CANINE T-REGULATORY CELLS IN AGING AND CANCER: QUANTIFYING A UNIQUE T CELL SUBSET

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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presented by Kerry Rissetto, a candidate for the degree of Master of Veterinary Clinical Sciences

and hereby certify that, in their opinion, it is worthy of acceptance.

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Dr. Carolyn Henry

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DEDICATION

This Master's thesis (aka the last 2.5 years of my life) is dedicated to those that remained in my life despite it being consumed with this very document.

To my parents, Martine and Gene Rissetto, who dealt with limited phone time and only one visit a year, but who remained selflessly happy for me because they knew I was doing what I love and understand that this career demands more than the typical 8-5 job that all of their friends' children have. Thank you for loving me unconditionally and being my biggest fans. I am so lucky to have you for parents.

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ABSTRACT

T regulatory lymphocytes (Tregs), identified by the markers CD4, CD25, and FoxP3, are an essential part of the immune system and promote tolerance with the purpose of minimizing auto-immune disease. However, if Tregs are overactive, they allow host susceptibility to infectious diseases and cancer, allowing these disease processes to escape normal immune surveillance. There are numerous human studies documenting not only an increase %Tregs in cancer patients, but also correlation with negative prognostic factors and overall survival time. At the time that this research was generated, there were only three veterinary papers examining Tregs in canine cancer patients and they only evaluated CD4+FoxP3+ Tregs because, until recently, there was no commercially available anti-canine CD25 antibody.

By cloning and transfecting canine CD25 into CD25 negative HeLa cells, we definitively validated the anti-human CD25 antibody for use in the dog and then quantified CD4+CD25+FoxP3+ Tregs in healthy dogs and dogs with osteosarcoma. Our data revealed no major differences in %Treg between healthy dogs and those with bone cancer. However, by definitively validating the use of the anti-human CD25 antibody, there is now a more specific way to identify this unique T cell subset and evaluate these cells in a variety of different diseases.

CHAPTER 1: INTRODUCTION

The study of cancer and, specifically, advances in its treatment, has improved greatly over the past 20 years. However, the chemotherapy standard of care in most patients rarely offers a cure for most patients mostly due to dose-limiting normal tissue toxicity as well as the development of various resistance mechanisms. New treatment modalities are necessary if further success in median survival times is to be achieved. Immunotherapy has been studied extensively in humans and has had variable success in clinical applications. The relationship between cancer and the immune system is complex, and attempts at immunotherapy have often failed for unknown reasons.

T regulatory lymphocytes (Tregs) are a specific subset of CD4+ helper T cells that modify the activity of cytotoxic effector T cells (CD8+ T cells) in addition to other helper T cells, dendritic cells, and Natural Killer cells. They are essential for preserving peripheral tolerance and immune homeostasis by preventing auto-immunity and regulating inflammation induced by pathogens. Tregs can have both beneficial and injurious effects and must be evaluated in relation to the primary disease process to truly understand their function. The primary goal of Treg function is to prevent autoimmune and chronic inflammatory diseases such as Type I diabetes, pemphigoid disease, asthma, and inflammatory bowel disease.[1-18] However, at the same time overzealous activity of this highly specialized T cell subset can suppress normal anti-viral[19-26] or anti-tumor immune responses and Tregs have even been found in increased numbers in cancer patients.[27-41] The accountability of Tregs in cancer pathogenesis is rapidly moving to the forefront of targeted therapy research as we learn more and more about their role in

tumor progression. In fact, multiple human studies have correlated increased Treg percentages with decreased survival and remission times.[30,32,42-46]

There have been encouraging studies documenting an improved anti-tumor response after specifically downregulating or targeting tumor-specific Tregs.[47-51] With these early yet promising advances, it is clear that understanding the origin, function, and development of Tregs in association with various disease processes may be crucial to potentially targeting this unique subset of cells in the future to exploit the host's own immune response.

One of the most powerful analytical tools for identifying and selectively harvesting Tregs is flow cytometry, a technique for quantifying and qualifying markers on, or in, cells in a fluid suspension. Multiple physical and chemical parameters can be evaluated simultaneously at a rate of thousands of cells per second, and subsequently cells of interest can then be sorted out and used for further experiments. A flow cytometer directs a beam of single wavelength laser light into a thin stream of sample fluid. The light then scatters from particles that constitute cells in suspension depending on size and complexity. This scattered light is picked up by various detectors situated in different positions around the beam. The detector parallel to the beam detects "forward Scatter" (FSC) and the detector perpendicular to the beam detects "side scatter" (SSC). There are also several detectors that recognize fluorescent chemicals found in or on the cells in suspension. The combination of scattered and fluorescent light is analyzed and portrayed as collection of dots on a plot with each dot representing one cell. The flow cytometer will provide a FSC vs. SSC plot, with FSC correlating with cell volume and SSC correlated with cell complexity (i.e. nuclear shape, granules, etc). The flow

cytometer will also provide evaluation of fluorescence using either a histogram (for a single fluorescent marker) or a dot plot (if evaluating two or more fluorescent markers simultaneously). Once a forward/side scatter plot is configured, the cell population of interest is extracted as a subset for further analysis. This subset extraction is called "gating," or telling the cytometer which population to evaluate further for other markers/fluorescence.[52-53] The investigator can then make conclusions based on the quantified populations or, of even greater utility, sort the populations, extracting the cell subset of interest for further functional experiments.

In veterinary medicine, we are just scratching the surface of Treg investigation, especially as it pertains to cancer. Tregs are characterized by clusters of differentiation (CD) on their cell surface, specifically CD4 and CD25, in combination with the intracellular protein FoxP3. At the time that research was generated for this thesis, there was no commercially available anti-canine CD25 antibody for use in the flow cytometric identification of Tregs, and thus the three prior published canine cancer Treg studies used only CD4 and FoxP3 positivity to define Tregs in their study subjects.[54-56] It was therefore important for our research to optimize the correct identification of Tregs.

Because the main goal of this research was to investigate T regulatory cells in canine cancer patients, it was necessary to first evaluate the effect of age on the immune system, specifically on the Treg subset. Cancer most often occurs in geriatric patients, thus, it was imperative to evaluate the effect of aging on Tregs in healthy individuals, prior to making comparisons in tumor bearing patients. The concept of immune senescence describes the gradual deterioration of the immune system's efficiency that occurs in all species in parallel with the aging process. There are multiple studies

documenting a significant difference in T cell subsets in the spleen and thymus of old and young mice[57-61] as well as humans[62-64] while other studies specifically noted an increased percentage of Tregs in the blood, spleen, and lymph nodes of aged mice with various suppressor abilities.[65-66] Regardless of the responsible mechanisms/cell subsets, impaired central tolerance and a reduction in the diversity of the T cell repertoire may be key factors in immune dysregulation in the elderly, and peripheral mechanisms for immune regulation may become increasingly important. At the present time, the effects of aging on Tregs have only been studied in murine and human subjects and it is necessary to establish a basic understanding of Treg trends in our companion animal patients so that these trends may be taken into account when diseases processes, such as cancer, are studied.

The goals of this Master's project were to validate the commercially available anti-human CD25 antibody for use in our canine flow cytometry assay to more accurately identify canine Tregs. We then wanted to determine if there was a difference in CD4+CD25+FoxP3+ Treg percentages between healthy young, middle aged, and elderly dogs to use as a baseline for comparison when quantifying Tregs in diseased patients of different ages. This is especially important in canine cancer patients as this disease typically affects elderly patients. Finally, we wanted to compare %Tregs in the peripheral blood and tumor draining lymph node of cancer bearing dogs with those of healthy controls to determine if a difference exists. If a difference truly exists, this may be exploited to specifically target these cells to allow our patients to mount a more potent anti-tumor immune response.

CHAPTER 2: TREG ORIGIN, FUNCTION, AND IDENTIFICATION

2.1 Treg Origin

In general, there are two subsets of Tregs which differ in their origin, antigen specificity, and effector mechanisms. The first subset, thymic or naturally derived Tregs, develops during the normal early stages of fetal and neonatal T-cell development and maturation in the thymus. These polyclonal cells develop when CD4+ thymocytes react with self-antigens in the thymus.[67-68] Once generated, the Tregs are released into the periphery where they function to prevent the activation of other, self-reactive T cells that have the potential of developing into detrimental effector cells.[69] Unlike classic T cell subsets which express CD25 only transiently when activated, natural Tregs maintain high levels of CD25 likely due to continuous exposure to peripheral autoantigens in the periphery under non-inflammatory conditions.[70-71] These natural Tregs continually survey for self-antigens in healthy individuals under normal conditions.

The second subset, adaptive Tregs, come about as a result of mature T cell activation under conditions of sub-optimal antigen exposure and/or the necessary dendritic cell co-stimulation.[72-73] Like natural Tregs, adaptive Tregs arise from the thymus but can be derived from both classical T-cell subsets and naturally derived Tregs. They also express CD25 but their level of expression varies with the specific disease and location.[74] In essence, adaptive Tregs differ from their naturally derived counterparts not by their origin, but by their need for further activation and functional differentiation according to the context of their antigen exposure.[75-77]

2.2 Treg Function

Though there is some controversy surrounding the exact means by which Tregs modulate effector cell function, most studies support one or more of four major mechanisms: secretion of inhibitory cytokines, induction of cytolysis, disruption of metabolic function, and modulation of dendritic cell maturation or function. The major suppressive cytokines presumably utilized by Tregs are interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), which inhibit the effector activity of both CD4+ and CD8+ T cells [**Figure 1**]. These cytokines, when released from Tregs, may also play a role in subsequently stimulating adaptive/induced Treg cell populations. Despite the well-defined suppressive role of these cytokines, their specific responsibility in the function of thymic-derived naturally occurring Tregs is still controversial based on studies supporting contact-dependent modulation (the requirement of cell to cell contact in order for suppression to occur).[15,17] In contrast to most in vivo studies, in vitro work suggests that IL-10 and TGF-β may not be essential for Treg function.[15,17,78-79] In various disease models, suppression of effector T cells appears to be cytokine dependent but there is much disagreement of whether or not the production of these cytokines by Tregs themselves is required for suppression.[80-88] Several studies have also found a significant difference in the molecular form of the cytokine, for example, membrane tethered TGF-β appears to play a crucial role in maximal Treg function while secreted TGF-β does not, supporting the "contact dependence" theory.[89-90] More recently, IL-35 was added to the Treg inhibitory cytokine repertoire and appears to be required for maximum Treg activity.[91-92] It is crucial to remember that though IL-10, IL-35, and TGF-β all play a role

in Treg function, their utilization in different disease settings varies and requires further elaboration.

Cytolytic killing is well known to be the main function of Natural Killer (NK) cells and CD8+ cytotoxic T lymphocytes (CTLs). However, activated naturally occurring Tregs were recently found to express granzyme B and perforin and selectively inhibited effector cells in vitro.[93-95] Furthermore, in murine models, Tregs derived from melanoma and lymphoma tumor microenvironments could induce NK and cytotoxic T cell death in a granzyme B- and perforin-dependent fashion.[96] [**Figure 2**] Therefore Tregs may directly destroy effector cells in addition to simply modulating their activity.

Several forms of "metabolic disruption" have been described for Treg function. One proposed mechanism is that of cytokine "deprivation," that is, the Treg cell utilizes its IL-2 receptor (IL-2R=CD25) to consume any local IL-2, thus depriving effector T cells of their major stimulatory cytokine and potentially even inducing deprivation mediated apoptosis.[17,97-98] However, given that IL-2 binding to the Treg IL-2R can actually cause overall Treg suppressive activity, further research is necessary to elucidate the precise effect of IL-2 binding to CD25.[99] Another suggested form of disruption is the generation of adenosine, which, upon binding to the adenosine receptor 2A, suppresses effector cell function and enhances Treg function.[100-103] A third and final mode of metabolic disruption is the transfer of cyclic AMP (cAMP), an inhibitory second messenger, into target effector cells via membrane gap junctions.[104] **Figure 3** describes the three common forms of metabolic disruption.

Tregs also indirectly suppress effector cells by targeting the dendritic cells (DC) required for their activation. Intravital microscopy has documented cell-to-cell contact among Tregs and DCs in vivo and this contact is thought to function to attenuate effector cell activation.[105-106] The Treg binds to the effector cell CD80/86 molecule via its co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA-4) and results in DC expression of the regulatory molecule indoleamine 2,3-dioxygenase (IDO). [**Figure 4**] [15,107-109] IDO is the first and rate-limiting enzyme of tryptophan catabolism, thus causing depletion of tryptophan which can cause halted growth of microbes as well as T cells. Other studies suggest that Tregs have the capability of down-regulating DC co-stimulatory molecules CD80/86, thereby impeding effector cell activation.[110]

Until more research is done evaluating Treg function, a single mechanism of suppression cannot be defined and it is reasonable to assume that Tregs have multiple mechanisms of action and utilize these mechanisms appropriately in their disease context.

2.3 Identification of Tregs:

Tregs can be identified both phenotypically and functionally. Surface markers CD4 (conferring a helper cell phenotype) and CD25 (the alpha chain of the IL-2 receptor) and the intracellular transcription factor, FoxP3, define the most commonly accepted phenotype. Phenotypic evaluation, however, can be challenging because each of these proteins are found to be upregulated in other (non T-reg) T-cells that have been activated by a variety of mechanisms. Initially, rodent studies found that the Forkhead Box 3 (FoxP3) transcription factor uniquely identified a highly enriched Treg population.[111-116] FoxP3 is the protein product of the *foxp3* gene which maps to the p arm of the human X-

chromosome, specifically X*p*11.23.[117] The FoxP3 protein serves as a transcriptional repressor of nuclear factor of activated T-cells (NFAT) and nuclear factor-kappa B (NFκB), which leads to the suppression of interleukin (IL)-2 secretion.[118-119] Mice that lack a functional FoxP3 protein (due to a spontaneous loss-of-function mutation) develop scurfy, a fatal auto-immune-like disease characterized by hyper-functional CD4+ T cells.[111,120] It is for this reason that the FoxP3 protein is often referred to as "scurfin." Humans that lack functional FoxP3 develop a severe immune-mediated disease involving immunodysregulation, polyendocrinopathy, and enteropathy, X-linked syndrome (IPEX), which closely resembles mouse scurfy.[18,117,121-122] However, although FoxP3 appears to be required, it is not sufficient for Treg function, because in human studies, FoxP3 could be induced in activated CD4+ T cells that did not possess suppressive activity.[123-128] This finding suggests that FoxP3 expression alone does not confer a Treg phenotype. In 1995, Sakaguchi and colleagues determined that Treg suppressor activity was confined to the subset of T cells that constitutively expressed CD25, the alpha chain of the IL-2 receptor.[9] However, non-regulatory T cells were also found to express CD25, thus suggesting the need for functionality assays to truly determine Treg phenotype. In 2005, Dannull et al found that FoxP3 was expressed almost exclusively in the subset of CD4+ cells expressing CD25 at high levels.[48] In addition, CD4+CD25+(high) cells were found to have distinct regulatory activity over CD4+CD25- and CD4+CD25+(low) cells.[129-130] Finally, Zhao and coworkers documented that the majority of $CD4^{+}CD25^{+}$ T cells are $Foxp3^{+}$ and very few $CD4^{+}CD25^{-}$ T cells express Foxp3 in young mice.[131] Thus, in most recent literature, Tregs are (at minimum) defined by the phenotype CD4+CD25+FoxP3+ in addition to functionality assays to truly assess suppressive behavior.

CHAPTER 3: CLONING OF CANINE CD25 AND VALIDATION OF THE ANTI-HUMAN CD25 ANTIBODY

At the time of generating the data for this thesis, Treg studies in canine cancer [54-56] had been limited to only the CD4+FoxP3+ phenotype because of a lack of a commercially available anti-canine CD25 antibody. There are rare publications suggesting the cross-reactivity of the anti-human CD25 antibody with the canine CD25 protein based on documenting increased antibody activity in mitogen stimulated T cells. [132-134] Almost simultaneous to the research described in this chapter, Mizuno et al found the anti-human CD25 antibody to be cross-reactive with the canine protein.[135] The goal of this chapter is to describe validation of the anti-human CD25 antibody to provide a more specific marker to distinguish canine Tregs.

3.1 RNA isolation from lymph node

A popliteal lymph node was removed in a sterile fashion within 5 minutes after euthanasia from an apparently healthy dog euthanatized in an animal shelter for reasons related to population control. Five 2 mm sections were placed into sterile tubes, immediately submerged in liquid nitrogen and stored at -80^{o}C for 1 week. Isolation of RNA from a single section of lymph node tissue was performed using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) according to a previously described protocol.[136] Briefly, the lymph node tissue was homogenized in a reagent containing phenol and guanidine thiocyanate and the simultaneous isolation of RNA, DNA and proteins was accomplished in a single step by a liquid-phase separation (the upper phase contained the RNA, the interphase contained DNA, and the lower phase contained proteins). The RNA was then

resuspended in 100 ul of nanopure water for RNA quantification based on UV absorbance (NanoDrop spectrophotometer; Thermo Scientific, Waltham, MA).

3.2 Reverse transcription and generation of CD25 cDNA

Reverse transcription was performed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions using either oligo(dT) or random primers. Subsequently, three sets of forward and reverse primers (Invitrogen, Carslbad, CA) were designed based on sequences flanking the canine CD25 gene (Broad Institute Dog Genome Sequencing Project, GenBank accession number NW_876291.1; Table 1), using the algorithm of Integrated DNA Technologies (www.idtdna.com) to optimize annealing temperatures and to minimize the formation of primer dimers. The primers were used to amplify the CD25 cDNA using Pfu enzyme with the following heat cycle conditions: 1. 94 ^{o}C for 2 min 2. 94 ^{o}C for 30 sec 3. 55 ^{o}C for 30 sec 4. 68 ^{o}C for 2 min 5. Repeat step #2 for 34 cycles 6. 68 ^{o}C for 10 min. PCR products were separated by agarose gel electrophoresis to visualize the expected product of approximately 800 bp. The product of primer pair #1 was selected and used in the remainder of the experiment. The primer pair is given in **Figure 5**.

3.3 Cloning of the CD25 cDNA

A terminal adenosine base was added to the PCR products by incubation with 0.5 ul Taq enzyme and 0.2 mM dNTPs at 72^{o}C for 10 min. The PCR products were then cloned into the Topoisomerase Vector, pCR2.1 Topovector (Invitrogen, Carsbad, CA) according to manufacturer's instructions. Subsequently, two ul of topovector reaction

mix (containing PCR product) was used to transform 50 ul of competent E. coli cells (One Shot® MAX Efficiency® DH5α™-T1^{R} Competent Cells, Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Twenty ul of the transformed cells and 40 ul of 5-bromo-4-chloro-3- indolyl-β-galactopyranoside (Xgal) were plated on ampicillin agar plates . The topovector contains an ampicillin resistance gene and thus only those E. coli colonies that had been successfully transfected would survive and proliferate on the plate. The topovector contains the insertion site for the PCR product within the *lacZ* gene encoding the β-galactosidase enzyme. Thus, when plated with X-gal, a blue E. coli colony indicates an intact *lacZ* gene i.e. the absence of the CD25 PCR product, while the lack of blue color (i.e. white colony) indicates the disruption of the *lacZ* gene with our PCR product. After incubation overnight at 37 ^{o}C, 10 white E. coli colonies were harvested and subcultured in 2 ml of 2x YT media and 50 ug/ml of ampicillin. Plasmid DNA was subsequently isolated using a small scale preparation protocol.[137] Agarose gel electrophoresis was used to determine the appropriate restriction pattern after digestion of the plasmid DNA using the EcoRI restriction site (New England BioLabs, Ipswich, MA). Several candidate clones were sequenced (University of Missouri DNA Research Core), and the identity of the clones was confirmed by alignment with published dog CD25 sequences (GenBank accession numbers NW_876291.1, AACN010128068.1, AACN010012922.1). One clone with the complete 807 base pair sequence of the canine CD25 cDNA was chosen for subsequent experiments (GenBank: GU207463.1)[**Figure 6**].

3.4 Expression of canine CD25 in mammalian cells

In order to conduct expression studies, the CD25 sequence was subcloned into the mammalian expression vector, pCMVtag2 (Stratagene products, Agilent technologies, La Jolla, CA), using BamHI and EcoRI restriction sites engineered into the amplification primers. The resulting construct, pCMV-CD25, was verified by restriction enzyme digestion and sequence analysis. Column-purified plasmid DNA (PureLink HiPure Plasmid DNA purification Kit, Invitrogen , Carslbad, CA) was used for subsequent transfection experiments. Two ul of the pCMV Tag2C expression vector was used to transform 50 ul of DH5αcompetent E. coli according to manufacturer's instructions. These cells were cultured in Kanamycin to exploit the kanamycin resistance gene engineered into the pCMV Tag2C expression vector. A large scale preparation of these E. coli cultures was performed to isolate purified plasmid DNA (both pCR 2.1 Topovector/CD25 insert and the pCMV Tag2C expression vector) following the specific Maxi preparation protocol (PureLink HiPure Plasmid DNA purification Kit, Invitrogen , Carslbad, CA). The DNA was then resuspended in Tris EDTA (TE) (Invitrogen, Carlsbad, CA) TE buffer for quantification via UV absorbance.

Restriction enzyme digestion of pCR2.1 Topovector/CD25 insert and pCMV vector was then performed Ecor1 (New England BioLabs,® Ipswich, MA) BamH1 (New England BioLabs,® Ipswich, MA), and heating at 37 ^{o}C for 3 hours. Agarose gel electrophoresis was again performed to separate the CD25 insert from the Topovector and to visualize the linearized pCMV vector. The ~807 bp band (CD25) and the 4.2 kb band (linearized PCMV vector) were cut out of the agarose using a sterile 11 blade.

To extract the DNA from the gel, the DNA containing agarose segments were centrifuged over a glass wool pellet at 10,000 rpm for 5 minutes. Butanol was added and the tube centrifuged to adsorb the water and ethidium bromide remaining with the DNA. The supernatant was discarded and 400 ul of chloroform added and the tube centrifuged at 10,000 rpm for 30 seconds. The supernatant was transferred to a separate tube and 10 ul of 3.2 M Tris HCL + 500 ul of 100% of ethanol added for overnight precipitation in a -20^{o} C freezer.

Ligation of the expression vector and CD25 was performed by combining 100 ng of vector and 20 ng of CD25 insert with 1 ul of DNA ligase and incubating at room temperature for 1 hour. Five ul of ligated vector/CD25 insert was then added to 75 ul DH5αcompetent E coli cells The E. coli cells were plated onto Kanamycin agar plates and incubated at 37^{o} C at 5% CO2 overnight. Transformed E. coli colonies were harvested in 2 mls of 2Xyt media and 2 ul of kanamycin (1X concentration) and incubated overnight at 37^{o} C in a plate shaker. This was followed by a small scale preparation to isolate “impure” plasmid DNA from bacteria as described above. Finally, Ecor1 and BamH1 digestions were run on a 1% agarose gel at 100 V for 30 minutes to ensure correct size of the construct. Confirmation of the canine CD25 gene was performed by DNA sequencing at the University of Missouri DNA Research Core.

For transfection experiments, HeLa cells (ATCC, Manassas, VA), a human cervical cancer cell line, were cultured in a 6 well plate until approximately 90% confluency. HeLa cells have been well characterized and are known to be CD25 negative. Between 0 and 6 ug of pCMV-CD25 was mixed with OPTI-MEM and Lipofectamine 2000 at a DNA-reagent ratio of 1:3. Sonicated salmon sperm DNA was

added where appropriate to adjust the total DNA content for each reaction. After 30 min, the complexes were added to the cells. Successful transfection was ascertained by co-transfection of 0.5 ug of a gfp (green fluorescent protein) expression plasmid. Following incubation at 37^{o} C at 5% CO2 for 24 hours, the plates were washed with enzyme free dissociation buffer (GIBCO, Invitrogen, Carlsbad, CA) and the cells collected and processed for flow cytometry.

3.5 Confirmation of CD25 expression using flow cytometry

HeLa cells were aliquoted at 1 X 10^6 cells/well after counting on a hemacytometer and plated in a 96 well round-bottom plate. The cells were then washed 3 times with fluorescence-activated cell sorting (FACS) buffer (PBS with 3% FBS and 0.09% sodium azide). The cells were incubated with either 10 ul of anti-human CD25 monoclonal antibody (#RO811, clone ACT-1, Dako , Carpinteria, CA) or 10 ul of mouse IgG1 R-phycoerythrin conjugated isotype control (clone 15H6, Southern Biotech, Birmingham, Alabama) in 40 ul of FACS buffer. The same antibodies were added to non-transfected HeLa cells for use as a control to check for baseline human CD25 expression. Cells were incubated in the dark at 4 ^{o}C for 60 minutes and then washed 3 times as above, but using ice cold FACS buffer and a 4 ^{o}C centrifuge. Flow cytometry was performed on a Cyan ADP Flow Cytometer (Becton Dickenson, Franklin Lakes, NJ).

3.6 Expression of CD25 on non-transfected and transfected HeLa cells: The human anti-CD25 antibody recognizes the canine CD25 protein

Flow cytometric analyses of the non-transfected human HeLa cells demonstrated a lack of surface expression of CD25 using the anti-human CD25 antibody [**Figure 7**]. In contrast, the canine CD25-transfected HeLa cells showed expression of the canine CD25 protein which was recognized by the anti-human CD25 antibody. Additionally, by increasing the amount of CD25 expression vector used, a dose response was noted with a corresponding increase in the percentages of CD25 PE positive cells so that 0.5 ug of transfected CD25 resulted in 40.11% expression and 2 ug of transfected CD25 resulted in 71.06% expression [**Figure 8**].

CHAPTER 4: TREGS AND AGING CHANGES

4.1 Immune senescence

The immune system has a complex and very dynamic organization that changes constantly throughout the host's life. Immune senescence is the gradual deterioration of the immune system's efficiency in appropriate responses that occurs in parallel with the aging process. This process occurs in all species regardless of life expectancy and has been found to be more a product of relative life span rather than chronological time. Immune senescence is manifested as reduced immune function due to the functional decline of memory cell populations, as well as an increased susceptibility to infections and malignancy due to the decline in production and function of lymphocyte effector populations. There are multiple studies documenting a significant difference in T cell subsets in the spleen and thymus of old and young mice[57-61] as well as humans,[62-64] suggesting a shift from immune surveillance and homeostasis to dysregulation and often, dysfunction.

The mechanisms of this process are poorly understood. One theory suggests that thymic involution and decreased peripheral trafficking[138] are likely contributing factors to the contraction of the T-cell receptor (TCR) repertoire and the emergence of oligoclonal T cell populations in aged mice[139] as well as humans.[140] This process results in an overall decreased TCR diversity and thus, dysfunctional immune surveillance. Another potential mechanism is the accumulation of Tregs resulting in a decline in lymphocyte effector function. Numerous studies have documented an increased Treg percentage in the peripheral blood of aged humans[141-142] and mice.[131] However, the correlation

between increased Tregs and immune dysfunction may not be that simple as Tregs may preferentially inhibit certain effector cells differently than others. Zhao et al demonstrated an increased percentage of CD4+CD25+ cells expressing FoxP3 in the blood, spleen, and lymph nodes of aged mice. However, in functionality assays, the investigators demonstrated that Tregs from aged mice had a significantly decreased ability to inhibit delayed hypersensitivity reactions and IL-2/IFN-γ production but not the cell proliferation of effector T cells.[131] This evidence may support increased incidence of both auto-immune disease (due to decreased Treg suppression of IL-2 and IFN-γ production) as well as malignancies (due to increased Treg suppression of anti-tumor lymphocyte proliferation) in aged individuals. Other studies further complicate the immunologic picture preventing a clear understanding of exactly how Tregs in aged individuals contribute to a higher incidence of disease. Tsaknaridis et al[143] documented a decreased function of CD4+CD25+ Tregs while Nishioka et al[144] documented that though Tregs were slightly increased in number in aged mice they retained similar suppressive function to those of young mice. Interestingly, Nishioka and colleagues found that the CD4+CD25- T cells in the aged mice were hyporesponsive to paracrine signals implicating T helper cell hypoactivity rather than an increased suppressive activity by Tregs as the main reason for immune pathology associated with aging.

Regardless of the responsible mechanisms/cell subsets, impaired central tolerance and a reduction in the diversity of the T cell repertoire may be key factors in immune dysregulation in the elderly, and peripheral mechanisms for immune regulation may become increasingly important. At the present time, the effects of aging on Tregs have only been studied in murine and human subjects. Because the ultimate goal of this

Master's project was to determine if Treg percentages in canine cancer patients differed from those of healthy controls, it was imperative that we define expected reference values for healthy control patients of different ages, especially considering that the majority of canine cancer patients are elderly. The goals of Chapter 4 were to use the validated cross-reactive mouse anti-human CD25 antibody to identify canine Tregs in peripheral blood and peripheral lymph nodes, and to demonstrate a significant difference in Treg percentages between healthy young, middle aged, and elderly dogs. This information may be useful as a baseline for comparison when quantifying Tregs in diseased dogs of different ages.

4.2 CD4+CD25+FoxP3+ staining of whole blood and peripheral lymph nodes of dogs of different ages

Thirty-nine dogs belonging to the students, faculty, and staff of the University of Missouri Veterinary Medical Teaching Hospital, were determined to be healthy based on history from the owner (lack of gastrointestinal, urinary, neurologic, and respiratory signs) and no detectable abnormalities on physical examination, complete blood count, and serum biochemical profile. All dogs were enrolled in this study with informed client consent. Procedures were approved by the University of Missouri - Animal Care and Use Committee. Lymphocytes from a mandibular lymph node were obtained by aspiration using a 22- gauge needle and incubated in 500ul RPMI-1640 (Invitrogen™, Calsbad, CA). At the same time, 0.5 ml of whole blood was obtained using a 22-gauge needle and vacutainer system and placed in 1 ml EDTA tubes. Samples were processed for flow cytometry within 24-48 hours of collection.

Erythrocytes in whole blood and lymph nodes (when present) were lysed using water followed by the addition of 2X PBS. Cells (peripheral blood mononuclear cells; PBMCs) were then transferred to a 96-well plate at a concentration of 10^6 cells/well, then washed three times in FACS buffer, and incubated with the appropriate antibodies. Whole blood and lymph node cells from one dog were used for appropriate gating and compensation: a well with unstained cells only in 200 µl FACS buffer, a well with rat anti-canine CD4 fluorescein isothiocyanate (FITC) stained cells only (clone YKIX302.9; AbD Serotec, Raleigh, NC), a well with anti-human CD25 PE stained cells only, a well with anti-mouse/rat FoxP3 allophycocyanin (APC) stained cells only (clone FJK-16s, eBioscience, San Diego, CA) and wells with each of two isotype control stained cells: mouse IgG1 PE isotype (clone 15H6, Southern Biotech, Birmingham, Alabama) control and rat IgG2a APC isotype control (clone eBR2a, eBioscience, San Diego, CA). From each study dog, 10^6 cells/well were used for triple staining with anti-canine CD4+ FITC, mouse anti-human CD25+ PE, and anti-mouse/rat FoxP3 APC. Details on the cross-reactivity of the anti-mouse/rat FoxP3 antibody have been published previously.[54]

Cell pellets in the 96-well plate were first stained for the surface markers CD4 and CD25 using 10 ul of the rat anti-canine CD4 FITC antibody and 10 ul of the mouse anti-human CD25 PE antibody in 30 ul FACS buffer on ice for 60 minutes in the dark. Plates were then washed twice with FACS buffer and permeabilized overnight using the eBioscience FoxP3 staining kit (eBioscience, San Diego, CA) according to manufacturer's instructions. Cells were again washed and incubated with 5 ul of the anti-mouse foxP3 APC antibody in 45 ul FACS buffer on ice for 60 minutes in the dark. Cells were again washed twice in FACS buffer and resuspended to a final volume of 400 ul

FACS buffer and 1% formalin in a 12X75 polypropylene tube (#352003, Becton, Dickinson, and Company, Falcon, Franklin Lakes, NJ). All assay preparations were performed by the same individual (KCR).

A CyAn ADP flow cytometer was used for analysis: lymphocytes were first gated on a forward versus side scatter plot and that gate was used to display double positive stained cells (CD4+ CD25+ T cells and CD4+FoxP3+ cells) on an FL1 vs FL2 plot or an FL1 vs. FL8 plot, respectively. The CD4+CD25+ T cells were then applied to a histogram of FoxP3 APC to obtain quantification of the triple positive cells (CD4+CD25+ lymphocytes expressing FoxP3). The number of CD4+CD25+ lymphocytes expressing FoxP3 was also divided by the number of peripheral blood lymphocytes in the gate on the forward versus side scatter plot to provide the %CD4+CD25+FoxP3+ lymphocytes. Finally, cells positive for CD4 were gated on a FITC histogram and were subsequently applied to a FL2 vs. FL8 plot to obtain the % of CD4+ T helper cells that are also CD25 and FoxP3 positive. A minimum of 10,000 events per gate were collected per tube. All flow cytometric assays and subsequent analyses were performed by the same individual (KCR).

4.3 Statistical analysis

Only those samples with a minimum of 10,000 events per gate were evaluated statistically. Statistical analysis was performed to determine if a significant difference between percentages of Tregs, using all previously defined combinations of markers, was present. Non-parametric statistical analysis (Rank Sum tests) was used to compare percentage of Tregs between the defined age groups. A p value <0.05 was considered significant.

4.4 Results of flow cytometric analysis of peripheral blood and mandibular lymph node Tregs

The signalments of 39 healthy dogs, divided into three age strata (< 2 years, 2-6 years, and >/= 7 years) are given in **Table 1**. Five lymph node samples from the healthy dogs were not used due to the low cellularity and insufficient number of events per gate. Representative scatter plots for Treg identification in peripheral blood lymphocytes are shown in **Figure 9**; scatter plots were set up in a similar fashion for lymph node cells **Figure 10**. The median percentages of CD4+CD25+ Tregs, CD4+FoxP3+ Tregs, CD4+CD25+ lymphocytes expressing FoxP3+, CD4+CD25+FoxP3+ Tregs, and CD4+ cells expressing both CD25 and FoxP3 for each subgroup of the healthy controls are shown in **Table2**. There was a significantly decreased % CD4+CD25+ T lymphocytes expressing FoxP3 (triple positive) within the blood of older dogs when compared to the blood of the other two age groups ($p < 0.001$). There were no other significant differences among age groups for any other Treg population.

CHAPTER 5: TREGS IN CANCER BEARING DOGS AND HEALTHY CONTROLS

5.1 Tregs in Human Cancer

In human medicine, the role of Tregs in cancer pathogenesis has been well studied. Based on our knowledge of this unique T cell subset, it is not surprising that an increased Treg proportion correlates with a negative outcome. [30,32,42-46] In numerous human clinical cancer studies, there is a documented increase in the Treg proportion among lymphocytes in peripheral blood[29,34-38,40-41,45,145] and tumor draining lymph nodes of tumor bearing patients when compared to healthy controls.[29,34-35] Further studies investigating the tumor microenvironment showed a higher percentage of Tregs in the malignant ascites of ovarian cancer patients compared to patients with non-malignant ascites, in glioblastoma multiforme tumors compared to normal brain tissue, in invasive breast carcinoma compared to normal breast tissue, and in gastric adenocarcinoma compared to normal gastric mucosa.[30-31,44,146-147] Among tumor infiltrating lymphocytes (TILs), Tregs were higher in later stage compared to earlier stage ovarian carcinomas and in invasive breast carcinoma when compared to carcinoma in situ.[30,147] When survival times were evaluated in mesothelioma and breast carcinoma patients, increased Tregs correlated with decreased overall survival.[44,147-148] In addition, an increased Treg percentage of TILs in breast carcinomas, correlated with tumor invasion, size, microvessel density (MVD), higher grade, nodal positivity, estrogen receptor negativity in addition to a higher risk of relapse and shorter remission times.[44,147]

Not only do Tregs have the potential to predict advanced stage of disease and overall prognosis, but they may offer a novel treatment target. With the recent finding

that the downregulation of tumor specific Tregs allows human patients to mount a more potent anti-tumor response, it seems logical that this immunologic subset be further explored in veterinary medicine as well.[47-51]

5.2 Canine Osteosarcoma

Osteosarcoma (OSA) is the most common primary bone tumor in dogs comprising approximately 85% of all skeletal tumors.[149-153] It is an aggressive bone tumor that is both locally and systemically aggressive. Approximately 77% of OSA occurs in the appendicular skeleton making amputation an excellent treatment option.[150] However, even when there is no visible evidence of pulmonary metastasis, it is likely that 90% of patients have microscopic metastasis at the time of diagnosis. This theory is based on studies showing an approximately 10% one year survival rate for those patients undergoing amputation alone with the majority of patients dying due to metastatic disease.[149,154-155] Thus, for canine osteosarcoma, surgical removal of the primary tumor is considered only palliative, and adjunctive treatment to slow metastatic disease and thus prolong survival is always warranted. Clinical trials consistently report improved survival times compared to amputation alone (10-12 months vs.4-6 months) when adjuvant chemotherapy is administered after the primary tumor is removed via amputation.[156-169] However, the relatively consistent one year median survival time in a multitude of clinical trials suggests that a more targeted therapeutic approach is warranted to overcome the one-year survival threshold associated with amputation and chemotherapy. Currently, there are few other adjuvant therapies with the potential to further prolong the life of our canine osteosarcoma patients.

Due to the correlation of increased Tregs and negative prognosis in the human literature, a study determining the prognostic significance of increased Tregs in veterinary cancer patients is warranted. Up to this point, our spectrum of prognosticators for canine appendicular osteosarcoma is limited to location,[160,166,170-172] presence of metastasis, alkaline-phosphatase positivity,[166,173-176] lymph node involvement,[177] and age.[155,166] Investigation of Tregs in osteosarcoma may provide not only an additional prognosticator, but enable better monitoring of treatment efficacy to allow tailoring of aggressive therapy accordingly. In essence, Tregs have potential to act as biomarkers for monitoring disease progression and treatment failure. What is unique about this "biomarker" is that it may also provide opportunity as a treatment target. If we can modulate the canine immune system to suppress the tumor recruited Tregs, we may allow the patient's own tumor specific T cell response to more efficiently target the tumor cells.

Though Tregs are becoming a more common topic of research, there is not nearly enough information in veterinary medicine. In 2007, Biller et al proved that dogs with various tumor types (4 oral melanoma, 3 osteosarcoma, 2 mast cell tumor, and one soft tissue sarcoma) had a significantly increased proportion of CD4+FoxP3+ Tregs in their peripheral blood and tumor draining lymph nodes compared to the blood and lymph nodes of healthy control subjects.[54] In 2009, O'Neill et al. also documented an increase in % CD4+FoxP3+ Tregs in the blood and tumor draining lymph nodes of canine cancer patients (14 sarcomas, 7 carcinomas, 7 lymphomas, and 6 MCT) compared to those of healthy controls. However, when evaluating the tumor types by etiology, only patients with carcinomas had an increased %Tregs compared to healthy controls. [56] Finally, in another 2009 paper by Horiuchi et al, dogs with metastatic tumors also had a significantly

higher CD4+FoxP3+ Treg% compared to those without metastatic disease.[55] It is important to note, however, that none of these studies evaluated CD4+CD25+FoxP3+ Tregs due to the lack of a canine specific antibody.

The purpose of this study in dogs with spontaneously developing osteosarcoma is to determine the percent of CD4+CD25+FoxP3+ Tregs in blood, tumor-draining lymph nodes, and unrelated lymph nodes (and to compare to healthy dogs), and determine whether treatment affects the % Tregs in peripheral blood and lymph nodes. This pilot information will help us determine if Tregs are important in the body's response to this bone cancer, and will set the stage for future treatments to target this population of immune cells.

5.3 CD4+CD25+FoxP3+ Staining of Whole Blood and Peripheral Lymph Nodes of Dogs with Osteosarcoma

Sixteen dogs with appendicular osteosarcoma (OSA) presenting to the Oncology Service at the Veterinary Medical Teaching Hospital, University of Missouri, were determined to have no other life-threatening or systemic inflammatory disease processes based on physical examination, complete blood count, and serum biochemical profile and no evidence of macroscopic pulmonary metastatic disease on thoracic radiographs. All dogs were enrolled in this study with informed client consent. Procedures were approved by the University of Missouri - Animal Care and Use Committee. Lymphocytes from a submandibular lymph node and the tumor draining lymph node were obtained by aspiration using a 22- gauge needle and incubated in 500ul RPMI-1640 (Invitrogen™, Calsbad, CA). At the same time, 0.5 ml of whole blood was obtained using a 22-gauge needle and vacutainer system and placed in 1 ml EDTA tubes. Samples were processed for flow

cytometry within 24-48 hours of collection. The flow cytometry assay was performed as written above for the healthy control patients and results were evaluated using the same statistical analyses. An additional comparison was made between dogs with osteosarcoma and healthy controls (from Chapter 4). All flow cytometric assays and subsequent analyses were performed by the same individual (KCR).

5.4 Results of Flow Cytometric Analysis of peripheral blood, tumor draining lymph node and mandibular lymph node Tregs of Osteosarcoma patients

The signalments of the 16 Osteosarcoma patients are provided in **Table 3**. Among the OSA patients, one "unrelated" lymph node and six "tumor draining" lymph nodes were removed due to low cellularity and insufficient number of events per gate. Representative scatter plots for Treg identification in peripherally blood lymphocytes and lymph node cells, are as shown in **Figure 9** and **10**, respectively. The median percentages of CD4+CD25+ Tregs, CD4+FoxP3+ Tregs, CD4+CD25+ lymphocytes expressing FoxP3+, CD4+CD25+FoxP3+ Tregs, and CD4+ cells expressing both CD25 and FoxP3 in the blood, unrelated and tumor draining lymph nodes of osteosarcoma patients are shown in **Table 4**. There was a significant decrease in the % of CD4+CD25+FoxP3+ Tregs in the tumor draining lymph nodes of OSA patients compared to the unrelated lymph node in the same patient ($p=0.049$).

5.5 Comparison of Treg percentages between healthy controls and osteosarcoma patients

There were no significant differences found in any of the Treg populations in peripheral blood between osteosarcoma patients and the healthy controls.

CHAPTER 6: DISCUSSION

The study of Tregs has increased incrementally in the last 5 years with new manuscripts being added weekly to the already growing list of Treg publications. There is no denying the importance of this unique T cell subset, especially since it can apply to a variety of pathologic diseases, specifically viral disease, autoimmune disease, and cancer. Based on many years of research, we know that dysfunctional or underdeveloped Tregs predispose to autoimmune disease and overactive or excessive Tregs results in progressive viral disease and neoplasia with a dose dependent relationship that corresponds with stage of disease and prognosis. However, with the ability to perform cell sorting and specifically target Treg populations, cancer researchers have found preliminary success in downregulating these cells to allow the host to mount a more potent immune response.

In the last part of the 20th century, it was discovered that simply depleting Tregs in murine models could potentially induce tumor regression. In 1999, two studies found that after the administration of a monoclonal anti-CD25 antibody to tumor bearing mice, a decrease in CD4+CD25+ T cells (assumed to be Tregs) was noted in the peripheral lymphoid tissues. More importantly, however, the anti-CD25 activity resulted in regression of various different syngeneic tumors.[50,178] Shimzu and colleagues found that when athymic nude BALB/c mice received CD4+CD25+ depleted splenic cell suspensions (via cell sorting), subsequent tumor cell transplants grew initially but then regressed within a 1 month period. Furthermore, this study found a potent induction of cytotoxic lymphocytes in the splenic cell suspensions that were depleted of CD4+CD25+ cells. [50]

6.1 Potential Future Utility of Treg Targeting

More recent studies have evaluated the potential synergistic effects of Treg depletion in addition to cytotoxic chemotherapy. Based on previous work suggesting decreased survival times in mesothelioma patients with increased percentages of CD4+CD25+ cells among TILs,[148] Anraku et al created a murine model of intrathoracic malignant mesothelioma for use in treatment trials. The mice were depleted of Tregs via administration of an anti-CD25 monoclonal antibody, either before or after tumor cell injection. The study found that depletion of Tregs only provided survival benefit if performed prior to tumor induction. However, the addition of pemetrexed chemotherapy (a folate anti-metabolite) to Treg blockade (even after tumor induction) was synergistic and resulted in decreased tumor-infiltrating Tregs, increased IL-2 production, dendritic cell maturation, and increased CD8+ tumor-infiltrating T cells.[179]

Previous phase I clinical trials have suggested that autologous vaccines consisting of tumor RNA transfected dendritic cells were efficacious at inducing a potent cytotoxic T lymphocyte immune response.[180-181] However, if this treatment approach is undertaken without disrupting the strong regulatory pathway, the cytotoxic immune response will be impeded, resulting in only a short lived anti-tumor response. In 2005, Prasad and colleagues exploited the unopposed immune systems of Treg depleted mice, finding them to have prolonged tumor immunity after a tumor antigen loaded dendritic cell vaccine.[182] This was then translated to human oncology when Dannull and colleagues successfully eliminated CD25-expressing Tregs in renal carcinoma patients using a conjugate of recombinant IL-2 and diphtheria toxin (DAB_{389}IL-2) to target and kill cells expressing high levels of CD25. This depletion resulted in improved stimulation of tumor-specific

cytotoxic T cells when patients were subsequently vaccinated with autologous tumor RNA dendritic cell vaccines.[48]

Currently at the University of Pennsylvania, School of Medicine, the novel anti-human CD25 antibody daclizumab is being used in a phase I clinical trial in human patients with metastatic breast cancer. Preliminary results suggest a marked and durable (> 5 weeks) elimination of CD25+FoxP3+ Tregs in the peripheral blood of patients. When these patients are then administered an anti-tumor peptide vaccine during their Treg nadir, they show a successful generation of a cytotoxic T lymphocyte response.[51] Though this adjunctive treatment is still in phase I trials, if promising data continues to be found, this antibody may offer a novel/adjunct treatment modality for cancer patients as they undergo various other forms of therapy.

Interestingly, previously accepted cancer treatment modalities are now being discovered to affect Treg activity as well. Imatinib mesylate (Gleevec, STI571) a selective tyrosine kinase inhibitor, has been used to treat chronic myelogenous leukemias and gastrointestinal stromal tumors in humans by competitively binding to the active kinase domain (ATP-binding pocket) preventing substrate binding.[183-184] Recently, in vitro exposure of murine spleen and lymph node Tregs to clinical doses of imatinib, resulted in decreased Treg cell viability and immunosuppressive function. When mice were treated with imatinib for 7 days, there was an obvious decrease in splenic and lymph node Treg numbers and function when compared to untreated mice. When A20 lymphoma cells were transplanted into the mice, those treated with imatinib had improved responses to a dendritic cell vaccine.[49]

Radiation therapy may also have more beneficial effects than previously thought. Low dose total body irradiation in naïve mice resulted in not only a decreased percentage of Tregs, but an increase in CD4+ and CD8+ effector cells. More importantly, these mice experienced an increased anti-tumor immune response following dendritic cell vaccine and subsequent tumor cell transplant.[185]

It is quite clear that the therapeutic depletion of Tregs improves responses to cancer immunotherapy and may improve survival times of various cancers previously thought to be refractory to treatment. With osteosarcoma (OSA) being the most common canine bone tumor and having an aggressive systemic behavior, it poses a significant therapeutic challenge to veterinary oncologists. Since various canine osteosarcoma treatments have yet to consistently prolong patient survival past a median of 10-12 months, investigation of immune dysregulation in these patients is a reasonable next step. Currently, there are few other adjuvant therapies with the potential to further prolong the life of canine osteosarcoma patients, making immunotherapy appealing for further study. In addition, this disease is strikingly similar to its human counterpart and may serve as an excellent comparative model.

By cloning and transfecting the canine CD25 cDNA and subsequently expressing the canine CD25 protein in human HeLa cells, we were able to document the cross-reactivity of an anti-human CD25 monoclonal antibody in a flow cytometric assay. While other studies [132-134] have suggested this antibody recognizes canine CD25, only this and the simultaneously published research by Mizuno et al, have rigorously documented cross-reactivity with the human antibody.[135] This has important implications for veterinary immunology and translational research in that the presence of CD25 is essential in

identifying the unique T regulatory lymphocyte subset and the dog serves as a model for a number of human conditions in which this regulatory cell plays a critical role. Proper identification of Tregs will be useful for understanding the immune response (or lack thereof) in a variety of diseases including autoimmune, allergic, viral and neoplastic diseases. To date, there have only been three papers discussing Tregs in cancer bearing dogs and all have used only CD4 and FoxP3 positivity to define the Treg phenotype [54-56] In human studies, FoxP3 can be induced in activated CD4+ T cells that do not possess suppressive activity, suggesting that FoxP3 expression is not sufficient to specifically define the Treg phenotype.[123-128] The results of our study confirm that there is a more specific marker to identify this important and understudied population of cells in the dog.

In human and murine studies, a significant change in T lymphocyte subsets has been noted as patients age.[131,141-142] Because osteosarcoma is often a disease of older dogs,[149-153] we first sought to rule out the effect (if any) of aging on the %Tregs in our healthy control group. We found that the percentage of most Treg populations did not significantly increase with age. In fact, the only significant change was a decreased % CD4+CD25+ T lymphocytes expressing FoxP3 within the blood of older dogs compared with the other two age groups. Moreover, the % Tregs in the peripheral blood and lymph nodes of dogs with OSA did not differ significantly from those of healthy controls. However, within the OSA patients, tumor draining lymph nodes had significantly less CD4+CD25+FoxP3+ Tregs than the unrelated lymph nodes. A lack of an increase in Tregs in dogs with OSA is in agreement with a prior study which documented no increases in blood and lymph node %CD4+FoxP3+ Tregs in dogs with sarcomas, despite an increase in % CD4+FoxP3+ Tregs in the blood and tumor draining lymph nodes of

various canine cancer patients when evaluated collectively.[56] That is, only dogs with carcinomas, and not with OSA, had an increased %CD4+FoxP3+Tregs compared to healthy control dogs. Our reasons for selecting OSA patients as a target population included availability of cases, relatively homogeneity of disease, and the relatively consistent standard of care available for treatment. It is not entirely surprising that there were not significant changes in percentages of Tregs in dogs with OSA, as changes in function of Tregs may actually play a more important role in immune system modulation than changes in percentages of Tregs. This remains to be explored in the future, and the identification of a cross-reactive CD25 antibody in the dog will be critical for isolating Tregs for functionality assays. The fact that the tumor-draining lymph node of the osteosarcoma patients had fewer Tregs than the unrelated lymph node may actually fit with newer theories on Treg activity in human cancers. In these studies, Tregs seem to migrate preferentially and predominantly to the tumor mass/ascites and work by inhibiting extranodal effector T cell function rather than naive T cell priming in the lymph nodes (at least in later stages of tumors).[30] Thus, one might theorize that the osteosarcoma patient tumor-draining lymph nodes were being depleted of their Tregs due to recruitment/migration into the tumor mass.

As with most veterinary studies, small case number is often a potential reason for the lack of significance between groups. Another potential confounding factor in this study is the use of the submandibular lymph node as an internal control. We chose this lymph node since it is the most consistently palpable node and the easiest to harvest adequate numbers of lymphocytes for our assay; however, due to its proximity to the oral cavity, there is a strong potential for immune reactivity which may affect the %Tregs. A

final potential reason for the lack of significance between age groups is the discrepancy between quantification of Tregs and their functionality, as mentioned previously. The inhibitory abilities of Tregs are not entirely dependent on the total number of cells and functionality assays are required to delineate the relationship between function and quantity.

The study of cancer and, specifically, advances in treatment, have improved greatly over the past 20 years; however, standard chemotherapy rarely offers a cure for most patients primarily due to a combination of dose-limiting normal tissue toxicity and continually emerging resistance mechanisms. New treatment modalities are essential if further success in survival is to be achieved. Immunotherapy has been studied extensively in humans and has had variable success in clinical applications. The relationship between cancer and the immune system is complex, and attempts at immunotherapy have often failed for unknown reasons. T regulatory cells may be one of the reasons for these failed attempts at immunomodulation in the treatment of various cancers. Thus, further investigation of this unique helper T cell subset is warranted.

The study of T regulatory cells in the dog carries much potential for expanding not only our knowledge of the immune response to various diseases but may provide insight into how to dampen or enhance that response to the advantage of our patients. With a newly validated cross-reactive CD25 antibody, functionality studies on magnetically or fluorescently sorted canine lymphocyte populations can now be performed to assess function of Tregs. Further studies evaluating Tregs in cancer patients with other, potentially more immunogenic tumors, are indicated. In addition, comparing Tregs in patients with different stage tumors and prognoses, as well as the effect of local

and systemic treatment on Tregs over time, may provide a clinically relevant use for quantifying this unique subset of T cells in order to predict case outcome and inform case management decisions.

6.2 Conclusion

Cloning and transfection of the canine CD25 gene followed by expression of the canine CD25 protein has allowed definitive confirmation of cross-reactivity of an anti-human CD25 antibody in a flow cytometric assay. The use of this antibody in canine cancer has not been performed previously and facilitates identification of a very specific subset of T cells, the T regulatory lymphocyte. Using blood or cells from the lymph nodes, the flow cytometric assay to identify %CD4+CD25+FoxP3+ Tregs was not useful to discriminate dogs that were healthy from dogs with OSA. In other words, OSA patients do not appear to have any increased percentage of Tregs compared to healthy controls. As this does not rule out increased effector function of these Tregs, future studies focusing on Treg functionality assays may provide additional information.

FIGURES

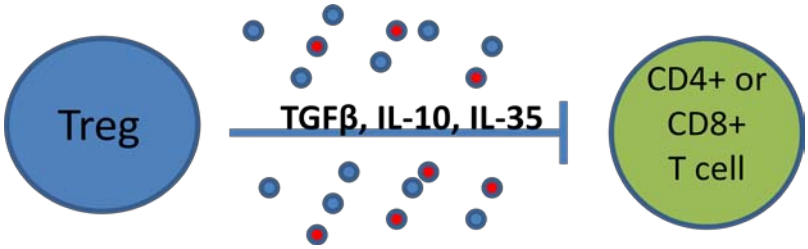

Figure 1: Inhibitory cytokines released by T regulatory cells.

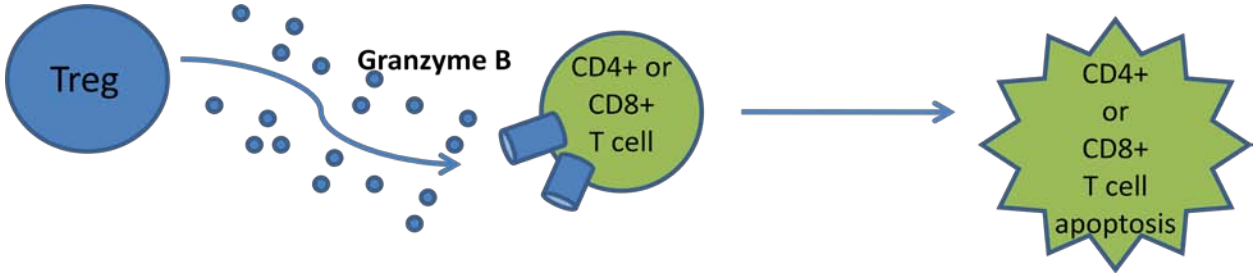

Figure 2: Cytolytic destruction via granzyme B released by T regulatory cells.

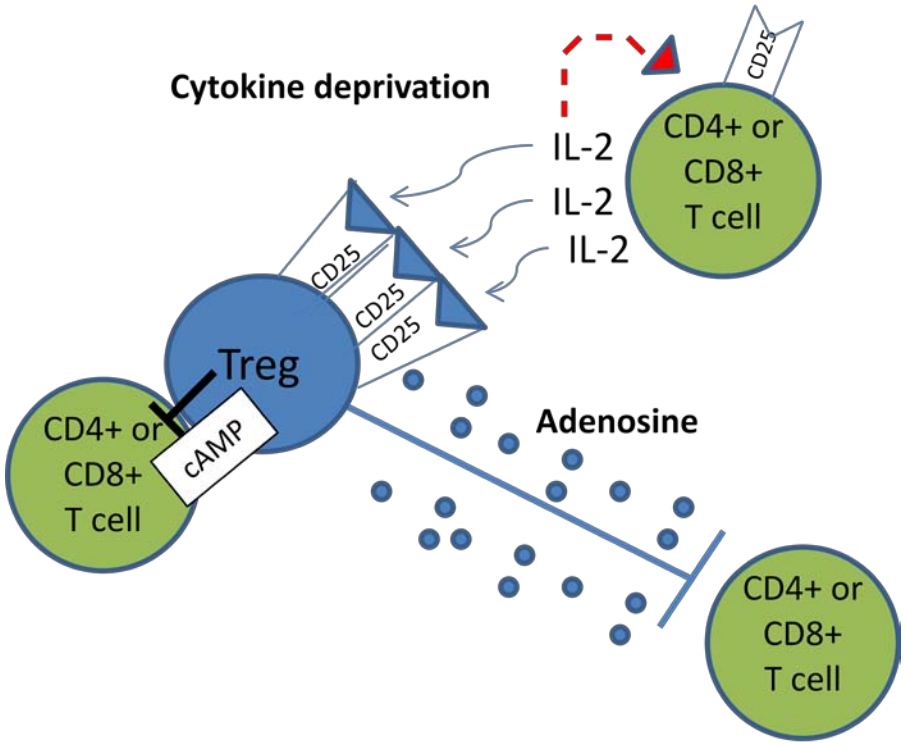

Figure 3: Metabolic disruption of effector T cells caused by T regulatory cells: Adenosine release, cytokine deprivation, and cAMP signaling.

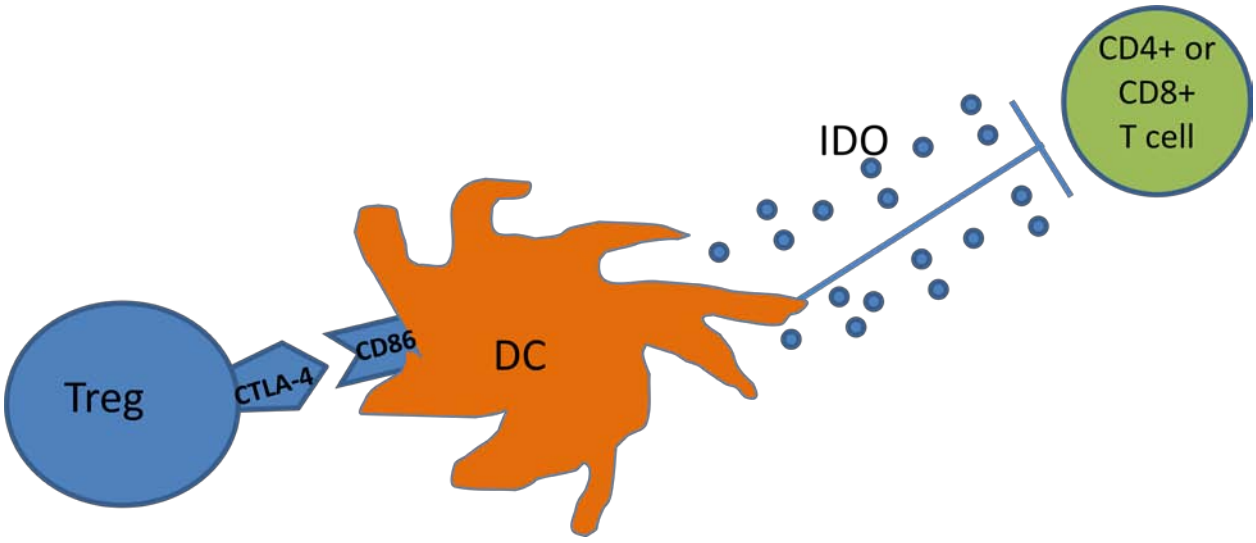

Figure 4: Indirect dendritic cell targeting by T regulatory cells.

Forward: 5'-GGATCCTGGAGCCATGTTTGCTGATGTG

Reverse: 5'-GAATTCGATTGTTCTACTCTTCCTCCTTCTCCG

Figure 5: The sequences of the forward and reverse CD25 specific primers used for PCR to obtain CD25 cDNA.

atggagccat gtttgctgat gtggggaatc cttacattca tcacagtatc tggctacacg
acagacctct gtgatgatga cccaccaaac ctcaaacacg ccacattcaa agctctcaca
tacaagacag gcacggtgtt aaattgtgat tgtgagaggg gcttccgcag aataagcagc
tacatgcatt gtacaggaaa ctctagccat gcttcctggg aaaacaaatg tcggtgcaaa
agtgtatccc cagagaacag aaaaggaaaa gttaccacta aacctgagga acagaaggga
gaaaacccca cagaaatgca gagccaaacg ccgcccatgg acgaagttga ccttgtaggt
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gtggggcaga cacttcacta ccagtgcatg cagggattca cagccctcca cagaggtcct
gccaagagca tctgcaaaac catctttggg aagaccagat ggacgcagcc cccgctcaag
tgcataagtg aaagtcagtt tccagatgac gaagagcttc aagcaagcac tgatgctcct
gctgggaggg acacttcgtc tcccttcata acgacaagta ctccagattt ccacaaacac
acagaagtgg ctacaaccat ggagtcattc atattcacga ccgagtacca gatagcagtg
gcgagctgcg tcctcctgct gatcagcatc gtcctgctga gtgggctcac ctggcagcgg
agaaggagga agagtagaac aatctag

Figure 6: The 807 bp DNA sequence of our CD25 clone chosen for further experiments.

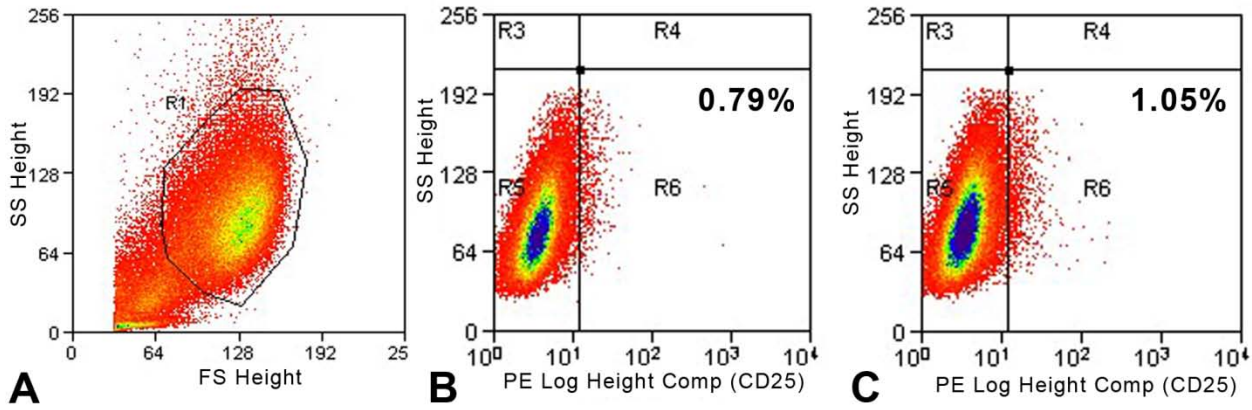

Figure 7: Non-transfected HeLa cells (a human cervical cancer cell line) were used in a flow cytometric assay to determine surface expression for CD25 using a mouse anti-human CD25 PE antibody. Numbers represent the percentage of positive cells in each quadrant. (A) A forward/side scatter plot demonstrates gating of the HeLa cell population for further analysis. (B) A scatter plot of the non-transfected HeLa cells stained with the mouse IgG1 PE isotype control antibody shows minimal reactivity. (C) A scatter plot of the non-transfected HeLa cells stained with the anti-CD25 PE antibody also shows minimal reactivity.

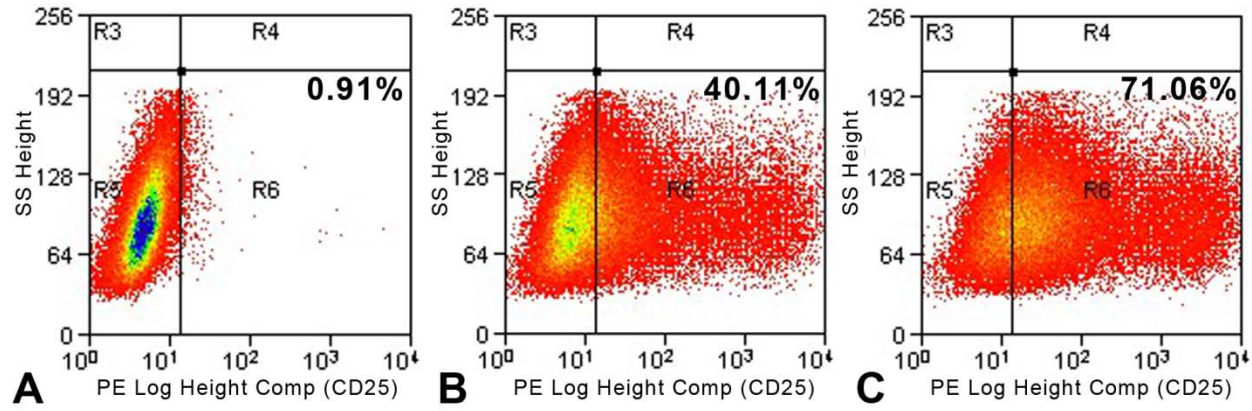

Figure 8: HeLa cells were transfected with canine CD25 and subsequently evaluated for surface expression of CD25 using a mouse anti-human CD25 PE antibody. Numbers represent the percentage of positive cells in each quadrant. (A) A scatter plot of the canine CD25-transfected HeLa cells stained with the mouse IgG1 PE isotype control antibody documents minimal reactivity. (B) A scatter plot of the canine CD25-transfected HeLa cells using 0.5ug of pCMVCD25 plasmid documents cross-reactivity of the anti-human CD25 antibody. (C) A scatter plot of the canine CD25-transfected HeLa cells using 2ug of pCMV-CD25 plasmid demonstrates a dose–response effect. Further increases in the amount of pCMV-CD25 plasmid (4ug and 6ug) did not result in higher cellular expression for CD25 (data not shown).

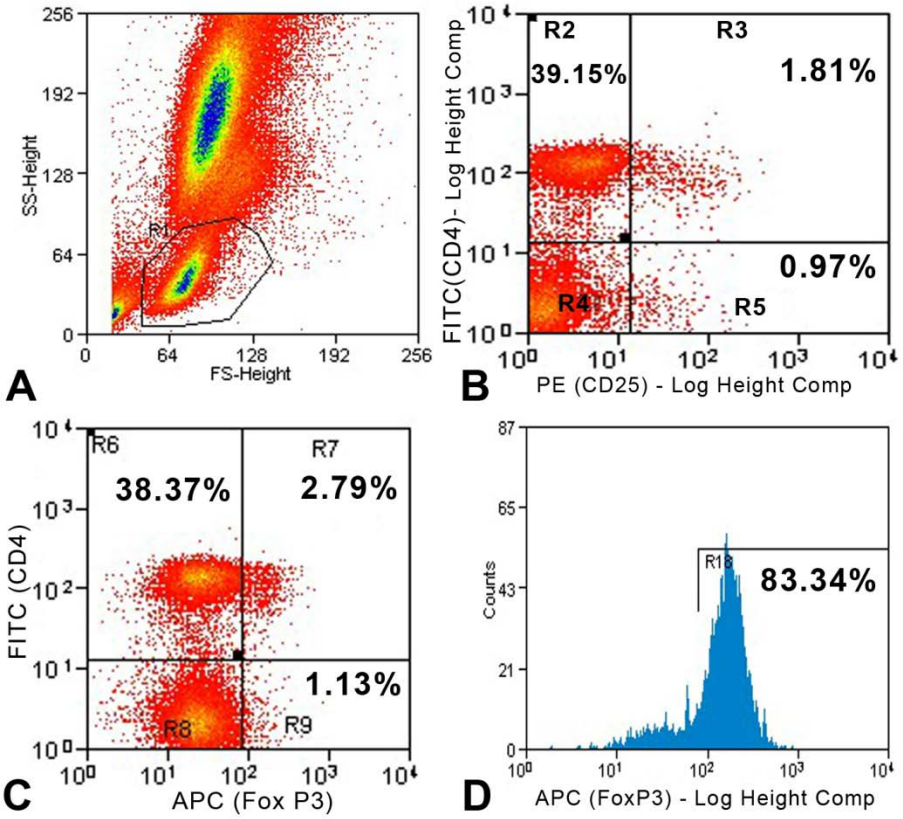

Figure 9: Peripheral blood mononuclear cells (PBMCs) were used in a three-color flow cytometric assay to identify surface expression of CD4 and CD25, and the intracellular transcription factor FoxP3 in healthy dogs of various ages. Results of PBMC staining from a representative dog are shown. (A) Cells were first gated on a forward/side scatter plot to specify the lymphocyte population for further analysis. (B) A PE vs. FITC scatter plot demonstrates staining for CD25 vs. CD4 positivity, respectively. Double positive cells are shown in R3 and are used in subsequent analysis of CD4+CD25+ cells expressing FoxP3. (C) An APC vs. FITC scatter plot demonstrates FoxP3 vs. CD4 positivity, respectively. (D) A histogram of Fox P3 gated on the CD4+CD25+ population (R3) noted in (B) shows CD4+CD25+ cells expressing FoxP3. Additionally, the absolute numbers of CD4+CD25+ cells expressing FoxP3 were divided by the absolute numbers of lymphocytes in the FSC/SSC gate (A) to give the CD4+CD25+FoxP3+ cells (data not shown).

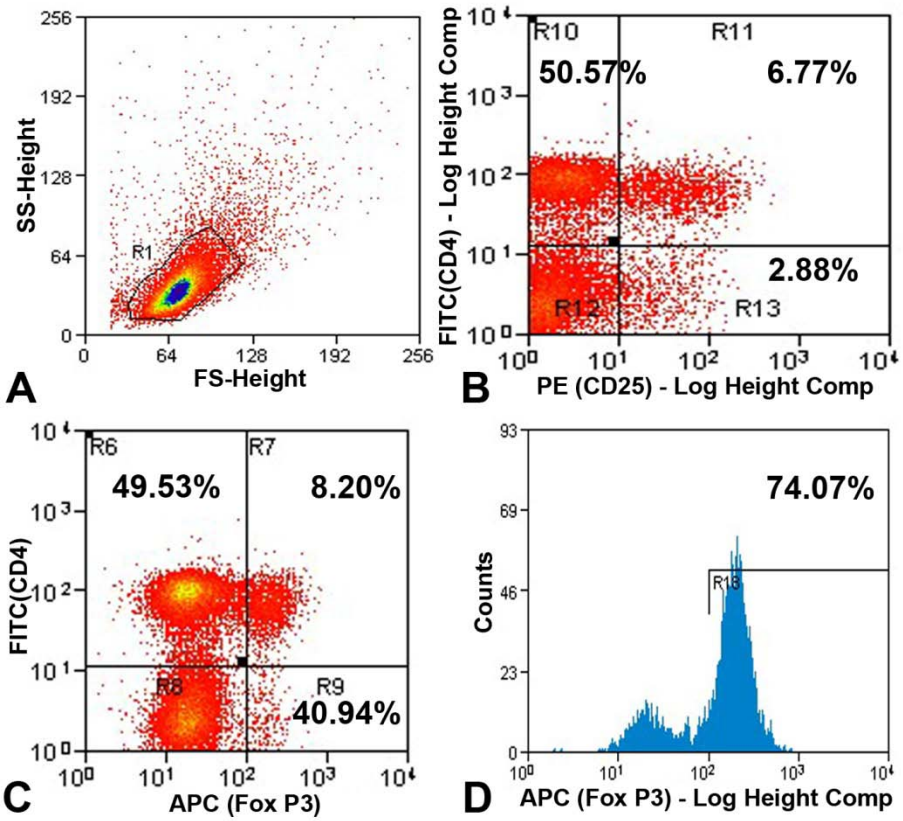

Figure 10: Lymph node cells were used in a three-color flow cytometric assay to identify surface expression of CD4 and CD25, and the intracellular transcription factor FoxP3 in healthy dogs of various ages. Results of lymph node cell staining from a representative dog are shown. (A) Cells were first gated on a forward/side scatter plot to specify the lymphocyte population for further analysis (NOTE: this is the majority of cells since the source is a lymphoid organ). (B) A PE vs. FITC scatter plot demonstrates staining for CD25 vs. CD4 positivity, respectively. Double positive cells are shown in R11 and are used in subsequent analysis of CD4+CD25+ cells expressing FoxP3. (C) An APC vs. FITC scatter plot demonstrates FoxP3 vs. CD4 positivity, respectively. (D) A histogram of Fox P3 gated on the CD4+CD25+ population (R11) noted in (B) shows CD4+CD25+ cells expressing FoxP3. Additionally, the absolute numbers of CD4+CD25+ cells expressing FoxP3 were divided by the absolute numbers of lymphocytes in the FSC/SSC gate (A) to give the CD4+CD25+FoxP3+ cells (data not shown).

TABLES

	Controls < 2 yrs	Controls 2-6 yrs	Controls >/= 7 years	HEALTHY CONTROLS (ALL)
Cases	11	16	12	39
Spayed females/Intact females	3/1	9/0	6/0	18/1
Castrated males/Intact males	7/0	5/2	6/0	18/2
Median Age (yr)	1.15	4	8	4.21
Range (yr)	0.9-2	3-6	7-13	0.9-13
Mean age +/- SD	1.25 +/- 0.5	4.04 +/- 1.02	8.83 +/- 2.38	4.81 +/- 3.44
Median weight (kgs)	24.4	23.4	23.4	23.6
Mean weight +/- SD	25.4 +/- 10.2	24.5 +/- 8.8	25.8 +/- 9.6	24.6 +/- 9.3
Breeds	French bulldog (3), Golden Retriever(3), Great Dane (2), mixed breed (1) Labrador (1), Newfoundland (1)	Mixed breed (7), Labrador (3), Greyhound (2), German Shepherd (1), American Staffordshire Terrier (1), Brittany Spaniel (1), Hound (1)	Pointer,(2) Weimeraner (2) Labrador (2), German Shepherd (1), Collie (1), Mixed breed (1),English Spaniel (1), Cocker Spaniel (1), Dogue de Bordeaux (1)	Mixed breed (9), Labrador (6), French Bulldog (3), Golden Retriever (3), German Shepherd (2), Great Dane (2), Pointer (2), Weimeraner (2) Greyhound (2), American Staffordshire Terrier (1), Newfoundland(1), Brittany Spaniel (1), Hound (1), Dogue de Bordeaux (1), , Cocker spaniel (1), Collie (1), English spaniel (1),

Table 1: Healthy control patient signalment.

	Controls < 2 yrs	Controls 2-6 yrs	Controls >/= 7 years	HEALTHY CONTROLS (ALL)
BLOOD				
CD4+CD25+	2.12%	1.80%	2.49%	1.98%
CD4+CD25+FOXP3+	1.00%	0.98%	1.09%	0.98%
CD4+FOXP3+	2.17%	2.81%	2.50%	2.63%
CD4+CD25+ lymphocytes expressing FoxP3	55.29%	53.23%	*32.20%	47.01%
CD4+ cells expressing CD25 and FoxP3	1.97%	1.67%	2.5%	1.97%
LYMPH NODE				
CD4+CD25+	8.11%	5.55%	9.14%	7.15%
CD4+CD25+FOXP3+	5.79%	4.33%	6.16%	5.36%
CD4+FOXP3+	9.05%	8.20%	9.92%	9.25%
CD4+CD25+ lymphocytes expressing FoxP3	72.62%	73.19%	66.79%	71.68%
CD4+ cells expressing CD25 and FoxP3	9.54%	8.66%	11.97%	9.62%

* There was a significantly decreased % CD4+CD25+ T lymphocytes expressing FoxP3 within
the blood of older dogs when compared to the blood of the other two age groups ($p < 0.001$).

Table 2: The median % Tregs in the peripheral blood and unrelated lymph nodes within each age group of the healthy controls

OSA PATIENTS	
Cases	16
Spayed females/Intact females	10/0
Castrated males/Intact males	6/0
Median Age (yr)	8
Range (yr)	3.9 -10.25
Mean age +/- SD	7.74 +/- 2.12
Median weight (kgs)	37.9
Mean weight +/- SD	40.9 +/- 12.8
Breeds	Mixed breed (4), Mastiff (3), American Staffordshire Terrier (2), Labrador (2), Alaskan Malamute (1), Sharpei (1), Doberman (1), Newfoundland (1), Golden Retriever (1)

Table 3: Osteosarcoma patient signalment

OSA PATIENTS	
BLOOD	
CD4+CD25+	2.20%
CD4+CD25+FOXP3+	0.84%
CD4+FOXP3+	3.37%
CD4+CD25+ lymphocytes expressing FoxP3	39.68%
CD4+ cells expressing CD25 and FoxP3	1.7%
LYMPH NODE	
CD4+CD25+	8.47%
CD4+CD25+FOXP3+	5.66%
CD4+FOXP3+	8.59%
CD4+CD25+ lymphocytes expressing FoxP3	70.41%
CD4+ cells expressing CD25 and FoxP3	10.39%
TUMOR DRAINING LYMPH NODE	
CD4+CD25+	7.19%
CD4+CD25+FOXP3+	**4.25%
CD4+FOXP3+	9.16%
CD4+CD25+ lymphocytes expressing FoxP3	63.18%
CD4+ cells expressing CD25 and FoxP3	8.15%

**There was a significant decrease in the % of CD4+CD25+FoxP3+ Tregs in the tumor draining lymph nodes of Osteosarcoma patients compared to the unrelated lymph nodes in the same patients (p=0.049).

Table 4: The median % Tregs in the peripheral blood, unrelated, and tumor draining lymph nodes of osteosarcoma patients.

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