2003). Despite these potential species-specific differences in the regulation of FoxP3 expression, identification of CD4⁺FoxP3⁺ T cells has greatly facilitated the study of Treg. Treg have also been identified in cats using an antibody specific for feline CD25 (Vahlenkamp et al., 2004).

Inhibition of the development of effective anti-tumor immunity by Treg is well-documented in humans and mice with cancer (Baecher-Allan and Anderson, 2006; Beyer and Schultze, 2006). A number of clinical studies have demonstrated increased numbers of Treg in humans with a diverse range of malignancies such as melanoma, ovarian carcinoma, hepatocellular carcinoma and squamous cell carcinoma of the head and neck (Curiel et al., 2004; Ormandy et al., 2005; Schaefer et al., 2005; Viguier et al., 2004). Increased numbers of Treg, especially within tumor tissues and in tumor-draining lymph nodes, is a strong negative prognostic factor for some types of cancer. In some cases, the number of Treg predict survival and response to treatment better than traditional prognostic factors such as stage and grade of tumor (Alvaro et al., 2005; Curiel et al., 2004; Ichihara et al., 2003; Wolf et al., 2005).

At present, there are no available antibodies to surface determinants such as CD25 in dogs that can be used to identify Tregs. Therefore, we conducted studies to determine whether a cross reactive anti-murine FoxP3 antibody could be used to identify Treg in dogs. We found that the FoxP3 antibody specifically recognized intracellular FoxP3 in canine CD4⁺ T cells. In functional studies, we also found that FoxP3 expression was markedly upregulated in canine CD4⁺ T cells by stimuli known to elicit expansion of human Treg in vitro. Our results suggest that FoxP3 expression occurs predominantly within the Treg population of CD4⁺ T cells in dogs. Therefore, FoxP3 expression may serve as a useful marker for identification and quantitation of Tregs in dogs. Our preliminary data also suggest that Treg may play a role in dogs with cancer.

2. Materials and methods

2.1. Study population and sample collection

For analysis of Treg in normal dogs, blood and lymph node aspirates were collected from 10 healthy control dogs. For analysis of Treg in dogs with cancer, blood and lymph node aspirates were collected from patients ($n = 10$) at the Colorado State University Veterinary Teaching Hospital and from Veterinary Cancer Specialists in Englewood, CO. These studies were approved by Animal Care and Use Committees in place at both participating institutions.

Approximately 4 ml of anticoagulated whole blood was obtained via jugular venipuncture and PBMC were separated by Ficoll density centrifugation. Lymph node aspirates were obtained by fine needle aspiration and placed in complete tissue culture medium at 4 °C prior to analysis by flow cytometry. For control dogs, lymph node samples were obtained from the sub-mandibular lymph nodes. In dogs with cancer, where possible, lymph node aspirates were obtained from the nearest lymph node draining the tumor. In addition, a second lymph node aspirate was obtained from a non-tumor draining lymph node, usually the sub-mandibular lymph node.

2.2. Flow cytometric analysis

PBMC or cells obtained by lymph node aspiration were added at 5×10^5 and 1×10^6 per well in 96-well round bottom plates (Costar, Corning Corporation, Acton, MA) and then immunostained for surface expression of CD4 and CD8, using appropriate

concentrations of FITC-conjugated anti-dog CD4 (clone YKIX302.9; Serotec, Raleigh, NC) and PE-conjugated anti-dog CD8 (clone YCATE55.9, Serotec), as described previously (Walter et al., 2006). The staining was done at 4 °C for 30 min, with antibodies suspended in FACS buffer (PBS with 2% FBS and 0.1% sodium azide).

After washing to remove unbound antibodies, intracellular detection of FoxP3 was performed using a cross-reactive, directly conjugated murine FoxP3 antibody (PE conjugated, anti-mouse/rat FoxP3, clone FJK-16s, eBioscience, San Diego, CA) and the permeabilization and fix/perm buffers that accompany the antibody (FoxP3 Staining Set, eBioscience). A directly conjugated rat IgG2a antibody (PE) was used as the isotype control (eBioscience). A series of experiments (data not shown) were done to determine optimal conditions for intracellular FoxP3 staining in which a range of FoxP3 antibody concentrations, as well as various incubation times and temperatures, were evaluated. Specifically, we found that optimal intracellular staining was achieved when the cells were resuspended in fix/perm buffer overnight at 4 °C following surface staining. The next day, cells were washed twice with permeabilization buffer and incubated with anti-mouse/rat FoxP3 antibody (1 µg per 1×10^6 cells) diluted in FACS buffer for 30 min at 4 °C. Following two additional washes in permeabilization buffer, the cells were resuspended in FACS buffer and kept at 4 °C prior to analysis.

Flow cytometric analysis was done using a CyAn MLE flow cytometer (Dako-Cytomation, Ft. Collins, CO) and analysis was done using Summit software (Dako-Cytomation). Analysis gates were set on the live lymphocyte population based on typical forward and side scatter characteristics (Faldyna et al., 2001). For lymphocytes obtained from PBMC, the percentage of Treg was calculated based on the percentage of FoxP3⁺CD4⁺ cells within the overall CD4⁺ T cell population. Absolute Treg numbers in peripheral blood were calculated based on the total lymphocyte count determined on the same day by automated cell counter (Advia, Bayer Diagnostics, Tarrytown, NY).

2.3. Quantitation of canine Foxp3 by real-time PCR

Primers for FoxP3 detection were designed based on the canine FoxP3 sequence, using Primer3 software (Rozen and Skaletsky, 1998). The forward primer sequence was 5'-AAACAGCACATTCCCAGAGTTC-3' (sense) and the reverse primer sequence was 5'-AGGATGGCCCAGCGGATCAG-3' (antisense). The

sequences of the primer pair for the house keeping gene hypoxanthine phosphoribosyltransferase (HPRT) were 5'-TGCTGCATTCCTGAAGTCTTTAT-3' (sense) and 5'-TCACAAGTCAAACAACAATCCAC-3' (antisense). Primers were purchased from Invitrogen (Carlsbad, CA.) and were resuspended in distilled water and stored at -20 °C. For quantitative real-time PCR (RT-PCR) analysis, RNA was extracted from PBMC or lymph node aspirates (approximately $1-3 \times 10^6$ total cells) using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First strand cDNA was generated using a kit containing M-MLV Reverse Transcriptase and First-Strand buffers (Invitrogen). For each PCR reaction, 3 µl of cDNA template was added to iQ SYBR Supermix (Bio-Rad, Hercules, CA) containing the primer pair for either FoxP3 or HPRT. Amplification was carried out in 40 µl volumes using the iQ iCycler system (Bio-Rad). Negative control reactions were performed using either mock RNA or water. All amplification reactions were performed in duplicate, and the average threshold cycle (C_t) numbers of the duplicates were used to calculate the relative mRNA expression of FoxP3 normalized to HPRT expression in each sample. Normalized FoxP3 expression was then compared between control and treated samples from multiple in vitro experiments.

2.4. Quantitation of canine TGF-β and IL-10 by quantitative real-time PCR

For quantitation of canine IL-10 and TGF-β mRNA, primer sequences were obtained from published sources (Nuttall et al., 2002). For IL-10, the primer sequences were 5'-GTCCCTGCTGGAGGACTTTAAGA-3' (sense) and 5'-TGGTTCGGCTCTCCTACATCTCG-3' (antisense). For TGF-β, the primer sequences were 5'-AGTTAAAAGCGGAGCAGCATGTGG-3' (sense) and 5'-GATCCTTGCGGAAGTCAATGTAGAGC-3' (antisense). RNA extraction, cDNA preparation and RT-PCR were done as described above.

2.5. In vitro expansion and induction of Treg

Canine lymphocytes were activated and cultured in IL-2 and TGF-β, using protocols previously described for in vitro expansion of human and murine Treg (Chen et al., 2003; Fu et al., 2004; Levings et al., 2001). For these experiments, PBMC were obtained from blood collected from three healthy dogs. For each dog, PBMC were cultured at 2×10^6 /ml in complete medium in 24-well plates (Costar). Tissue culture medium consisted of Modified Eagle's medium (Gibco-Invitrogen, San

Diego, CA) supplemented with nonessential and essential amino acids (Gibco) with penicillin and streptomycin (Sigma–Aldrich, St. Louis, MO) and 10% FBS (Gemini Bioproducts, West Sacramento, CA). Some cultures were placed in complete medium supplemented with recombinant human TGF- β and IL-2 (Peprotech, Rocky Hill, NJ) with or without the addition of ConA (Sigma–Aldrich) as a T cell activation stimulus. These culture conditions have been shown previously in experiments with murine T cells to favor the *in vitro* induction of Treg (Chen et al., 2003; Fu et al., 2004). The following culture conditions tested were evaluated: Condition 1—cells cultured in complete tissue culture medium only; Condition 2—addition of 2 ng/ml rhTGF- β and 100 U/ml rhIL-2; Condition 3—addition of 5 μ g/ml ConA only; Condition 4—addition of 2 ng/ml rhTGF- β , 100 U/ml rhIL-2 and 5 μ g/ml ConA. The cultures were harvested on day 5 and the samples were split for flow cytometric analysis of FoxP3 expression and quantitative RT-PCR analysis of canine FoxP3, TGF- β and IL-10 mRNA expression.

2.6. Statistical analyses

Differences between two groups were compared using the Mann–Whitney *U*-test, which assumes non-Gaussian distribution of data. Comparisons between multiple groups with small sample sizes were done using one-way analysis of variance for non-parametric data (Kruskal–Wallis test), followed by Dunn’s multiple comparisons test. Comparisons between multiple groups with larger sample sizes were done by ANOVA with Tukey’s multiple means comparison. Statistical analyses were done using Prism software (GraphPad, San Diego, CA). For all comparisons, a *p*-value ≤ 0.05 was considered significant.

3. Results

3.1. Identification of canine Treg by intracellular expression of FoxP3

Given the critical role of the FoxP3 transcription factor in the development and function of regulatory T cells and the fact that the FoxP3 sequence is highly conserved between diverse species, we first investigated whether a cross-reactive murine FoxP3 antibody could detect the FoxP3 protein within CD4⁺ canine lymphocytes (Fontenot and Rudensky, 2005; Yagi et al., 2004; Ziegler, 2006). To address this question, staining protocols for FoxP3 detection were optimized and

blood and lymph node aspirates from normal dogs and dogs with cancer were evaluated for CD4 and FoxP3 expression. In Fig. 1, representative flow cytometry results for FoxP3 expression in CD4⁺ lymphocytes from a dog with cancer are shown. The specificity of FoxP3 staining was assessed by staining with an irrelevant isotype-matched antibody and also by assessing FoxP3 expression by non-CD4⁺ T cells. Although staining for FoxP3 was consistently highest in CD4⁺ T cells, there was some detectable FoxP3 expression in non-CD4⁺ lymphocytes (see Fig. 1). Further analysis revealed that the majority of the CD4⁺FoxP3⁺ population was CD8⁺ (data not shown). This finding does not necessarily indicate non-specific FoxP3 staining, as CD8⁺FoxP3⁺ Treg have also been described in humans and in mice (Hori et al., 2003; Khattry et al., 2003; Ziegler, 2006). Thus, these experiments indicated that the anti-murine FoxP3 antibody was cross-reactive with a protein expressed primarily in a subset of CD4⁺ T cells of dogs.

3.2. *In vitro* expansion of dog CD4⁺FoxP3⁺ Treg using T cell activation plus TGF- β and IL-2

The preceding experiments indicated that FoxP3 expression could be detected specifically in dog CD4⁺ T cells, but did not address the issue of whether these cells behaved functionally as Treg. For example, Treg in

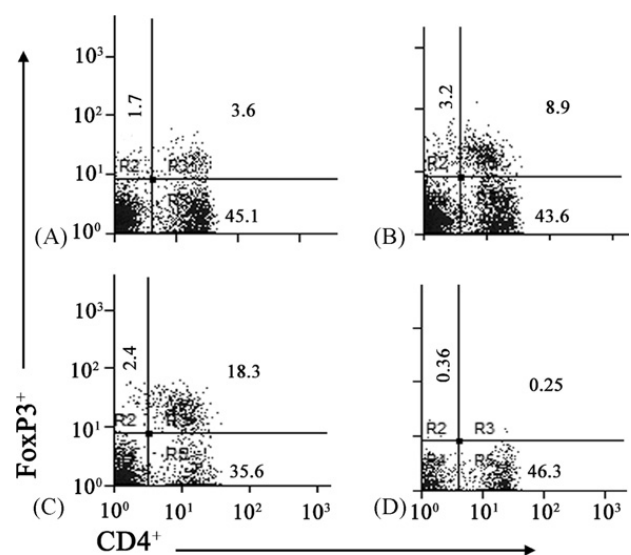


Fig. 1. Identification of canine Tregs using FoxP3 antibody. Lymphocytes were obtained from blood and lymph nodes of a dog with oral melanoma and immunostained for expression of FoxP3 and CD4, as described in Section 2. Representative dot plots of FoxP3 expression by CD4⁺ T cells from (A) blood; (B) an uninvolved lymph node; (C) tumor-draining lymph node are shown. In (D), staining of blood from the same dog with an irrelevant isotype-matched antibody (control for FoxP3 staining) is shown. The percentages of lymphocytes contained within each quadrant are given.

mice, cats, and humans can be enriched by cell sorting and added to cultures of activated T cells, where they exert a strong inhibitory effect on proliferation and cytokine production. However, in the case of dogs, the absence of any available antibodies for detection of surface expression of CD25, GITR, CTLA-4, CD127, or CD103 make it nearly impossible to sort cells and do these types of add-back experiments.

Therefore, we took a more indirect approach to demonstrating that FoxP3 expressing CD4⁺ T cells in dogs behaved functionally as Treg. In mice and humans, Treg can be expanded in vitro from activated PBMC following addition of exogenous TGF- β and IL-2 (Bacchetta et al., 2002; Chen et al., 2003; Fu et al., 2004). In mice, FoxP3 mRNA expression in activated T cells increases within 12 h after cytokine addition and is maximal after about 96 h of culture (Fu et al., 2004). Therefore, we set up in vitro cultures of dog PBMC under conditions that have been shown previously to induce expansion of Treg in mice and humans. For these cultures, we evaluated FoxP3 expression by flow cytometry and also FoxP3, TGF- β , and IL-10 mRNA expression by quantitative RT-PCR.

We found that culture of activated T cells in TGF- β and IL-2 led to a 2 to 3-fold increase in the percentage of CD4⁺FoxP3⁺ T cells as detected by flow cytometry (Fig. 2). While there was an increase in FoxP3 expression in T cells that were activated only (i.e. exposure to ConA, but not TGF- β or IL-2), the increase in FoxP3⁺ cells in cultures incubated with ConA plus TGF- β and IL-2 was significantly greater ($p = 0.01$, Fig. 2C).

We also observed a 45 to 250-fold increase in mRNA levels for FoxP3 in cultures containing ConA, IL-2 and TGF- β (Table 1). The increased expression of FoxP3 mRNA in these cultures was also accompanied by significant increases in mRNA expression for TGF- β (2 to 4-fold increase) and IL-10 (7 to 14-fold increase). Although increased expression of FoxP3 mRNA was detected in cultures containing ConA alone, the increase was significantly less than for cultures

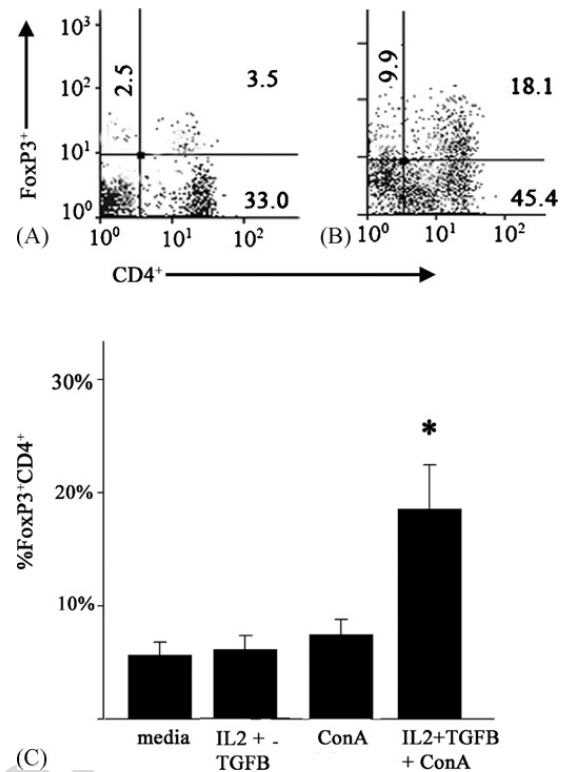


Fig. 2. In vitro expansion of FoxP3⁺CD4⁺ T cells by T cell activation and culture in IL-2 and TGF- β . PBMC were collected from normal dogs and placed in culture in complete medium for 5 days, with or without the indicated supplements. After 5 days in culture, the cells were collected and immunostained for FoxP3 and CD4 expression and for quantitation of FoxP3 mRNA expression. Cells were cultured in medium alone, with IL-2 and TGF- β alone, with ConA alone, or with ConA plus IL-2 and TGF- β , as described in Section 2. Representative dot plots for flow cytometric analysis of cells cultured in medium alone (A) or medium plus ConA and IL-2 and TGF- β (B) are shown. In (C), the mean percentage (\pm S.E.) of FoxP3⁺CD4⁺ lymphocytes present in cultures maintained under the indicated conditions was calculated and plotted. Culture in ConA plus IL-2 and TGF- β resulted in a significant increase ($p = 0.01$) in the percentage of FoxP3⁺CD4⁺ T cells, compared to other culture conditions, as determined by ANOVA. The data represent the pooled results of five independent experiments (asterisk (*) denotes significant difference relative to the other three treatment groups).

containing ConA plus IL-2 and TGF- β . Also, as expected from the results of rodent and human studies, cytokine addition alone without TCR activation did not result in significant increases in FoxP3 (protein or

Table 1

Effect of in vitro activation and culture in IL-2 and TGF- β on expression of FoxP3, TGF- β , and IL-10 mRNA in normal canine PBMC

Culture condition	FoxP3 ^a	TGF- β ^a	IL-10 ^a
Media only	–	–	–
IL-2 + TGF- β	9.5 (\pm 6.1)	3.1 (\pm 1.2)	1.6 (\pm 0.75)
ConA	11.7 (\pm 8.3)	2.6 (\pm 2.1)	1.9 (\pm 0.9)
ConA + IL-2 + TGF- β	147 (\pm 102) [*] ($p < 0.01$)	7.2 (\pm 2.0) [*] ($p < 0.05$)	14.0 (\pm 7.0) [*] ($p < 0.01$)

Asterisk (*) denotes significant difference in mean fold increase in expression, compared to the other three treatment groups, as determined by ANOVA.

^a Mean (\pm S.E.) fold increase in mRNA expression of FoxP3, TGF- β , or IL-10, as compared to media control.

Table 2

Treg populations in blood and lymph nodes of healthy dogs and dogs with various forms of cancer

Patient population	Number of dogs	%Treg in peripheral blood ^a	%Treg in non-draining LN ^a	%Treg in draining LN ^a
Healthy dogs	10	4.3 (±0.7)	9.8 (±2.4)	ND
Oral melanoma	4	11.1 (±2.1)	12.0 (±0.5)	19.5 (±8.0)
Osteosarcoma	3	7.2 (±1.9)	10.8 (±0.5)	ND
Mast cell tumor	2	6.3	10.0	15.5
Soft tissue sarcoma	1	5.5	11.6	16.4

^a Mean (±S.E.) percentage of FoxP3⁺CD4⁺ T cells (Tregs); ND = not done.

mRNA) or IL-10 or TGF- β mRNA expression (Table 1). These data indicated therefore that culture of canine T cells under conditions known to promote the in vitro generation of Treg in human T cells stimulates a significant increase in the number of canine FoxP3⁺CD4⁺ T cells. This treatment was also associated with significant upregulation of expression of TGF- β and IL-10 mRNA. These results provide support therefore for our assertion that FoxP3 expression is associated with the Treg phenotype in dogs as well as in humans and rodents.

3.3. Dogs with cancer have increased Treg in their peripheral blood and tumor-draining lymph nodes

To assess the biological usefulness of Treg identification using the FoxP3 antibody, we evaluated Treg numbers in dogs with cancer. We chose this population of dogs to study because previous studies in mice and humans have shown that Treg numbers are often elevated in humans with cancer (Baecher-Allan and Anderson, 2006; Beyer and Schultze, 2006). For this analysis, 10 dogs with several different common cancers of dogs were investigated and their results compared to those obtained from 10 age-matched, healthy control dogs (Table 2). The percentages and numbers of FoxP3⁺CD4⁺ Tregs in lymph nodes and peripheral blood were determined using flow cytometry.

We found that the percentage of Treg and the absolute number of Treg were significantly higher in the peripheral blood of dogs with cancer than healthy dogs (Fig. 3). For example, the mean percentage of Treg in peripheral blood in all 10 dogs with cancer was 7.5%, compared to 4.3% in the 10 normal dogs. The absolute numbers of Treg in peripheral blood of dogs with cancer were also significantly increased (mean of 173 cells per μ l blood in dogs with cancer versus 98 cells per μ l blood in control dogs, $p < 0.04$). These results are consistent with previous observations in humans and mice and provide further evidence for the specificity of using FoxP3 expression to identify canine Treg.

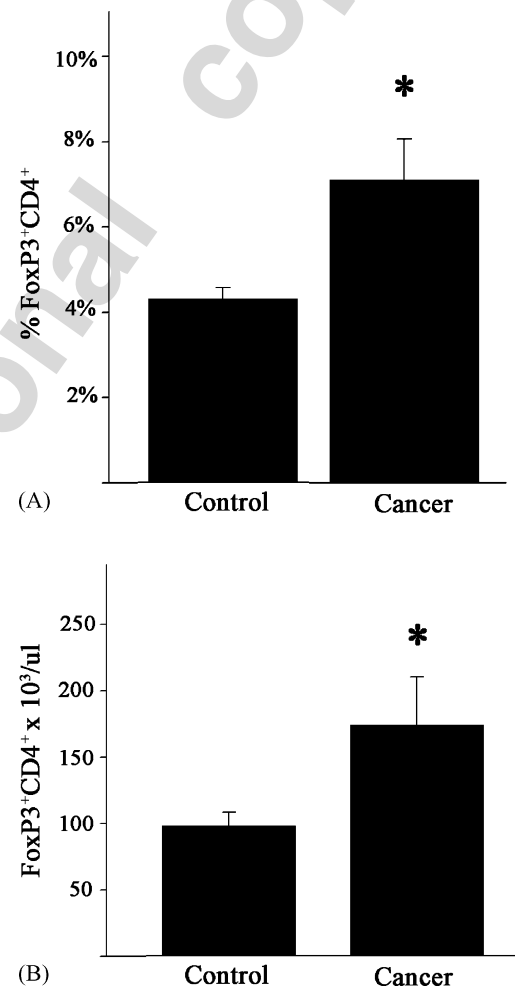


Fig. 3. Relative and absolute numbers of Treg in peripheral blood in dogs with cancer and healthy control dogs. Blood was collected from 10 healthy, age-matched dogs and from 10 dogs with cancer (see Table 2) and immunostained for enumeration of Treg, using FoxP3 and CD4 expression and flow cytometric analysis, as described in Section 2. The mean percentage (±S.E.) of Treg in blood of normal dogs and dogs with cancer was plotted in (A) and the mean of the absolute numbers (±S.E.) of Treg was plotted in (B). The percentage of Treg in blood was significantly increased in dogs with cancer compared to healthy control dogs (4.3% vs. 7.1%; $p = 0.0005$), as assessed by the Mann–Whitney U -test. The absolute numbers of Treg in blood were also significantly higher (98×10^3 vs. $173 \times 10^3/\mu$ l; $p = 0.04$) in dogs with cancer, compared to healthy control dogs (asterisk (*) denotes significantly different relative to control dogs).

Previous studies had found higher numbers of Treg in lymphoid tissues of humans and cats than in blood (Liyanage et al., 2002; Vahlenkamp et al., 2004). Therefore, Treg populations in lymph node aspirates of dogs were compared to blood Treg populations. We found that in both normal dogs and dogs with cancer the percentages of Treg were significantly higher in lymph nodes than in blood ($p < 0.001$) (Figs. 3 and 4, and Table 2).

Next, we assessed whether lymph nodes draining tumors had more Tregs than lymph nodes not draining the primary tumor (non-tumor draining lymph nodes) in dogs with cancer. The percentage of Tregs in the tumor draining lymph nodes of seven dogs with cancer (excluding three dogs with osteosarcoma where a tumor draining lymph node was no longer present due to limb amputation) was significantly higher ($p = 0.04$) than the percentage of Treg in the non-tumor draining lymph nodes of the same dogs (Fig. 4). However, we found that the percentage of Tregs in the non-tumor draining

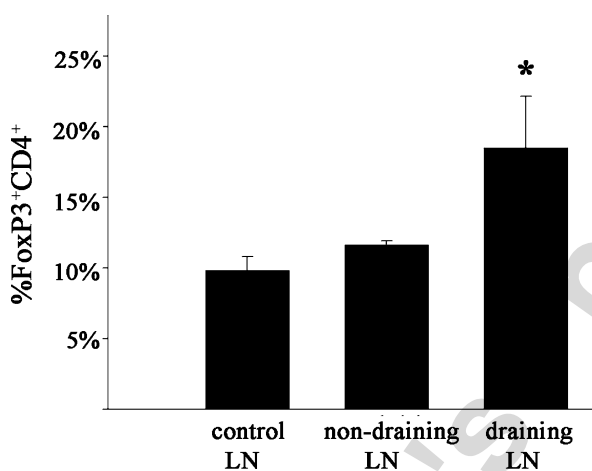


Fig. 4. FoxP3⁺CD4⁺ T cells in lymph nodes of dogs with cancer and healthy dogs. The percentage of FoxP3⁺CD4⁺ T cells (Tregs) was determined in lymph node aspirates from 10 normal dogs and 10 dogs with cancer, using flow cytometry, as described in Section 2. In dogs with cancer, lymph node aspirates were obtained from the tumor-draining lymph node (seven dogs, excluding dogs with osteosarcoma, Table 2) and from a lymph node not draining the primary tumor (10 dogs). The mean percentages (\pm S.E.) of FoxP3⁺CD4⁺ T cells in lymph nodes from normal dogs (normal LN) and in lymph nodes not associated with the primary tumor in dogs with cancer (non-draining LN) and in lymph nodes draining the primary tumor in dogs with cancer (draining LN) were compared. The mean percentage of FoxP3⁺CD4⁺ T cells was significantly higher ($p = 0.03$) in tumor-draining lymph nodes of dogs with cancer than in the non-draining lymph nodes from the same dogs, as assessed by Mann–Whitney *U*-test. In addition, the mean percentage of FoxP3⁺CD4⁺ T cells was significantly higher in tumor-draining lymph nodes of dogs with cancer than in the lymph nodes of healthy control dogs ($p = 0.01$) (asterisk (*) denotes significant difference relative to control LN and relative to non-tumor draining LN).

lymph nodes of dogs with cancer (11.1%) was not significantly different from the percentage of Tregs in lymph nodes of healthy control dogs (9.8%; see Fig. 4 and Table 2). Thus, it appears that Treg preferentially accumulate within the tumor draining lymph nodes of dogs with cancer, as has been reported previously for humans with metastatic melanoma (Viguier et al., 2004). These results also suggest the possibility that the major source of increased Tregs in the blood of dogs with cancer may be the Tregs that accumulate in the tumor draining lymph nodes.

Finally, we also conducted a preliminary assessment of whether particular tumor types might be associated with higher numbers of Tregs. For this analysis, we compared dogs with melanoma ($n = 4$) and dogs with osteosarcoma ($n = 3$) to healthy control dogs ($n = 10$). We found that dogs with melanoma had significantly ($p < 0.01$) more Tregs in their blood (11.1% versus 4.3%) than control dogs, as assessed by Kruskal–Wallis test and Dunn's multiple means comparison (Fig. 5). When Tregs in aspirates from the same lymph node (sub-mandibular lymph nodes) of all three groups of dogs were compared, we also found that dogs with melanoma had significantly ($p < 0.05$) more Tregs (19.5%) than both control dogs (9.8%) and dogs with osteosarcoma (10.8%). However, it should be noted that the sub-mandibular lymph node was the tumor draining lymph node for dogs with oral melanoma. Therefore, we also compared the percentages of Tregs in non-tumor draining lymph nodes for all three groups of dogs (Fig. 4). In this case, we did not observe any significant

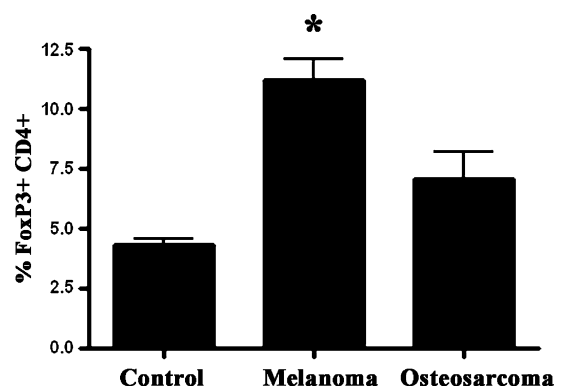


Fig. 5. Comparison of Treg percentages in blood of dogs with melanoma or osteosarcoma and in healthy control dogs. The percentage of FoxP3⁺CD4⁺ T cells (Treg) in blood of healthy control dogs ($n = 10$), dogs with oral melanoma ($n = 4$), and dogs with osteosarcoma ($n = 3$) was determined by flow cytometry, as described in Section 2. The mean percentage (\pm S.E.) of FoxP3⁺CD4⁺ T cells was plotted for each population of dogs. Dogs with melanoma had significantly more Tregs than control dogs ($p < 0.01$), as assessed by the Kruskal–Wallis test (asterisk (*) denotes significant differences relative to control dogs).

differences ($p = 0.20$) in Tregs in lymph nodes between the three groups of dogs. These results suggest therefore that certain cancers may be associated with higher numbers of Tregs in the blood and lymph nodes of dogs. Moreover, the results also indicate that the most informative sites to sample for evaluation of Treg numbers in dogs with cancer are the blood and the tumor draining lymph nodes.

4. Discussion

The major findings to emerge from these studies were that a cross reactive FoxP3 antibody can also be used to detect dog FoxP3 and to identify canine Treg *in vivo*. We found that Treg numbers were significantly higher in peripheral blood of dogs with cancer and that Treg may preferentially accumulate within the tumor-draining lymph nodes of dogs with cancer. In addition, it appears that certain types of tumors in dogs, especially highly malignant tumors, may be associated with higher numbers of Tregs.

In mice, FoxP3 is specifically expressed by most Treg and can be used to distinguish CD4⁺CD25⁺ Treg cells from CD4⁺CD25⁺ non-regulatory T cells. These non-regulatory T cells upregulate CD25 expression, but not FoxP3 expression, upon activation (Fontenot et al., 2003; Hori et al., 2003). Conflicting reports exist, however, concerning the specificity of human FoxP3 expression for identification of Tregs. For example, Yagi et al. demonstrated that TCR stimulation of naïve human CD4⁺CD25⁻ T cells failed to elicit FoxP3 expression and that FoxP3 expression was limited to T cells with a regulatory phenotype (Yagi et al., 2004). Other investigators however have shown that FoxP3 expression is increased in activated non-regulatory T cells (Morgan et al., 2005; Walker et al., 2003). Because of these discrepancies, evaluation of human Treg currently relies on analysis of expression of multiple Treg-associated cell surface markers, including CD25, GITR, and CTLA-4, in addition to assessment of intracellular FoxP3 expression. A recent study also suggests that evaluation of CD127 expression in conjunction with CD4 and CD25 expression may be even more specific than previous markers (Liu et al., 2006). The lack of antibody reagents for assessing expression of these molecules in dogs currently precludes Treg analysis using multiple surface markers. Therefore, at present assessment of FoxP3 expression may be the best available method of enumerating Treg in dogs.

The definition of Treg also depends in part on demonstration of typical immunosuppressive functions.

For example, studies of feline CD4⁺CD25⁺ Treg have shown that *in vitro* activation of purified Treg with LPS and rhIL-2 induced strong suppressive activity against ConA-stimulated CD4⁺CD25⁻ T cells (Vahlenkamp et al., 2004). The lack of available antibodies to surface determinants such as CD25 on dog T cells however prevented us from performing these types of experiments. (Levings et al., 2001; Thornton and Shevach, 1998). Therefore, as an alternative we investigated whether Treg expansion could be elicited *in vitro* by stimuli known to expand the numbers of Treg in human T cells. For example, in humans and mice naturally occurring CD4⁺CD25⁺FoxP3⁺ Treg can be expanded *in vitro* from PBMC when cultured in the presence of IL-2 and TCR stimulation (Levings et al., 2001; Thornton and Shevach, 1998). Signalling through the high affinity IL-2 receptor CD25 has been recently shown to be critical to Treg growth and differentiation (Almeida et al., 2002; Antony and Restifo, 2005). Addition of TGF- β to activated T cells may also be used to further expand Treg through induction of FoxP3 expression (Chen et al., 2003; Fu et al., 2004). In our studies with dog lymphocytes, we observed an increase in FoxP3 mRNA and protein expression in activated lymphocytes cultured in IL-2 and TGF- β . Similar to observations described by Morgan and others, we found that TCR activation alone induced small increases in FoxP3 expression by CD4⁺ as well as by some CD4-T cells (Allan et al., 2005; Morgan et al., 2005; Walker et al., 2003). However, the expression was significantly greater when the activated T cells were also cultured in IL-2 and TGF- β (Fig. 2). Thus, in contrast to murine Treg, our findings suggest that the regulation of FoxP3 expression in dog Treg is more similar to the regulation of FoxP3 expression in human Treg. Our assertion that FoxP3 expression occurs predominantly within the canine Treg population is further strengthened by finding concurrent significant increases in IL-10 and TGF- β mRNA expression in appropriately activated cells, a result not expected if activation were to lead to the development of cells without a Treg phenotype.

The availability of a diagnostic reagent to reliably identify Treg in dogs should greatly facilitate future investigations into the role of Treg in important diseases of dogs, including autoimmune diseases and chronic infections and cancer. The observation that the dogs with cancer in this study had increased numbers of Treg in their peripheral blood and tumor-draining lymph nodes compared to healthy dogs is remarkably similar to findings for human cancer patients. Our preliminary observations suggesting that more malignant tumors may be associated with higher numbers of Tregs also

indicates that evaluation of Treg numbers may have important prognostic value. We anticipate that further investigations into the role of Treg in various malignancies will offer important insights into immunological control of cancer. These studies may well lead to newer tools for assessing the immunopathogenesis of cancer in dogs and manipulating the immune system to control cancer.

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