THESIS

REGULATORY T CELLS IN CANINE CANCER

Submitted by

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ABSTRACT

REGULATORY T CELLS IN CANINE CANCER

Numbers of regulatory T cells (Treg) are increased in some human malignancies and are often negatively correlated with patient disease-free interval and survival. Canine Treg have previously been identified in healthy and cancer-bearing dogs and have found to be increased in the blood of dogs with some types of cancer. Moreover, some preliminary research indicates that increased numbers of Treg and decreased numbers of $CD8^+$ T cells in the blood of dogs with osteosarcoma (OSA) are associated with decreased survival. The aim of this project was to examine Treg distributions in dogs with cancer compared to normal dogs and to determine any association between Treg numbers and patient outcome. Additionally, we sought to determine if treatment with daily low-dose (metronomic) chemotherapy would decrease Treg in dogs with cancer.

Pre-treatment Treg populations in blood, tumors, and lymph nodes of dogs with OSA were assessed and compared with Treg in blood and lymph nodes of a healthy, control population of dogs. No significant changes in Treg numbers in the blood or lymph nodes of the OSA-bearing dogs compared to the control population were identified. The dogs with OSA with treated with amputation of the affected limb followed by adjunctive chemotherapy. Treg numbers in the blood or tumor were not associated

with outcome but an elevated ratio of $CD8^+$ T cells to Treg in the tumor was associated with a prolonged disease-free interval and increased survival. These findings suggest that changes in number of Treg and effector T cell subset may provide prognostic information for canine OSA.

In mice and humans with advanced cancer, the administration of metronomic (daily low dose) cyclophosphamide (CYC) selectively decreases circulating Treg numbers and inhibits their function. Protocols containing metronomic CYC likely have anti-tumor properties in dogs as well. Despite wide use of metronomic CYC in veterinary medicine, its effects on canine Treg have not previously been reported. Dogs with soft tissue sarcoma (STS) were administered CYC at 12.5 mg/m^2 or 15 mg/m^2 orally once daily for 28 days. Whole blood samples and tumor biopsies were obtained on days 0, 14, and 28 to assess changes in T lymphocyte subsets, circulating endothelial cells, and tumor microvessel density (MVD). Administration of CYC at 12.5 mg/m^2/day significantly decreased the number of Treg from day 0 to 28, but there was no change in the percentage of Treg or tumor MVD. In dogs that received CYC at 15.0 mg/m^2/day, both the number and percent of Treg as well as tumor MVD were significantly decreased over 28 days. These findings suggest that metronomic dosing of CYC may have immunomodulatory and antiangiogenic effects in dogs with cancer.

Taken together the work described in this thesis support the theory that Treg are altered in dogs with cancer and that these changes may have prognostic value. Additionally, Treg can be decreased in dogs with cancer though administration of metronomic doses of CYC; the therapeutic benefit of Treg depletion has yet to be evaluated in cancer bearing dogs.

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CHAPTER 1

Literature Review and Project Rationale

Overview of regulatory T cells

The existence of immune cells with suppressive activity was first proposed in 1971 but the concept of regulatory T cells (Treg), as they came to be known, was not fully accepted until decades later with the identification of a population of $CD4^+$ T cells that highly expressed CD25 and prevented autoimmunity in mice.[1,2] Further research has elucidated that natural Treg arise from the thymus and function to maintain tolerance to self-antigens, thereby preventing autoimmune diseases.[3] Natural Tregs are thought to exert their inhibitory effects via direct cell to cell contact in a cytokine independent fashion.[4] Treg can be induced in the periphery secondary to increased amounts of the cytokine IL-2; this population is known as adaptive Treg. Adaptive Treg are frequently up regulated secondary to inflammatory processes such as infection and neoplasia, and there is increasing evidence that this increase in Treg with cancer may play a role in inhibition of anti-tumor immunity. [4]

Treg were initially identified as $CD4^+$ T cells that also highly express CD25, the alpha chain of the IL-2 receptor.[2] These surface markers were found to be specific for Treg in mice living in pathogen-free conditions, but $CD4^+CD25^+$ T cells were

determined to be non-specific for human Treg. CD25 is an early marker of T cell activation and up to 30% of $CD4^+$ cells in the peripheral blood may express low levels of CD25 due to continuous antigenic exposure.[5] Treg were determined to have higher CD25 expression as compared to the low expression of CD25 by transiently activated T cells but more specific means of Treg detection were needed.[5] The intracellular transcription factor, forkhead box P3 (FoxP3), was identified as a highly specific marker of murine Treg and subsequent work supports that FoxP3 is up regulated in human Treg as well.[6,7] These $CD4^+CD25^+FoxP3^+$ Treg constitute about 5-10% of $CD4^+$ T cells in the peripheral blood of humans and defining characteristics include existence in an anergic state and active inhibition of effectors T cells, dendritic cells, NK cells, and B cells via cell-to-cell contact and in a dose dependent manner.[8] There is some recent evidence that a population of $CD8^+FoxP3^+$ Treg may exist as well; discussion here will focus on $CD4^+CD25^+FoxP3^+$ Treg as this populations of cells has been more widely researched.[9]

Regulatory T cells and Cancer

There is great interest in the role that Treg may play in inhibition of anti-tumor immune responses. Treg exert their immunosuppressive effects primarily through the secretion of transforming growth factor- β (TGF-β) and interleukin-10 (IL-10). There are several inhibitory mechanisms of IL-10, including inhibition of cytotoxic T cell responses, down regulation of activated macrophages, and inhibition of dendritic cells; TGF-β inhibits $CD8^+$ T cell cytotoxic activity as well as suppresses NK cell anti-tumor responses.[10-14] This suppression of both the innate and adaptive immune responses

against tumor cells likely impacts the response to treatment and overall prognosis of cancer patients.

Treg are increased in the peripheral blood and lymph nodes and within tumors for a number of human malignancies, including ovarian carcinoma, gastric and esophageal cancer, pancreatic and breast cancer, colorectal cancer, melanoma, and hepatocellular carcinoma.[15-20] More importantly, increased numbers of Treg have been negatively associated with prognosis for several of these malignancies. This was first identified in ovarian carcinoma where increased Treg infiltration within the tumor is associated with decreased survival.[21] Since that time, similar inverse correlations with tumor recurrence and/or patient survival have been identified for Treg in the tumor of non-small cell lung cancer (NSCLC), blood of patients with hepatocellular carcinoma, and the in tumor draining lymph nodes in breast cancer patients.[15,22,23] Increases in Treg are often associated with changes in other effector T cell populations, namely decreases in $CD8^+$ T cells; alterations in these populations may also be expressed as ratios of $CD8^+$ T cells to Treg. Increased ratios of intratumoral CD8/Treg have similarly been associated with improved patient outcome for several malignancies.[24,25] While increased numbers of Treg are most frequently negative predictors of outcome in human cancer, this relationship does not always hold true. There are several studies which have shown no association between Treg and patient outcome, as well as several where there is a positive correlation between Treg and patient survival.[26-28]

Evidence that increased numbers of Treg may play a role in patient outcome in human malignancies has led to the investigation of the role of Treg in canine cancer. Until recently, antibodies against canine CD25 were not available and therefore, canine

Treg were defined as CD4+FoxP3+ T cells for much of this research.[29] $CD4^+$ $FoxP3^+$ Treg have been identified in normal and cancer-bearing dogs, and dogs with melanoma, osteosarcoma (OSA), and lymphoma have a significantly greater numbers of Treg in circulation than healthy dogs.[29-32] Additionally, these canine $CD4^+FoxP3^+$ cells have increased expression of IL-10 and TGF-β mRNA, supporting their immunosuppressive function.[29] Studies examining the prognostic implication of Treg are limited but dogs with OSA that have a high pre-treatment CD8/Treg ratio in the blood have a longer survival than dogs with a low CD8/Treg ratio.[31] Distribution of Treg and effector T cells in blood, lymph nodes, and tumors should be investigated further for different types of canine cancer and may give further insight into alterations of the immune system that occur with neoplastic processes.

As Treg generally correlate with poor patient survival, the next question becomes whether selective depletion of Treg may improve response to therapy and patient outcome. Some of the approaches that have been investigated to date include use of deniluekin diftitox, anti-CD25 antibody (PC61), and low dose chemotherapy. Denileukin diftitox is an IL-2-diptheria toxin fusion protein that binds to cells that express CD25 and is subsequently taken up into the cell where it inhibits protein synthesis resulting in cell death.[33] Anti-CD25 antibody specifically binds to the IL-2 receptor alpha chain on T cells and has been shown to decrease tumor growth and improve survival in murine models of cancer.[34] Finally, metronomic chemotherapy, defined as the frequent administration of low doses of antineoplastic drugs, has been investigated as means to decrease Tregs. While it has been utilized most frequently for its antiangiogeneic properties, there is accumulating evidence that metronomic chemotherapy also depletes Treg.

Metronomic Chemotherapy and Regulatory T cells

Chemotherapy has traditionally been administered to patients at doses that result in direct cytotoxicity to tumor cells; this manner of dosing cytotoxic agents is referred to as maximally tolerated dose (MTD) chemotherapy. MTD chemotherapy inhibits or kills rapidly dividing tumor cells, but it also can damage cells that turn over rapidly under normal homeostatic conditions resulting in significant adverse effects to the patient. Because of normal tissue toxicity, prolonged breaks are needed between doses of chemotherapy to allow for the recovery of these tissues, namely the gastrointestinal tract and bone marrow. There is some thought that these prolonged breaks may also allow the tumor cells to recover as well, thereby potentially reducing the efficacy of cytotoxic chemotherapy.[35]

In contrast, metronomic chemotherapy involves the administration of chemotherapy drugs at doses much lower than MTD without prolonged breaks between treatments and is generally not associated with severe systemic toxicities. Rather than causing direct cytotoxicity to tumor cells, metronomic chemotherapy has been demonstrated to inhibit tumor angiogenesis.[36] Angiogenesis is a process critical to tumor growth and progression; inhibition of this process has been theorized to improve tumor control.[37] Proposed mechanisms of antiangiogenesis induced by metronomic chemotherapy include targeting the drug-sensitive endothelial cell compartment of tumors, inhibiting mobilization of proangiogenic circulating endothelial cells from the bone marrow, and induction of the endogenous angiogenesis inhibitor thrombospondin.[38-40]

While the antiangiogenic properties of metronomic chemotherapy are well studied, there is accumulating evidence that the antitumor effects of metronomic chemotherapy may be also due to immunomodulation in part through Treg depletion. The association of Treg depletion and improved tumor immunogenicity in a murine model was first reported in 1999 by Shimizu et al.[41] Subsequent work in mice has demonstrated that metronomic dosing of cyclophosphamide (CYC), an alkylating nitrogen mustard compound, not only decreases numbers of Treg but also inhibits their immunosuppressive function.[42,43] Studies in human malignancies are limited but support earlier findings that metronomic CYC decreases numbers of Treg and restores effector T cell function.[44,45]

Delay of tumor growth and tumor regression secondary to metronomic CYC administration has been demonstrated in a number of rodent xenograft models.[46,47] Additionally, metronomic CYC may improve the efficacy of other treatment modalities, such as other antiangiogenic drugs, MTD chemotherapy, and immunotherapy.[48-51] Studies examining the effects metronomic chemotherapy in human cancers are limited to several small studies. In these studies, metronomic chemotherapy is generally combined with other agents such as steroids, non-steroidal anti-inflammatory drugs (NSAID), MTD chemotherapy, and/or immunotherapy; there is some preliminary evidence that these combination protocols may provide some clinical benefit to patients.[52-56]

Although metronomic chemotherapy protocols are widely used in veterinary oncology, studies examining their efficacy against canine cancer are limited. CYC is frequently used in these protocols primarily due to its aforementioned antiangiogenic and immunomodulatory properties. CYC has the advantage of oral administration and is relatively inexpensive. Most protocols consist of drug combinations, and have included a

combination of CYC with a NSAID, or alternating with etoposide.[57,58] While metronomic chemotherapy may have antitumor effects against canine splenic hemangiosarcoma and soft tissue sarcoma, the use of combination therapy precludes determination of the clinical effect of each drug. Additionally, CYC doses have been empirically selected and doses used ranged from 10 to 25 mg/m^2/day. Finally, the underlying mechanisms of tumor inhibition have not been investigated. Clearly, additional research is required to determine the mechanisms of action of metronomic chemotherapy and to determine an optimal dose to be used to treat dogs with cancer.

Concluding remarks

To address some of these gaps in our understanding of the role of Treg in canine cancer, the following chapters describe research performed to further elucidate the prognostic role of Treg in canine cancer as well as examine the effect of metronomic dosing of CYC on Treg. The second chapter evaluates distribution of Treg in blood, lymph nodes, and tumors of OSA-bearing dogs and compares the results to Treg in comparable tissues of healthy, control dogs. Additionally, numbers of Treg in these tissue compartments were examined to determine association with disease-free interval or survival for dogs with OSA. The third chapter investigates the effect of metronomic CYC therapy on circulating Treg in dogs with soft tissue sarcoma (STS). Two biomarkers of antiangiogenic activity, tumor microvessel density (MVD) and circulating endothelial cells, were also examined to determine if metronomic CYC inhibits angiogenesis in canine STS. Together, this research will provide further information regarding alterations

of Treg populations in cancer bearing dogs and assess whether metronomic dosing of CYC decreases Treg. The information gained here will ultimately guide future studies to determine if decreasing Treg in dogs results in anti-tumor activity and improved patient outcome.

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CHAPTER TWO

Alterations in T Cell Subsets in Canine Osteosarcoma and Association with Outcome

INTRODUCTION

The distribution of T lymphocyte subsets, including $CD4^+$ and $CD8^+$ effector T cells and regulatory T cells (Treg), is frequently altered in advanced cancers. These changes occur in blood, lymph nodes, and/or tumors and may include decreases in $CD4^+$ and $CD8^+$ T cells as well increases in Treg.[1-3] Treg are a distinct subset of $CD4^+$ T lymphocytes that function normally to prevent harmful autoimmune responses and have been shown in mouse models to impair anti-tumor immune responses.[4,5] Treg likely also impair anti-tumor immunity in humans, as they are frequently present in high numbers in human cancer patients and have been associated with a less favorable outcome for certain malignancies.[3,6-8]

There is limited information available regarding the role of Treg in cancer of companion animals. Canine Treg have been identified based on surface expression of CD4 and intracellular expression of the transcription factor, FoxP3; these $CD4^+FoxP3^+$ Treg constitute about 5% of $CD4^+$ T cells in healthy dogs.[9,10] In addition, Treg have been tentatively shown to be increased in the peripheral blood of dogs with various types

of carcinoma, oral melanoma, and osteosarcoma (OSA) as compared to healthy control dogs.[10-12] There is currently limited information regarding alterations in T cell subsets and their association with outcome in dogs with cancer.[11]

The purpose of this prospective study was to evaluate T cell subpopulations in the blood, lymph nodes, and tumor of dogs with OSA and investigate their association with clinical outcome. We hypothesized that dogs with OSA would have increased numbers of Treg in their blood and tumor draining lymph nodes as compared to blood and lymph nodes of healthy control dogs. We also hypothesized that OSA dogs with higher numbers of circulating Treg would have a shorter disease free interval (DFI) and overall survival (OS) as compared to dogs with lower numbers of circulating Treg.

MATERIALS AND METHODS

Inclusion Criteria

Dogs presenting to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) with histopathologically confirmed OSA of the appendicular skeleton were eligible for enrollment in this prospective clinical trial. Additionally, dogs with radiographic findings consistent with appendicular OSA but without biopsy confirmation were also eligible for enrollment. Exclusion criteria included presence of pulmonary metastasis at the time of diagnosis or treatment with chemotherapy or radiation therapy within three weeks of study enrollment. Glucocorticoids and non-steroidal anti-inflammatory drugs were allowable prior to enrollment and throughout the duration of the study. Dogs underwent amputation of the affected limb followed by chemotherapy

consisting of either single agent carboplatin, alternating carboplatin and doxorubicin, or single agent doxorubicin. Dogs receiving single agent carboplatin chemotherapy were treated at a dosage of 300 mg/m^2 intravenously (IV) once every three weeks for a total of four treatments. Dogs that received alternating treatments of doxorubicin and carboplatin received chemotherapy once every three weeks for a total of six treatments; three doses of doxorubicin were administered at a dosage of 30 mg/m^2 IV and three doses of carboplatin were administered at dosage of 300 mg/m^2 IV. Dogs receiving single agent doxorubicin were treated at a dosage of 30 mg/m^2 IV once every three weeks for a total of five treatments. This study was approved by the Institutional Animal Care and Use Committee at Colorado State University, and signed informed consent was obtained from all owners prior to study entry.

Sample Collection

For analysis of Treg and effector T cells in normal dogs, blood samples were collected from 22 healthy control dogs. For dogs with OSA, whole blood was collected prior to surgery and again 18 to 24 hours after amputation. During surgery, a small tissue sample was obtained of the tumor and of the regional lymph node. Aspiration of a non-tumor draining lymph node was also performed prior to surgery and again 18 to 24 hours post-operatively. Collection of whole blood and an aspirate of a non-tumor draining lymph node were also collected one week after the first treatment with chemotherapy. Additionally, the pre-treatment T cell populations in the blood and lymph nodes of dogs

with osteosarcoma were compared to a T cells in the blood and lymph nodes of a population of age-matched healthy control dogs.

Sample Preparation

For evaluation of circulating Treg, PBMC were obtained from blood samples collected in EDTA tubes after lysis of red blood cells. PBMC were added at a concentration of approximately 1 x 10^6 cells per well in 96-well round bottom plates and then immunostained for surface expression of CD4 and CD8 using FITC-conjugated anti-canine CD4 mAB (clone YKIX302.9, AbD Serotec, Raleigh, NC) and Alexa 647-conjugated anti-canine CD8 mAB (clone YCATE55.9, AbD Serotec) following the method described previously.[13] Immunostaining for FoxP3 expression was conducted as previously described.[9] Briefly, after washing to remove unbound surface antibodies, intracellular detection of FoxP3 was performed using a cross-reactive, PE-conjugated murine FoxP3 antibody (clone FJK-16s, Mouse FoxP3 staining kit, eBioscience, San Diego, CA). A directly conjugated rat IgG_{2A} antibody was used as the isotype control.

Lymph node aspirates were processed prior to immunostaining by washing with Hank's balanced salt solution containing 2% fetal bovine serum through a 70 µm strainer. Tumor tissues were incubated in one to two milliliters of a 5 mg/ml collagenase solution containing collagenase (C-9891, Sigma, St. Louis, Missouri), DNase I (D4263, Sigma), trypsin inhibitor (T6522, Sigma), and minimal essential medium (11095, Invitrogen, Carlsbad, California) prior to being washed through a 70 µm strainer. The cells collected

from the lymph nodes and tumor were then added to 96-well plates and immunostained as described above.

Flow Cytometric Analysis

Flow cytometry was performed using a CyAn ADP flow cytometer (Dako-cytomation, Fort Collins, CO) and Summit software (Dako-cytomation) for data analysis. Analysis gates were set on the live lymphocyte population based on typical forward and side scatter characteristics.[14] The percentage of Treg was calculated by determining the percentage of $CD4^+FoxP3^+$ cells within the $CD4^+$ T-cell population. The percentages of $CD4^+$ and $CD8^+$ T cells were also determined. Absolute numbers of Treg, $CD4^+$ and $CD8^+$ T cells in peripheral blood were calculated based on the total lymphocyte count determined from a CBC run on an automated cell counter.

Statistical Analysis

Mean percentages and numbers of Treg, effector T cells, and the mean CD8/Treg ratio were compared between normal and OSA-bearing dogs by an unpaired, two-tailed t-test. Mean percentages and numbers of Treg, T effector cells, and the mean CD8/Treg ratio pre- and post-operatively as well as pre- and post-chemotherapy were compared by a paired, two-tailed t-test. Comparisons between multiple groups were done by one-way ANOVA with Tukey's multiple means comparison. Repeated measures ANOVA was used to further asses changes over time. Patients were censored from disease-free interval (DFI) and overall survival (OS) analysis if they were lost to follow-up; all dogs that died

or were euthanized were presumed to be dead of disease. DFI was defined as the time from amputation until the development of metastasis and ST was defined as the time from amputation until death. Median DFI and ST were calculated by the Kaplan-Meier product limit method and compared between groups by log rank analysis. Variables assessed for value as predictors of outcome included percentages and absolute numbers of Treg, $CD4^+$ T cells and $CD8^+$ T cells, the CD8/Treg ratio, and type of postoperative chemotherapy used. Statistical calculations were performed using a commercial software program (GraphPad Prism version 5 for Windows, GraphPad Software, La Jolla, CA). A *P* value of <0.05 was considered statistically significant for all analyses.

RESULTS

Nineteen dogs with histopathologically confirmed or clinically suspected OSA of the appendicular skeleton were enrolled in this clinical trial; all underwent amputation of the affected limb. Fourteen dogs fulfilled the study criteria. Of the remaining five dogs, two did not undergo treatment with adjuvant chemotherapy, two were lost to follow-up shortly after amputation, and one dog did not have histopathologic evidence of OSA. For the dogs that underwent treatment with adjuvant chemotherapy, six dogs received four cycles of carboplatin, seven dogs were treated with three cycles each of alternating carboplatin and doxorubicin for seven dogs, and one dog received five doses of doxorubicin.

Analysis of the pre-treatment T cell populations in blood and lymph nodes of the 18 dogs with histopathologically confirmed OSA were compared with 28 control dogs. The average age of dogs with OSA was 8.5 years (range, 5 to 12 years) and the average

of the control dogs was 7.8 years (range, 2 to 14 years); this difference was not statistically different ($P = 0.32$).

Treg Are Not Increased in Blood or Lymph Nodes of Dogs with OSA

Blood and lymph node samples were collected from control dogs and dogs with OSA and evaluated to determine the percentage of $CD4^+$ and $CD8^+$ T cells and Treg. Absolute numbers of Treg and $CD4^+$ and $CD8^+$ T cells in the blood were determined by the percentage of each cell type multiplied by the total number of lymphocytes as determined from the patient's complete blood count. Representative dot plots for Treg analysis from the blood of a healthy dog and a dog with OSA are shown in Figure 2.1. For this analysis, the percentage of Treg was expressed as the percentage of $CD4^+FoxP3^+$ cells within the overall $CD4^+$ T cell population.

Dogs with OSA did not have significantly different percentages or absolute numbers of Treg in the blood as compared to healthy control dogs (Figure 2.2). The mean percentage of Treg in the blood of dogs with OSA was 5.51 (± 2.13)% versus 4.46 (± 1.41)% in the blood of control dogs ($P = 0.052$). The mean absolute number of Treg in the blood was also not significantly different between dogs with OSA and control dogs (74.5 ± 43.4 cells/µL versus 74.6 ± 46.6 cells/µL, $P = 0.99$).

To assess whether Treg percentages were different in lymph nodes of normal dogs as compared to healthy control dogs, lymph node samples were obtained by fine needle aspiration of any assessable lymph node in control dogs as well as lymph nodes distant from the primary tumor location on dogs with OSA. Treg percentages were determined

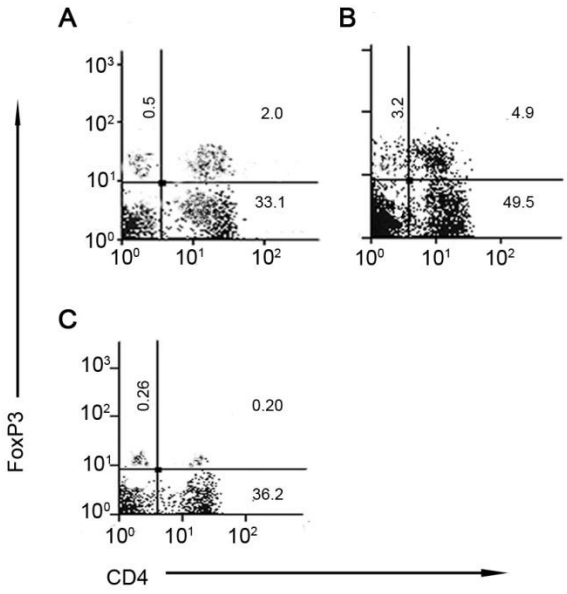

Figure 2.1: Flow cytometric analysis of regulatory T cells (Treg) in blood of dogs. Peripheral blood mononuclear cells from a healthy dog (A) and a dog with osteosarcoma (B) were immunostained for expression of CD4 and FoxP3, as described in "Materials and Methods." (C) Staining of blood form the same dog as in (A) with an irrelevant iso-type control for specificity of FoxP3 staining. The percentages of lymphocytes within each quadrant are given.

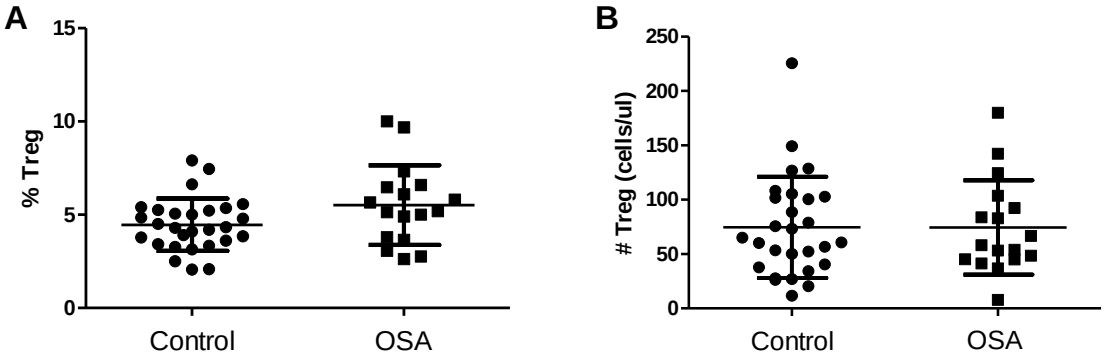

Figure 2.2: Treg in blood are not significantly different in dogs with osteosarcoma (OSA) compared to healthy control dogs. Blood samples of control dogs (n = 28) and dogs with OSA (n = 18) were analyzed with flow cytometry to determine the percentage and absolute numbers of Treg, as described in "Materials and Methods." The mean percentage (long horizontal line) of Treg in the blood of normal dogs and dogs with OSA was plotted in (A) and the mean of the absolute numbers (long horizontal line) was plotted in (B). Error bars show SD. Neither the percentage nor the absolute number of Treg was significantly different in dogs with OSA as compared to healthy control dogs (P = 0.052 and P = 0.99, respectively).

by flow cytometric analysis. The regional, tumor-draining lymph node was also sampled from dogs with OSA at the time of amputation to assess whether Treg were increased in the regional lymph node as compared to a lymph node distant to the tumor site. Treg percentages were similar in the regional lymph node and distant lymph nodes and also similar to Treg percentages found in healthy dogs (Figure 2.3). Thirteen of the 18 dogs with OSA had histopathologic evaluation of the regional lymph node at the time of amputation; two of these 13 dogs had evidence of lymph node metastasis of OSA.

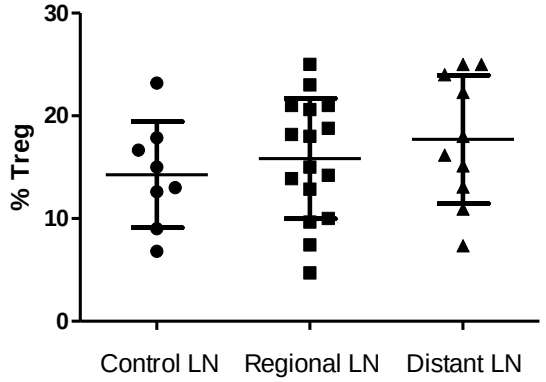

Figure 2.3: Percentages of regulatory T cells (Treg) in regional and distant lymph nodes are similar to those of healthy control dogs. The percentage of Treg was determined in lymph node (LN) aspirates from normal dogs (n = 8) and in LN distant from the primary tumor in dogs with OSA (n = 10). A portion of the LN nearest the tumor (regional LN) was sampled at the time of amputation for dogs with OSA (n = 16). The mean percentages of Treg (long horizontal line) were compared between the control dogs and regional and distant LN of dogs with OSA. Error bars show SD. No significant difference was detected between the three populations (P = 0.46).

The Distribution of T cell Subsets in the Peripheral Blood are Altered in Dogs with OSA

To assess whether dogs with OSA had changes in the distribution of their T cell subsets prior to therapy, the pretreatment percentages and absolute numbers of $CD4^+$ and

$CD8^+$ T cells in the blood of OSA-bearing dogs were compared to healthy control dogs. The percentage of $CD4^+$ T cells was increased in dogs with OSA as compared to the control dogs but absolute numbers of $CD4^+$ cells were not significantly different (Table 2.1). However, both the percentage and absolute number of $CD8^+$ T cells were significantly decreased in dogs with OSA as compared to control dogs (Table 2.1). Despite the decreases the $CD8^+$ T cells in dogs with OSA, there was no significant difference in the total lymphocyte count between the two groups.

Table 2.1: Percentages of T effector cells in blood, lymph nodes, and tumor in healthy control dogs compared to dogs with OSA. DLN, distant lymph node; RLN, regional lymph node.

Parameter	Site	Control Mean (± SD)	OSA Mean (± SD)	P-value
Total lymphocytes (cells/µL)	Blood	1,645 (807)	1,336 (558)	0.162
% $CD4^+$ T cells	**Blood**	**47.6 (10.4)**	**60.9 (14.9)**	**<0.001**
Total # $CD4^+$ T cells (cells/µL)	Blood	752 (339)	792 (372)	0.708
% $CD8^+$ T cells	**Blood**	**22.1 (8.74)**	**15.1 (8.55)**	**0.011**
Total # $CD8^+$ T cells (cells/µL)	**Blood**	**357 (203)**	**223 (169)**	**0.025**
% $CD4^+$ T cells	DLN	38.9 (9.37)	37.6 (17.9)	0.858
% $CD8^+$ T cells	DLN	13.1 (4.47)	13.8 (10.2)	0.857
% $CD4^+$ T cells	RLN	NA	37.4 (13.7)	
% $CD8^+$ T cells	RLN	NA	10.6 (5.42)	
% $CD4^+$ T cells	Tumor	NA	21.8 (16.4)	
% $CD8^+$ T cells	Tumor	NA	9.04 (8.24)	

Treg Percentages are Lowest in Peripheral blood in Dogs with OSA

To examine the distribution of T lymphocytes subsets in different compartments of dogs with OSA, the mean percentages of Treg, $CD4^+$, and $CD8^+$ T cells in regional and distant lymph nodes, peripheral blood, and the tumor of dogs with OSA were compared.

The mean percentage of Treg in the blood was significantly decreased as compared to the mean percentage of Treg in the tumor and regional and distant lymph nodes ($P < 0.0001$; Figure 2.4 C). There was no significant difference in mean percentage of Treg between both lymph node sites and the tumor.

$CD4^+$ T cells were found in greatest percentages in the blood as compared to the tumor and regional and distant lymph nodes in dogs with OSA. The mean percentage of $CD4^+$ T cells in the peripheral blood was 60.9 (± 14.9)% as compared to 37.4 (± 13.7)% in the regional lymph node, 41.6 (± 15.2)% in the distant lymph node, and 21.8 (± 16.4)% in the tumor ($P < 0.0001$). There was no significant difference in the mean percentages of $CD8^+$ T cells in the blood, either lymph node site, or tumor ($P = 0.12$).

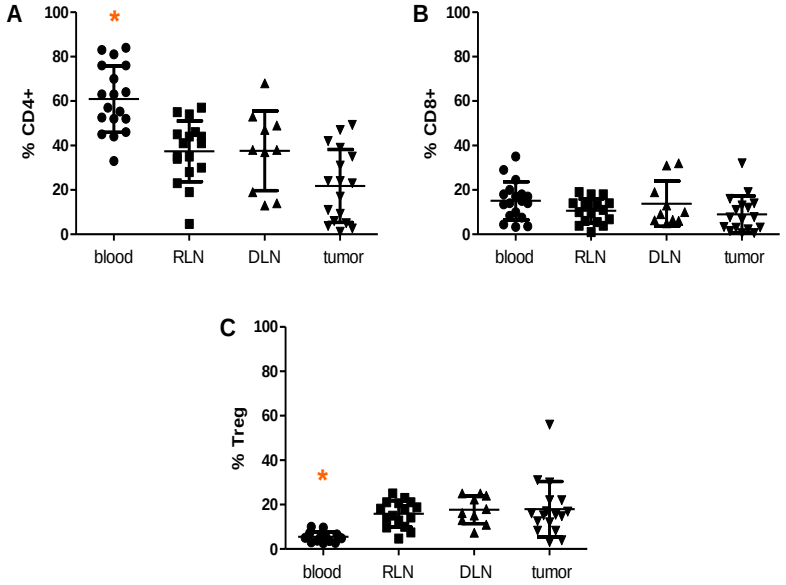

Figure 2.4: Variation in T lymphocyte subpopulations in blood, regional lymph nodes (RLN), distant lymph nodes (DLN), and tumor for dogs with osteosarcoma (OSA). The percentages of $CD4^+$ and $CD8^+$ and Treg were determined in blood, tumor, RLN and DLN for the 18 dogs with OSA. The mean percentages of Treg (long horizontal line) were compared between the tissue sites by one-way ANOVA and Tukey's multiple comparisons post-test. Error bars show SD. (A) There was a significant increase in the percentage of $CD4^+$ T cell in the blood as compared to the RLN, DLN, and tumor ($P < 0.0001$) but no difference was observed in the percentage of $CD8^+$ T cells between the four sites (B). (C) Treg were significantly lower in the blood as compared to the RLN, DLN, and tumor ($P < 0.0001$). *denotes significantly different mean percentage of cells from other tissue sites.

Alterations in T cell subsets of peripheral blood and LN post-operatively and after chemotherapy

To evaluate whether Treg and T effector cells were altered after treatment with surgery or chemotherapy, the mean pre-treatment percentages and absolute numbers of Treg and $CD4^+$ and $CD8^+$ T cells in the blood of dogs with OSA were compared to the post-operative and one week post-chemotherapy means using repeat measures ANOVA and Tukey's multiple comparisons post-test. While there were no significant differences between the mean percentage of pre-treatment, post-operative, and post-chemotherapy $CD4^+$ T cells, the absolute number of $CD4^+$ T cells in the blood was significantly decreased after amputation (Table 2.2). There was no difference in the mean percentage of $CD8^+$ T cells at any time point, but there was a significant increase in the mean absolute number of $CD8^+$ T cells between the post-operative time point and one week post-chemotherapy. A significant increase in the mean percentage and absolute number of Treg occurred between the post-operative and post-chemotherapy time points. Additionally, mean percentages of Treg post-chemotherapy were significantly increased from pre-treatment percentages.

There were insufficient distant lymph node aspirates obtained one week after chemotherapy, therefore only pre- and post-operative mean percentages of $CD4^+$, $CD8^+$, and Treg could be compared. There were no significant differences in the mean percentages of any of the T cell subsets pre- and post-operatively in the lymph node distant from the primary tumor in dogs with OSA (Table 2.2).

Table 2.2: Comparison of mean percentages of T effector cells in blood (n = 11) and distant lymph nodes (DLN; n = 10) in dogs with OSA pre-treatment (Pre-tx), post-operative (Post-op), and post-chemotherapy (Post-chemo).

Parameter	Site	Pre-Tx Mean (± SD)	Post-Op Mean (± SD)	Post-Chemo Mean (± SD)	*P*-value
Total lymphocytes (cells/µL)	**Blood**	**1,191 (511) ***	**655 (408) *†**	**1,353 (621)†**	**0.0008**
% $CD4^+$ T cells	Blood	60.1 (14.3)	54.7 (12.1)	50.1 (15.7)	0.147
Total # $CD4^+$ T cells (cells/µL)	**Blood**	**743 (421)***	**336 (177)***	636 (220)	**0.009**
% $CD8^+$ T cells	Blood	15.1 (9.10)	12.3 (11.1)	21.7 (12.7)	0.105
Total # $CD8^+$ T cells (cells/µL)	**Blood**	215 (178)	**89.9 (114)***	**291 (204)***	**0.046**
% Treg	**Blood**	**5.55 (2.00)***	**5.67 (1.87) †**	**8.52 (3.40) *†**	**0.016**
Total # Treg (cells/µL)	**Blood**	71.3 (41.9)	**38.2 (37.8)***	**98.8 (36.9)***	**0.019**
% $CD4^+$ T cells	DLN	41.6 (15.2)	41.6 (18.2)	NA	0.705
% $CD8^+$ T cells	DLN	11.2 (9.18)	7.76 (5.15)	NA	0.090
% Treg	DLN	16.9 (8.25)	167.0 (8.70)	NA	0.937

Symbols (*,†) indicate means that significantly different based on Tukey's multiple comparison test

Patient Outcome

For the 14 dogs that received adjuvant chemotherapy and outcome was known, the median disease free interval (DFI) was 253 days (range, 39 to 1252 days) and median overall survival (OS) was 348 day (range, 72 to 1252 days). There was no significant difference in either DFI or OS between dogs that received doxorubicin based chemotherapy and dogs that received single-agent carboplatin chemotherapy ($P = 0.91$ and 0.46, respectively).

Both elevated numbers of Treg and low CD8/Treg ratios have been found to be negatively associated with prognosis in human cancer patients.[6,15,16] For this reason, the relationship of T cell subpopulations as well as CD8/Treg ratio to DFI and OS were

investigated. The median percentage of Treg and the mean CD8/Treg ratio were calculated for blood, regional lymph node, and tumor. Additionally, the median absolute number of Treg was calculated for the blood as described previously in this section. Kaplan-Meier product limit analysis for DFI and OS were then performed by assigning outcome based on whether dogs were above or below the Treg and CD8/Treg ratio median values for each tissue compartment. There was no significant difference in outcome based percent Treg, absolute number of Treg, or CD8/Treg ratio in the blood or regional lymph node. However, dogs with high CD8/Treg ratios in the tumor had a statistically increased DFI of 822 days as compared to dogs with low CD8/Treg ratios with a DFI of 205 days ($P = 0.04$). The median OS was not reached for dogs with a high tumor CD8/Treg ratio compared to dogs with a low CD8/Treg ratio which had a median OS of 205 days (P = 0.02).

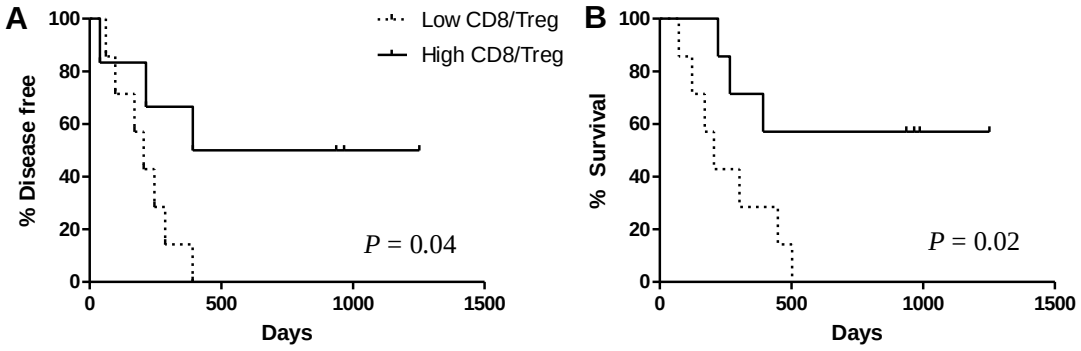

Figure 2.5: High CD8/regulatory T cell (Treg) ratio in the tumor is correlated with prolonged disease free interval (DFI) and overall survival (OS) in dogs with osteosarcoma (OSA). Kaplan-Meier graph depicting DFI (A) and OS (B) in dogs with OSA with a tumor CD8/Treg ratio equal to or higher (n = 6) or lower (n = 7) than the median value of 0.35.

DISCUSSION

Contrary to our hypothesis, Treg were not significantly increased in dogs with OSA as compared to healthy control dogs. This finding is in contrast to earlier work reported by our group where circulating Treg were increased in dogs with OSA but is supported by a recent study performed by Rissetto et al where dogs with OSA did not have increased Treg as compared to normal dogs.[9-11,17] One potential explanation for this discrepancy is the possibility that circulating Treg do not play a significant role in progression of OSA. The lack of published human studies prevents direct comparison with our data, but percentages of Treg are similar to those of other bone tumors in people such as non-metastatic Ewing sarcoma.[18] Interestingly, in patients with non-metastatic Ewing sarcoma Treg percentages were not elevated as compared to percentages observed in healthy individuals but Treg were significantly increased in the bone marrow of patients with metastatic disease.[18] While Treg have been shown to be increased in dogs with metastatic tumors, OSA has not been specifically investigated.[19]

Similar Treg populations in control and OSA dogs may also be due to concomitant medications the dogs received while on study. OSA causes significant bone pain necessitating treatment with oral pain medications and non-steroidal anti-inflammatory drugs (NSAID) are most commonly used. NSAID mitigate inflammation and associated pain through inhibition of a key enzyme in the arachadonic acid pathway, cyclooxygenase (COX), to decrease production of prostaglandin E_2 (PGE_2).[20] COX-inhibitors have been shown to decrease the percentage and function of Treg in a non-small cell lung cancer model in mice.[21] The isoform COX-2 has been found to be up

regulated in canine OSA; additionally, several human malignancies show increased tumoral COX-2 expression which correlates with increased Treg numbers.[21-23] In this study, 15 of the 18 dogs with OSA were treated with a NSAID prior to and concurrent with this study. It is possible that pre-treatment with a NSAID decreased Treg in OSA-bearing dogs prior to enrollment into this trial resulting in Treg numbers similar to the normal dog population. To date, there have been no studies performed evaluating the effect of NSAID on canine Treg.

Dogs with OSA had significantly decreased pre-treatment percentage and absolute number of $CD8^+$ T cells as compared to healthy dogs in this study. This finding is supported by previous work which also shows that dogs with OSA have decreased numbers of $CD8^+$ T cells two weeks post-amputation, prior to initiation of chemotherapy.[13] In contrast to the same study, which found that $CD4^+$ T cells were also decreased in dogs with OSA prior to starting chemotherapy, we found that dogs with OSA had an increased percentage, but not total number of $CD4^+$ T cells. This is likely due to the artificial inflation of $CD4^+$ T cell percentage secondary to the decrease in $CD8^+$ T cells as the absolute number of $CD4^+$ T cells was not altered. This difference in the percentage of peripheral CD4+ T cells is not likely clinically relevant as absolute numbers of CD4+ cells were similar between control and OSA-bearing dogs. Despite significant decreases in the percentage and absolute number of CD8+ T cells and increases in the percentage of CD4+ T cells, mean total lymphocytes counts were within normal limits for dogs with OSA and not significantly different from mean lymphocyte counts of healthy control dogs.

Distributions of T lymphocyte subsets are frequently altered in the regional (tumor-draining) lymph nodes of human malignancies as compared to lymph nodes distant from the tumor.[24-26] The distribution of Treg and $CD4^+$ and $CD8^+$ T cells was not significantly different in either the regional or distant lymph node of dogs with OSA as compared healthy control dogs. There are several possible explanations for this finding. It is possible that the lymph node sampled was not truly the draining lymph node as these could be difficult to identify and sample for dogs with proximal bone lesions. Another possible explanation is Treg accumulation in regional lymph nodes is not an important aspect of OSA progression as this tumor generally metastasizes hematogenously. OSA does occasionally metastasize to regional lymph nodes and this was documented in 2 of the 13 dogs for which histopathologic data was available for analysis in this study. Both of these dogs had percentages of Treg in the metastatic lymph node that exceeded the mean percentage of Treg in regional lymph nodes of dogs with OSA but this sample size is too small to subject to any meaningful statistical analysis.

Changes in T cell subsets were identified both post-operatively and one week after the first dose of chemotherapy. A significant decrease in the total lymphocyte count and number of $CD4^+$ T cells in the blood was observed after surgery. Post-operative lymphopenia is well documented in people and a study in pediatric patients found that total lymphocytes as well as $CD4^+$ T cells were significantly decreased 6 to 48 hours post-operatively.[27] It is possible that changes in total lymphocytes and $CD4^+$ T cell numbers in our study are secondary to surgical/anesthetic stress. $CD8^+$ T cells and Treg in the blood were found to be increased one week post-chemotherapy compared to the post-operative period. Information regarding changes in T cell population after

maximally tolerated dose chemotherapy is lacking, but there is some evidence that increases in $CD8^+$ T cell may be observed in tumor draining lymph nodes after combination chemoradiation.[28] Increases in Treg are often associated with advanced stage disease in humans but there is no evidence that the dogs in our study had early disease progression based on routine radiographic evaluation of the thorax. More work is need to determine if these increases are simply associated with a "rebound" of the bone marrow after treatment with myelosuppressive drugs.

Treg numbers or percentages in the blood, lymph node, or tumor were not associated with either DFI or OS, but the intratumoral (IT) CD8/Treg ratio was positively correlated to both DFI and OS. This finding is similar to human colorectal cancer where a high IT CD8/Treg ratio was associated with improved survival yet neither the IT percentage of Treg or $CD8^+$ correlated with outcome.[29] The positive association between high IT CD8/Treg ratio and survival has also been demonstrated for ovarian and gastric carcinoma.[15,30] The alterations of the tumor infiltrating lymphocytes (TIIL) may be a reflection of the patients' immune response to the tumor.

In conclusion, we found that an increased pre-treatment intratumoral CD8/Treg ratio was associated with prolonged DFI and OS for dogs with OSA. Circulating Treg in dogs with OSA were not significantly increased compared to healthy dogs in this study. These findings suggest that increased intratumoral Treg may be associated with more aggressive or advanced stage OSA but these intratumoral increases are not reflected in the peripheral blood. Because of the small sample size, results of this study should be considered preliminary. The effects of COX-2 inhibitors on canine Treg should be further

investigated before additional work is performed on OSA-bearing dogs to better understand the effects that NSAID therapy may have Treg of the dogs in this study.

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CHAPTER THREE

Low Dose Cyclophosphamide Selectively Decreases Regulatory T Cells and Inhibits Angiogenesis in Dogs with Soft Tissue Sarcoma

INTRODUCTION

Metronomic chemotherapy is defined as the continuous administration of chemotherapy drugs at doses that are significantly lower than conventional maximally tolerated dose (MTD) therapy.[1] In contrast to the direct cytotoxic effects of MTD chemotherapy, metronomic drug delivery inhibits tumor angiogenesis, a process critical for tumor development and progression.[2] There are several proposed mechanisms for these effects, including targeting of the drug-sensitive endothelial cell compartment of tumors, inhibiting mobilization of proangiogenic circulating endothelial cells from the bone marrow, and induction of the endogenous angiogenesis inhibitor thrombospondin-1.[3-5]

In addition to antiangiogenic properties, there is accumulating evidence that metronomic chemotherapy modulates the immune system of tumor bearing patients through inhibitory effects on regulatory T cells (Treg). Treg, a subset of $CD4^+$ lymphocytes that normally function to prevent autoimmunity, have recently been shown to inhibit antitumor immune responses.[6-8] Treg are frequently present in high numbers in

human cancer patients and increased numbers of Treg may be associated with a less favorable outcome for certain malignancies.[9-12] Treg are also increased in the peripheral blood of dogs with various types of cancer; however, the prognostic significance of this finding is unclear.[13-17] In mice and humans with advanced cancer, the administration of metronomic doses of cyclophosphamide (CYC) selectively decreases circulating Treg numbers and inhibits their function.[18-20] The effects of low dose CYC on canine Treg have not been previously reported.

Despite its frequent use in veterinary patients, metronomic chemotherapy has not been systematically evaluated and dosing schedules and drug combinations have been selected empirically.[21,22] Metronomic chemotherapy appears well tolerated in dogs and may have antitumor effects, but studies to identify optimal dosing, mechanisms of action, and biomarkers of drug activity are lacking. There are several challenges in designing effective metronomic chemotherapy protocols. First, most protocols tend to utilize a combination of drugs, each with differing mechanisms of action. The efficacy and therefore validity of using these drugs can only be determined once single drug effects are known; such experiments have not yet been performed. Secondly, dose optimization has been difficult to establish since low dose protocols are not defined by dose limiting toxicities (DLT) in the same manner as MTD chemotherapy. In addition, therapies that target tumor vasculature or a patient's immune system are unlikely to demonstrate a clinical benefit as quickly as high-dose, cytotoxic chemotherapy. Therefore, standard criteria for monitoring antitumor response may be inadequate for assessing efficacy of antiangiogenic or immune-modulating therapies.[23] Finally, clinically applicable

biomarkers of tumor response have not been identified, limiting the use of metronomic protocols as adjunctive therapies.

To address some of these challenges, we first performed a pilot study to evaluate whether low dose CYC administered at 10 mg/m^2/day altered Treg numbers or percentages in dogs with various types of cancer. We then performed a prospective clinical trial to evaluate the effects of low dose CYC on Treg numbers and percentages in circulation, tumor draining lymph nodes (TDLN), and tumor. To assess whether metronomic CYC has antiangiogenic activity in dogs, tumor microvessel density (MVD) and circulating endothelial cells (CEC) were evaluated as well. Our specific goal was to determine the dosage of CYC required to decrease Treg and inhibit tumor angiogenesis in dogs with soft tissue sarcoma (STS).

MATERIALS AND METHODS

Pilot Study

Dogs presenting to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) with histologically confirmed cancer were eligible for inclusion in this pilot study. Patients could either have gross or mircoscopic disease and metastatic cancer was allowable. All patients were required to have a Veterinary Co-Operative Oncology Group (VCOG) performance status of 0 or 1 (0, normal activity; 1, restricted activity (decreased activity from predisease status); 2, compromised (ambulatory only for vital activities, consistently defecates and urinates in acceptable areas), 3, disabled (dog needs to be force-fed, is unable to confine urination and defecation to acceptable areas); 4, dead). Previous therapy was allowable with a three week washout period from

chemotherapy or radiation therapy and a three day washout from any non-steroidal anti-inflammatory drug (NSAID). Information recorded included age, breed, sex, body weight, CBC results, tumor type and whether there was gross or microscopic tumor present. This study was approved by the Institutional Animal Care and Use Committee at Colorado State University, and signed informed consent was obtained from all owners prior to study entry.

Patients were administered CYC 10 mg/m^2/day at home for 28 days. The initial CYC dosage was selected based on previously published clinical studies examining metronomic chemotherapy for canine cancer where dosages of CYC ranged from 10 to 25 mg/m^2/day.[57,58] To improve accuracy of drug administration, CYC was compounded into 2.5 mg and 5 mg capsules. A complete blood count and whole blood for collection of peripheral blood mononuclear cells (PMBCs) were obtained at days 0, 14, and 28 after beginning CYC therapy. Circulating Treg were quantitated by flow cytometric analysis as outlined later in this section. To assess constitutional and gastrointestinal toxicoses, clients completed a patient quality of life questionnaire at each visit.

Inclusion Criteria for Prospective Clinical Trial

Dogs presenting to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) with histologically confirmed grade 1 or 2 STS were eligible for inclusion in this prospective clinical trial. This population was selected with the rationale that optimal treatment for low to intermediate grade STS was unlikely to be adversely affected by enrollment in a 4 week long study prior to pursuing definitive treatment. Patients were required to have a measurable and biopsy-accessible tumor, no evidence of metastatic

disease or significant biochemical or hematological abnormalities. Patients were also required to have a Veterinary Co-Operative Oncology Group (VCOG) performance status of 0 or 1. Previous therapy was allowable with a three week washout period from chemotherapy or radiation therapy and a three day washout from any non-steroidal anti-inflammatory drug (NSAID). Information recorded included age, breed, sex, body weight, CBC results, and tumor size, grade, and location. This study was approved by the Institutional Animal Care and Use Committee at Colorado State University, and signed informed consent was obtained from all owners prior to study entry.

Treatment Protocols and Sample Collection

For analysis of Treg and effector T cells in normal dogs, blood samples were collected from twenty-one healthy, age-matched dogs. Dogs with STS were enrolled in two cohorts. The first group was treated with CYC at a target dosage of 12.5 mg/m^2/day administered orally at home by the owners for 28 days; the second was treated with oral CYC at target dosage of 15.0 mg/m^2/day. The initial CYC dosages for this portion of the study were selected based on results of the pilot study. Again, CYC was compounded into 2.5 mg and 5 mg capsules to improve accuracy of drug administration

A complete blood count, whole blood for collection of peripheral blood mononuclear cells (PMBCs), aspirates of the TDLN, and tumor biopsies were obtained at days 0, 14, and 28 after beginning CYC therapy. Tumor biopsies were performed using local anesthesia and a needle core biopsy instrument in an aseptically prepared site on the tumor. The longest diameter of the tumor was monitored at each time point and tumor

response was assessed using Response Evaluation Criteria in Solid Tumours (RECIST).[24] Clients completed a patient quality of life questionnaire at each visit.

Sample Preparation

For evaluation of circulating Treg, PBMC were obtained from blood samples collected in EDTA tubes after lysis of red blood cells. PBMC were added at a concentration of approximately 1 x 10^6 cells per well in 96-well round bottom plates and then immunostained for surface expression of CD4 and CD8 using FITC-conjugated anti-canine CD4 mAB (clone YKIX302.9, AbD Serotec, Raleight, NC) and Alexa 647-conjugated anti-canine CD8 mAB (clone YCATE55.9, AbD Serotec) following the method described previously.[25] Immunostaining for FoxP3 expression was conducted as previously described.[13] Briefly, after washing to remove unbound surface antibodies, intracellular detection of FoxP3 was performed using a cross-reactive, PE-conjugated murine FoxP3 antibody (clone FJK-16s, Mouse FoxP3 staining kit, eBioscience, San Diego, CA). A directly conjugated rat IgG_{2A} antibody was used as the isotype control.

Lymph node aspirates were washed with a PBS buffer containing 2% fetal bovine serum through a 70 µm strainer prior to immunostaining. Tumor tissues were incubated in a collagenase buffer for 20 minutes before processing through the strainer. The cells collected from the TDLN and tumor were then added to 96-well plates and immunostained as described above.

To evaluate CEC, PBMC were obtained from blood samples collected in EDTA tubes after lysis of red blood cells. PBMC were added at a concentration of approximately 2 x 10^6 cells per well in 96-well round bottom plates and immunostained

for the surface markers CD3, CD45, and CD146 using FITC conjugated mouse anti-canine CD3 mAB (CA17.2A12, AbD Serotec) and rat anti-canine CD45 mAB (clone YKIX716.13, AbD Serotec), and PE conjugated CD146 mAB (clone P1H12, Millipore, Billerica, MA). For isotype staining, CD3-FITC, CD45-FITC, and a mouse IgG1-PE negative control (Millipore) were used to determine gating. Compensation controls were created using CD45-biotin and a secondary stain of the nuclear stain (LDS-751, Invitrogen) was added to cells.

Flow Cytometric Analysis

Flow cytometry was performed using a CyAn ADP flow cytometer (Dako-cytomation, Fort Collins, CO) and Summit software (Dako-cytomation) for data analysis. Analysis gates were set on the live lymphocyte population based on typical forward and side scatter characteristics.[26] The percentage of Treg was calculated by determining the percentage of $CD4^+FoxP3^+$ cells within the $CD4^+$ T-cell population. The percentages of $CD4^+$ and $CD8^+$ T cells were also determined. Absolute numbers of Treg, $CD4^+$ and $CD8^+$ T cells in peripheral blood were calculated based on the total lymphocyte count determined from a CBC run on an automated cell counter. CEC were defined as $CD45^-CD3^-LDS\text{-}751^+CD146^+$ and one million or more events were run to insure enough events for analysis.

Microvessel Density Assessment

Biopsy specimens were embedded in Tissue-Tek OCT compound (Ted Pella Inc, Redding, CA), snap frozen in liquid nitrogen, and stored at -80^{o}C until processing.

Tissues were cyrosectioned to a thickness of 4 μm and adhered to positively charged glass slides. Immunohistochemistry was performed using a mouse anti-human CD146 monoclonal antibody (clone P1H12, Millipore), that cross reacts with dog endothelium and has been validated as a measure of tumor of angiogenesis.[27,28] The slides were fixed in acetone for four minutes, allowed to air dry, and blocked with 5% donkey serum (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). The tissues were then incubated with a 1:100 dilution of the primary antibody followed by a 1:400 dilution of biotin-conjugated donkey anti-mouse IgG (Millipore). The slides were then incubated with strepavidin-HRP (Vector Laboratories, Burlingame, CA), followed by AEC substrate (Vector Laboratories), then hematoxylin counterstain. Normal canine liver and spleen were used as positive control tissues. Positive control samples were processed as outlined above and negative control samples were incubated with 1x PBS rather than the primary antibody.

To quantify tumor MVD, three to five photomicrographs for each time point were obtained at 20X magnification using a Zeiss AxioVision microscope and AxioVision Software v4.6 (Zeiss, Thornwood, NY). Computerized determination of the percentage of $CD146^+$ vessels positive within each 20x field was performed and averaged for each time point. The number of microvessels also included immunopositive endothelial cells or endothelial cell clusters regardless of the presence or absence of a lumen.[29,30] The final MVD value for each time point was calculated as the mean (± SD) percentage of microvessels for the 3 to 5 20x fields.

Statistical Analyses

Data regarding age and sex for a population of normal and STS bearing dogs were compared using an unpaired, two-tailed t-test. Data regarding age, sex, weight, and tumor size of dogs in the two CYC dose cohorts were also compared using an unpaired, two-tailed *t*-test. Changes in mean percentages and absolute numbers of Treg and MVD were compared using paired, one-tailed *t*-tests. Hematologic parameters and mean percentages and absolute numbers of $CD4^+$ and $CD8^+$ T cells were compared by paired, two-tailed *t*-tests. Statistical calculations were performed using a commercial software program (GraphPad Prism version 5 for Windows, GraphPad Software, La Jolla, CA). A *P* value of <0.05 was considered statistically significant for all analyses.

RESULTS

Pilot Study

Seven dogs were enrolled in the pilot study from April, 2008 to June, 2008 and these dogs received a mean CYC dose of 9.7 mg/m^2/day (range 8.7 to 11.1 mg/m^2/day; target dose 10.0 mg/m^2/day). Five dogs were castrated males and two were spayed females with an average age of 10.1 years (range 9.0 to 12.0 years). Five patients had measurable tumors and these included pulmonary metastasis of osteosarcoma, thyroid carcinoma, oral fibrosarcoma, pulmonary metastasis with an unknown primary tumor, and metastatic anal sac adenocarcinoma to the liver. Two dogs had mircroscopic tumors; these included an incompletely excised extraskeletal osteosarcoma and an incompletely excised oral amelanotic melanoma.

To determine the effects of metronomic CYC therapy on numbers of Treg in circulation, blood samples were collected on days 0, 14, and 28 and changes in the mean absolute number and percentage of Treg were analyzed. There was no significant change in the mean percentage or the mean absolute number of Treg over the 28 day period when CYC was administered at 10 mg/m^2/day (Figure 3.1). Additionally, there were no alterations in mean percentages or absolute numbers of $CD4^+$ and $CD8^+$ T cells as assessed by flow cytometry and no significant change in total leukocyte, neutrophil, lymphocyte, monocyte, or platelet counts as assessed by CBC on days 0, 14, and 28. There were no reported adverse events for any of the patients over the study period (data not shown).

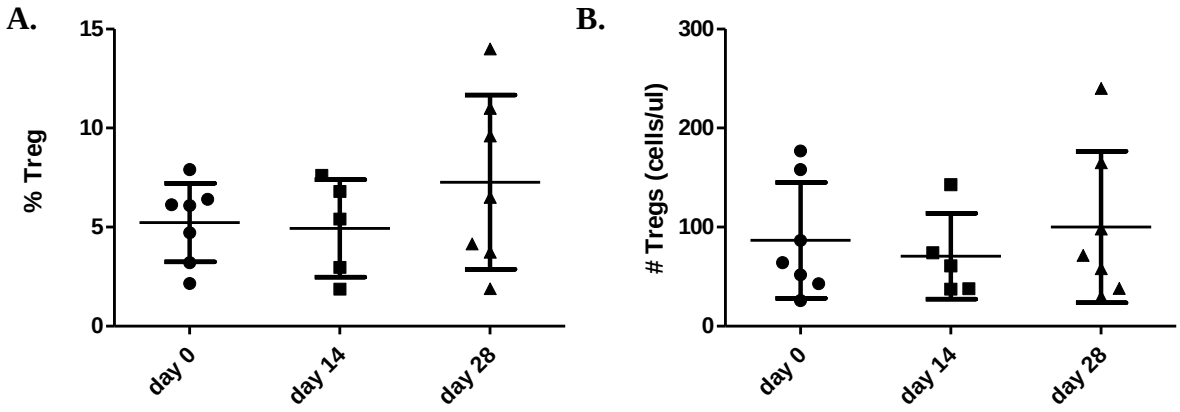

Figure 3.1: Cyclophosphamide (CYC) administered at 10 mg/m^2/day does not alter percentages or absolute numbers of Treg over 28 days. Relative and absolute numbers of regulatory T cells (Treg) in peripheral blood of dogs with various tumor types treated with metronomic CYC at a target dose of 10.0 mg/m^2/day (n = 7) are shown. The mean percentage (long horizontal line) of Treg for day 0, 14, and 28 was plotted in (**A**) and the mean of the absolute number (long horizontal line) of Treg day 0, 14, and 28 was plotted in (**B**). Error bars show SD. There was no significant decrease in the mean percentage or absolute number of Treg between any time points as assessed by unpaired, one-tailed *t*-test.

Study Patients

Eleven dogs were enrolled in this clinical trial; the first five dogs received a mean CYC dose of 12.4 mg/m^2/day (range 11.2 to 13.9 mg/m^2/day; target dose 12.5 mg/m^2/day) and the next six received a mean CYC dose of 15.8 mg/m^2/day (range 14.0 to 17.0 mg/m^2/day; target dose 15.0 mg/m^2/day). Five dogs were castrated males and six were spayed females with an average age of 9.9 years (range 6.9 to 12.8 years). There was no significant difference in age, sex, or weight between the two groups; similarly there was no significant difference in age or sex between the healthy dogs and those with STS (data not shown). Tumor biopsies were performed for all patients prior to enrollment: eight patients had grade 1 STS and three had grade 2 STS; 72.7% (8/11) of these tumors occurred at or distal to the elbow or stifle.

Average tumor maximal diameter was 9.3 cm with a range of 1.9 to 30 cm; there was no significant difference in tumor size between the two dose cohorts. For all dogs in the study, maximal tumor diameters remained stable during the treatment period, according to RECIST criteria. Additionally, no adverse events were reported for any of the patients over the 28 day study period.

Circulating Treg are Significantly Increased in Dogs with STS

Whole blood was collected from control dogs as well as dogs with STS and evaluated by flow cytometry to determine the percentage of $CD4^+$ and $CD8^+$ T cells and Treg in peripheral blood as described in Materials and Methods. Both the percentage and absolute number of Treg in peripheral blood were significantly increased in the dogs with STS prior to therapy as compared to healthy dogs (Figure 3.2). The mean

percentage of Treg for dogs with STS was 7.6 (± 3.0) % as compared to 4.8 (± 1.3) % for the normal dogs (P <0.001). The mean absolute number of Treg in peripheral blood was also increased in dogs with STS (107.8, SD ± 58.2 cells/μL) as compared to the normal dog population (35.4, SD ± 19.6 cells/μL; P <0.001). The total number and percentage of $CD4^+$ and $CD8^+$ cells did not differ significantly between the normal and diseased populations (data not shown).

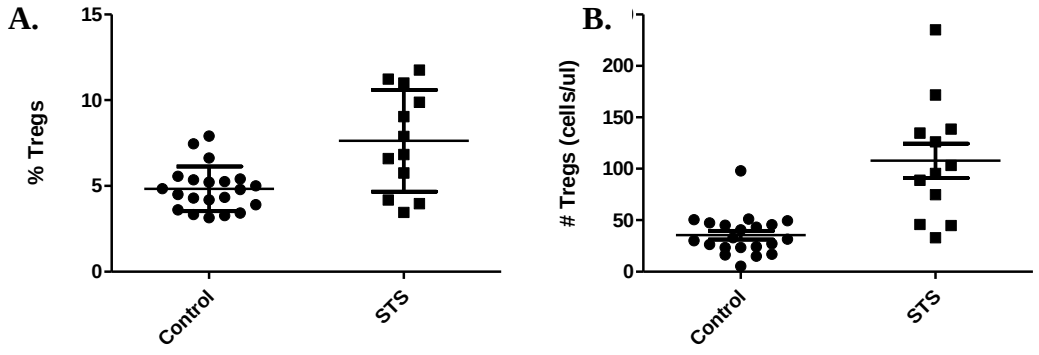

Figure 3.2: Circulating Tregs are increased in dogs with STS as compared to a healthy control population. Blood of control dogs (n = 21) and dogs with STS (n = 12) was analyzed by flow cytometry to determine the absolute number and percentage of Treg, as described in "Materials and Methods." The mean percentage (long horizontal line) of Treg in the blood of normal dogs and dogs with STS was plotted in (**A**) and the mean of the absolute number (long horizontal line) of Treg was plotted in (**B**). Error bars show SD. Both the percentage and the absolute number of Treg were significantly increased (P < 0.001) in dogs with STS compared with healthy control dogs as assessed by unpaired, two-tailed *t*-test.

TDLN samples were not obtained in sufficient number for statistical evaluation in this study due to the technical difficulty of aspirating normal canine lymph nodes. Additionally, insufficient numbers of tumor biopsies were obtained for intratumoral Treg assessement. Due to the sampling challenges encountered, the effect of low-dose CYC on Treg in tumor and TDLN was unable able to be assessed in this study.

Low Dose CYC Decreases Treg Numbers in Dogs with STS

To determine the effects of metronomic CYC therapy on numbers of Treg in circulation, blood samples were collected on days 0, 14, and 28 after beginning oral CYC and changes in the mean absolute number and percentage of Treg were analyzed at each time point for the two groups of dogs. For dogs with STS receiving CYC at a target dosage of 12.5 mg/m^2/day, there was no significant change in the mean percentage of Treg over the 28 day period (Figure 3.3A). A significant decrease in the mean absolute number of Treg was seen, however, from day 0 to 28 (P = 0.001; Fig 3.3B). The mean absolute number of pretreatment Treg was 78.9 cells/μL (± 43.1) which decreased to 54.1 cells/μL (± 43.7) after 28 days of CYC therapy. No significant change in the absolute numbers of Treg was observed between days 0 and 14 or days 14 and 28.

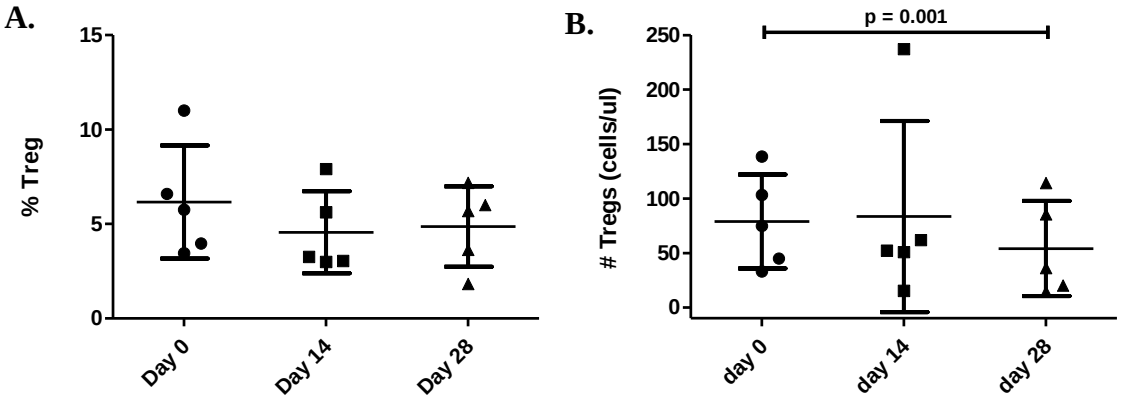

Figure 3.3: Absolute numbers of circulating regulatory T cells (Treg) are decreased between day 0 and 28. Relative and absolute numbers of Treg in peripheral blood of dogs with soft tissue sarcoma (STS) treated with metronomic cyclophosphamide (CYC) at a target dose of 12.5 mg/m^2/day (n = 5). The mean percentage (long horizontal line) of Treg for day 0, 14, and 28 was plotted in (**A**) and the mean of the absolute number (long horizontal line) of Treg day 0, 14, and 28 was plotted in (**B**). Error bars show SD. There was no significant decrease in the mean percentage of Treg between day 0 and 14, day 0 and 28, or day 14 and 28 as assessed by paired, one-tailed *t*-test. There was no significant decrease in the mean absolute number of Treg from day 0 to 14 or day 14 to 28. A decrease in the mean absolute number of Treg from day 0 to 28 (P = 0.001) was observed as assessed by paired, one-tailed *t*-test.

Dogs receiving CYC at a target dosage of 15.0 mg/m^2/day had significant decreases in both the absolute number and percentage of Treg in the peripheral blood during the 28 day study period (Figure 3). For this group of patients, a significant decrease in the mean percentage and mean absolute number of Treg occurred between days 14 and day 28 (7.4 ± 3.1 versus 5.8 ± 3.5%; P = 0.035 and 121.1 ± 64.1 versus 55.7 ± 27.9 cells/μL; P = 0.049).

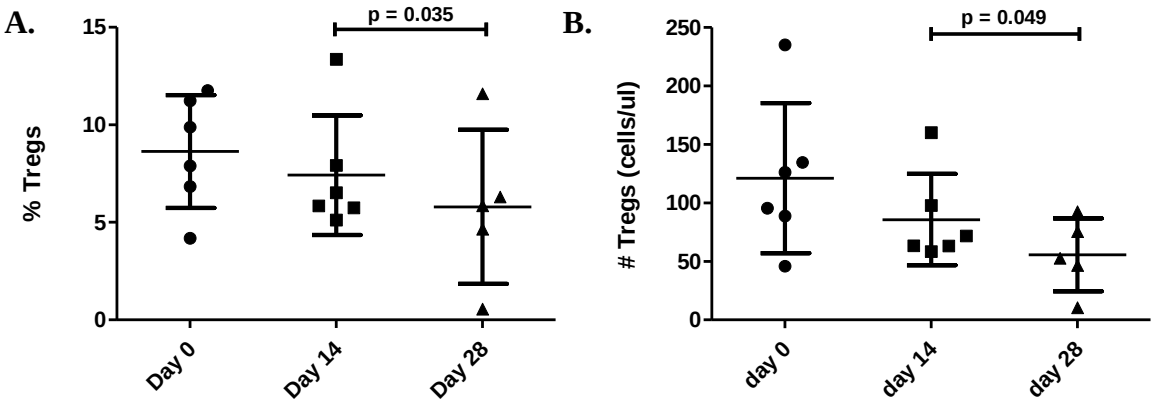

Figure 3.4: Metronomic cyclophosphamide (CYC) at a target dose of 15.0 mg/m^2/day (n = 6) decreases Treg in peripheral blood between days 14 and 28. The mean percentage (long horizontal line) of Treg for day 0, 14, and 28 was plotted in (**A**) and the mean of the absolute number (long horizontal line) of Treg day 0, 14, and 28 was plotted in (**B**). Error bars show SD. A significant decrease in the mean percentage of Treg and the mean absolute number of Treg was observed between day 14 and 28 (P = 0.035 and P = 0.049, respectively) as assessed by paired, one-tailed t-test.

Low Dose CYC Mediates Selective Decreases in Treg

To determine the effects of low dose CYC on leukocyte numbers, a complete blood count was performed at each study time point for all patients. There were no significant changes in the total numbers of neutrophils, lymphocytes, monocytes, or platelets in dogs with STS receiving CYC at 12.5 mg/m^2/day or 15.0 mg/m^2/day. To determine the effect of CYC on T-lymphocyte subsets, the CD4$^+$ and CD8$^+$ T cell

populations were also evaluated. Despite the decrease of Treg at the higher dosage of CYC, there were no changes in the absolute numbers or percentages of either $CD4^+$ or $CD8^+$ lymphocytes during the course of the clinical trial, suggesting that the effects of CYC were selective for the Treg subset of T-lymphocytes (Table 3.1).

Table 3.1: $CD4^+$ and $CD8^+$ lymphocyte subpopulations are not significantly altered in dogs with soft tissue sarcoma treated with metronomic cyclophosphamide for 28 days.

	12.5 mg/m^2 cohort (mean ± SD)			**15.0 mg/m^2 cohort (mean ± SD)**		
Parameter	**Day 0**	**Day 28**	***P* value**	**Day 0**	**Day 28**	***P* value**
% $CD4^+$	45.7 (± 13.1)	44.6 (± 7.9)	0.83	33.8 (± 6.4)	51.3 (± 16.7)	0.12
Total $CD4^+$ (cells/µL)	694 (± 492)	500 (± 274)	0.33	463 (± 169)	611 (± 326)	0.21
% $CD8^+$	26.9 (± 4.0)	25.6 (± 5.7)	0.61	20.5 (± 7.1)	17.6 (± 8.6)	0.31
Total $CD8^+$ (cells/µL)	396 (± 231)	318 (± 222)	0.12	270 (± 100)	204 (± 119)	0.15

Low-dose CYC Decreases Tumor MVD in Canine STS

Tumor MVD was evaluated at each time point to assess whether low dose CYC exhibits antiangiogenic properties in canine STS. This was assessed by measuring the tumor MVD in serial tumor biopsies for each patient. There were no significant changes in the mean tumor MVD between the three study time points in the tumors of dogs treated with 12.5 mg/m^2/day CYC (Figure 3.5A). However, in the dogs receiving CYC at 15.0 mg/m^2/day, the mean tumor MVD significantly decreased from a pretreatment mean of 2.6 (± 0.9)% to a mean of 1.3 (± 1.0)% on day 14 ($P = 0.015$). This decrease persisted through day 28 (1.8 ± 0.8%) and was also significantly lower than the MVD prior to therapy ($P = 0.004$, Figure 3.5B). The mean percent MVD was not significantly different between day 14 and day 28 in the 15.0 mg/m^2/day dose cohort.

Low-Dose CYC Does Not Alter CEC

CEC isolated from peripheral blood were analyzed at each time point in this study as another means of assessing antiangiogenic properties of low-dose CYC. As shown in Fig 3.6, there were no significant change in the absolute number or percentage of CEC at any time point for either the 12.5 mg/m^2/day or the 15.0 mg/m^2/day dose cohort.

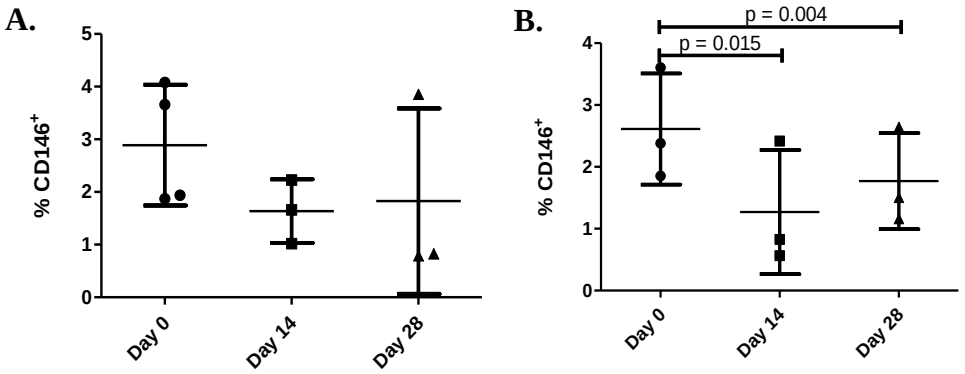

Figure 3.5: Tumor microvessel density (MVD) is significantly decreased in the 15.0 mg/m^2/day cohort. Percentage of CD146$^+$ cells in canine soft tissue sarcoma as an assessment of tumor MVD in dogs treated with metronomic cyclophosphamide (CYC). (**A**) The mean tumor MVD (long horizontal lines) on days 0, 14, and 28 for dogs treated with 12.5 mg/m^2/day CYC (n = 4) did not change significantly over the 28 day study period as assessed by an paired, one-tailed *t*-test. (**B**) The mean tumor MVD (long horizontal lines) in STS of dogs treated with 15.0 mg/m^2/day CYC (n = 3) significantly decreased from day 0 to 14 (P = 0.015) as well as from day 0 to 28 (P = 0.004). Error bars show SD.

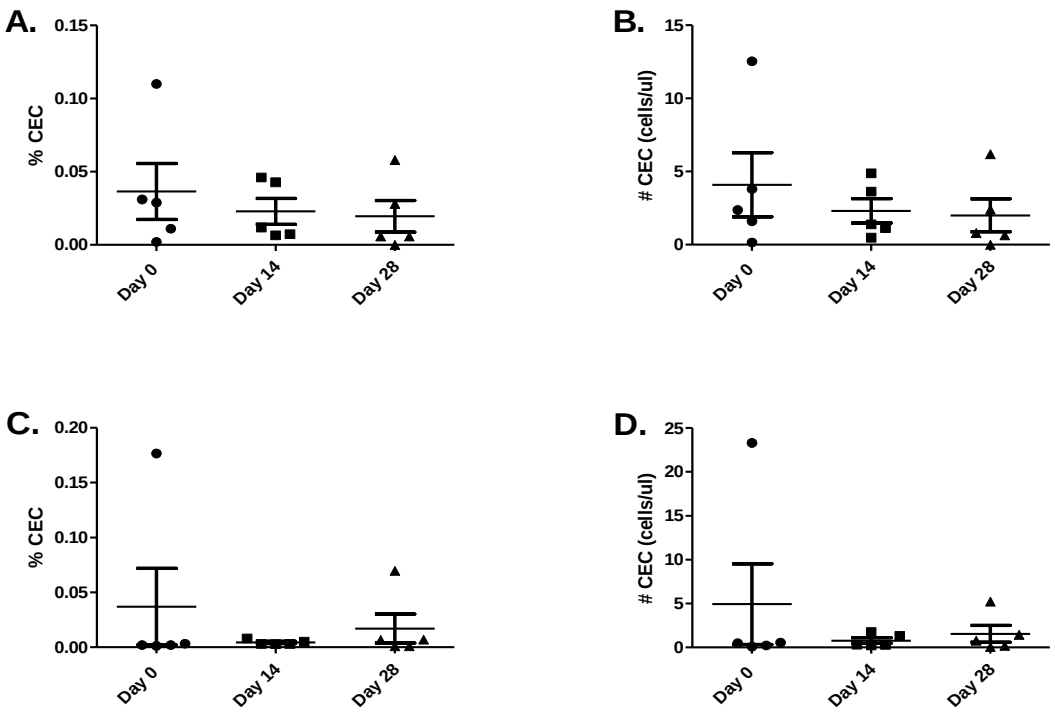

Figure 3.6: Circulating endothelial cells (CEC) are not altered in dogs with soft tissue sarcoma (STS) over 28 days of low dose cyclophosphamide in either dose cohort. The mean percentage (long horizontal line) of CEC for day 0, 14, and 28 was plotted in (A &C) and the mean of the absolute number (long horizontal line) of CEC was plotted in (B & D). The CEC data from the 12.5 mg/m^2/day cohort is shown in figures A & B and the 15.0 mg/m2/day cohort in C & D. There were no significant differences in CEC between any of the time points in any of the groups as assessed by paired, one-tailed *t*-test.

DISCUSSION

Several important findings emerged from this investigation. First, when compared with a population of healthy dogs, the blood of dogs with STS contained significantly increased numbers of Treg. When dogs with STS received oral CYC at a dosage of 15 mg/m^2/day, Treg numbers declined over the 28 day study period to that observed in healthy dogs. In addition, daily administration of CYC at 15 mg/m^2 was associated with a significant decrease in the density of blood vessels within tumor tissues from these patients, suggesting that metronomic delivery of CYC at this dosage has both immunomodulatory and antiangiogenic effects.

Previous work has established that Treg are increased in humans with a wide range of malignancies including melanoma, carcinoma of the ovaries, pancreas, breast and liver, and a number of hematologic malignancies; however, increases in Treg in humans with STS have not been previously described.[11,31] Recent reports suggest that Treg are also increased in the blood of dogs with various cancers including melanoma, lymphoma and osteosarcoma.[13-16,32] Although the results of the present study support the notion that circulating Treg are increased in dogs with cancer, conflicting reports exist in the veterinary literature, likely reflecting differences in experimental approach and the small numbers of dogs evaluated to date.[33]

A key finding of this study is that the administration of CYC at 15 mg/m^2/day over the 28 day study period selectively decreases absolute numbers and percent of Treg in the peripheral blood of dogs with STS. Dosages of CYC used in previously published studies ranged from 10 to 25 mg/m^2/day.[21,22] In two of these studies, metronomic chemotherapy appeared to have antineoplastic effects but the mechanism of action of the drugs used was not investigated.[21,22] Our data suggest that decreased numbers of circulating Treg may be one potential antitumor activity of metronomic CYC therapy. Recent work demonstrates decreased levels of intracellular ATP in murine and human Treg as compared to other CD4$^+$ T cells; lower levels of ATP and subsequent decreased glutathione levels contribute to low dose CYC Treg depletion.[34] Whether this is the case in canine Treg is not known.

Serial changes in tumor MVD were used in this study to assess the antiangiogeneic properties of metronomic CYC therapy. We found that MVD was significantly decreased over the 28 day study period when CYC was administered orally

at 15.0 mg/m^2/day to dogs with STS. This finding is consistent with previous work in other species that has established the antiangiogenic properties of metronomic dosing of CYC.[40,49,50] Our findings should be interpreted with caution, however, as tumor MVD may not be the most accurate assessment of response to antiangiogenic therapy.[37] Additionally, there was no significant change in the CEC population in this study suggesting that low-dose CYC may not inhibit angiogenesis by decreasing CEC. CEC comprise a small fraction of circulating cells and detection of clinical relevant changes may be difficult to accomplish with this small sample size. Further work should be performed in a larger population of dogs to more fully understand the possible effects of metronomic CYC on CEC. Combining evaluation of tumor MVD with other markers of angiogenesis such as vascular endothelial growth factor (VEGF) concentrations may provide greater understanding of the antiangiogenic properties of metronomic CYC in dogs with cancer.

Interestingly, significant decreases in both Treg numbers and tumor MVD occurred in dogs with STS treated with CYC at 15.0 mg/m^2/day. One explanation for this is that alterations in tumor immunity and angiogenesis occur at a similar dose of CYC. This finding is in line with previous investigations in murine tumor models in which metronomic delivery of drugs such as CYC and paclitaxel were associated with both immunomodulatory and antiangiogenic effects.[38] In a previously published study examining the impact of metronomic chemotherapy on incompletely excised STS in dogs, Elmslie et al. treated patients with 10 mg/m^2/day of CYC.[21] Although our preliminary work indicated that decreases in Treg numbers do not occur at this dosage it is possible that the addition of piroxicam in the Elmslie study had a synergistic effect on

Treg depletion. Our data also suggests that Treg decreases may occur at dosages less than 15.0 mg/m^2/day because of the decrease in the absolute numbers of Treg observed in dogs receiving 12.5 mg/m^2/day CYC; larger numbers of dogs will be required to optimize metronomic dosing of CYC.

Surface staining for CD4 and detection of the intracellular transcription factor FoxP3 were used in this study to identify canine Treg by flow cytometry similar to the method use in earlier studies.[13-15] In addition to these markers, CD25, the alpha chain of the IL-2 receptor, is also commonly used to identify Treg in other species. Recently both an anti-human CD25 antibody and a canine anti-CD25 antibody have been found to be useful in the identification of canine Treg.[33,39] These antibodies were not available for use in the dogs of the present study; however, future studies should include the use of one of these CD25 antibodies to facilitate identification of $CD4^+CD25^+FoxP3^+$ Treg.

No adverse events were reported for any of the patients in this study. This result is in contrast to previous studies examining metronomic chemotherapy protocols for canine malignancies that have reported that up to 23% of dogs develop grade 1 or 2 gastrointestinal toxicity and 10-22% of patients developed sterile hemorrhagic cystitis (SHC) when CYC is administered.[21,22] Both of these studies included the use of a non-steroidal anti-inflammatory drug (NSAID) so it is possible that the gastrointestinal signs were secondary to the NSAID rather than the low dose CYC. A limitation of the current study is that routine monitoring of urine was not performed to assess the presence of microscopic hematuria; however, clinical signs consistent with the development of SHC were not reported in any of the patients. This is may be due to the short duration of CYC

administration here as dogs receiving long term therapy CYC at the MTD have a reported incidence of up to 10%.[40]

In addition to the occurrence of SHC, there are other potential complications of Treg prolonged depletion that should be investigated. Under non-neoplastic conditions, Treg function to prevent autoimmunity and therefore it is possible that chronic Treg depletion could lead to the development of autoimmune diseases. Autoimmunity secondary to Treg depletion has been demonstrated in murine models; whether this may develop secondary to Treg modulation in dogs is currently unknown.[41,42]

In conclusion, we found that dogs with STS have significantly more Treg in circulation than healthy dogs. Additionally, CYC administered at 15.0 mg/m^2/day selectively decreased the number and percentage of Treg in the peripheral blood and appears to have some antiangiogenic properties in dogs with STS. Due to the small number of dogs in this study, these results must be considered preliminary but support further studies to better characterize the antitumor effects and optimal dosing of low dose CYC in canine cancer therapy. Additional studies will require longer treatment of a larger number of dogs to gain valuable information regarding potential biomarkers of clinical response to metronomic CYC therapy.

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CHAPTER 4

GENERAL CONCLUSIONS

The work contained within this thesis is intended to further the knowledge regarding the role of regulatory T cells (Treg) in canine cancer. In chapter two, we investigated the distribution of Treg in the peripheral blood, distant and tumor draining lymph nodes, and the tumor of dogs with osteosarcoma (OSA) as compared to the blood and lymph nodes of healthy control dogs. In addition, we also examined whether an association exists between Treg amounts in these tissue compartments and outcome of dogs with OSA. We demonstrated that a high intratumoral CD8/Treg ratio in dogs with OSA was positively correlated with disease-free interval (DFI) and overall survival (OS). This may indicate that Treg are increased and CD8+ T cells decreased in more advanced or aggressive OSA and that tumor infiltrating lymphocytes may have a role in the progression of this tumor type. We also found that circulating Treg are not increased as compared to Treg in the blood of healthy dogs. As this finding is in contrast to previously published results, it raises the question as to whether use of non-steroidal anti-inflammatory drugs (NSAID) may inhibit Treg as well. The concomitant use of NSAID in this study described in chapter two prevents definitive conclusions regarding the up regulation of Treg in dogs with OSA, but it identifies an area of further research regarding additional mechanisms of Treg inhibition in canine cancer. Finally, Treg were

not increased in distant or tumor draining lymph nodes in dogs with OSA. This finding may relate to the biologic behavior of canine OSA in which lymph node metastasis is uncommon. Alternatively, normal Treg amounts in lymph nodes of dogs with OSA may be additional evidence that Treg may not play an important role in progression of OSA, particularly when combined with the finding that circulating Treg are not increased in dogs with OSA. T lymphocyte subsets in normal canine lymph nodes as well as Treg in multiple tissue compartments of dogs with OSA needs to be evaluated in a larger number of dogs before definitive conclusions may be made.

In chapter three, we examined whether metronomic cyclophosphamide (CYC) has immunomodulatory and/or antioangiogenic properties in canine cancer. Dogs with soft tissue sarcoma (STS) where enrolled in this study as this tumor type was easily amenable to repeated biopsy for assessment of microvessel density (MVD) and delay of definitive therapy for a 28 days period would not negatively impact the outcome of these dogs. In this study, we found that circulating Treg in dogs with STS were increased as compared to healthy dogs. Alterations in Treg in the blood of dogs with STS have not been previously reported; Treg in the lymph nodes and tumors could not be consistently assessed in this study. We determined that metronomic CYC administered at doses of 15.0 mg/m^2/day selectively decreased Treg, whereas lower dose of 10.0 and 12.5 mg/m^2/day did not. Additionally, the higher dose of daily CYC decreased tumor MVD, but not circulating endothelial cells (CEC); this provides some preliminary information that metronomic CYC may inhibit angiogenesis.

As a whole, this work has identified alterations in Treg numbers in cancer bearing dogs and demonstrates that Treg levels may have prognostic value when evaluated in

combination with numbers of other effector T cells. Additionally, we have shown that Treg can be decreased with metronomic dosing of CYC. Decreases in Treg may be one of the anti-tumor mechanisms of metronomic CYC therapy; additional work needs to be performed to examine whether decreasing Treg in cancer-bearing patients correlates with improved outcome.

FUTURE DIRECTIONS

The work outlined in this thesis identified a number of additional areas of research regarding the role of Treg in canine cancer. One potential area of further study is the effect of NSAID use and subsequent cyclooxygenase-2 (COX-2) inhibition on Treg populations in canine cancer. NSAID are commonly used in cancer therapy, not only for pain control but for anti-tumor effects as well.[1,2] COX-2 is up regulated in many tumor type and this increased COX-2 expression has been associated with increases in Treg numbers as well.[3,4] Treg numbers were not significantly increased in dogs with OSA as compared to healthy control dogs in this work, but most dogs received therapy with NSAID prior to and during the clinical trial. Prospective studies examining changes in Treg in dogs that are treated with an NSAID alone, metronomic CYC alone, or a combination of the two should be performed to determine the effects of NSAID on Treg and to explore whether the combination of NSAID and CYC may have synergistic activity in decreasing Treg in cancer-bearing dogs.

Increased frequencies of Treg are commonly associated with shortened disease-free intervals and survival for a number of human malignancies.[5-8] This association does

not always hold true, however, particularly for hematologic cancers such as lymphoma where several studies have shown that increased frequencies of Treg are positively correlated with outcome.[9,10] A small population of dogs with lymphoma has previously been shown to have an elevated Treg/CD8 ratio as compared to other canine tumor types, but the prognostic significance of this finding is unknown.[11] Further work needs to be performed to assess Treg populations in larger numbers of dogs with various tumor types and to correlate these findings with patient outcome in order to better understand the role of Treg in canine tumor immunity.

Our knowledge regarding Treg distribution in canine cancer patients remains limited. Further investigation into numbers of Treg and other effector T cell populations in tumors and tumor draining lymph nodes (TDLN) would allow a better understanding of the role of Treg in cancer progression. Work performed in this thesis focused on dogs with sarcomas, which have not been widely researched in humans. As carcinomas tend to readily spread to the TDLN, it would be intersting to examine Treg frequency within blood, tumors, and the TDLN of dogs with sarcomas as compared to carcinomas. If differences in Treg distributions exist between sarcomas and carcinomas, this may speak to differences in the biologic behavior of these cancers.

This research supports the theory that Treg populations are altered in canine cancer and these changes may provide prognostic information for dogs. It is unknown whether Treg modulation may provide a clinical benefit for cancer-bearing dogs but we have provided preliminary evidence that metronomic dosing of CYC may decrease Treg frequencies in dogs with cancer. This work is a small step forward in understanding the

involvement Treg may have in canine cancer and, combined with previous research on canine Treg, sets the foundation for additional studies in this field.

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