Immune Checkpoint Molecule Expression in Canine Lymphoma and Canine Reactive Lymphoid Hyperplasia

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Academic Abstract

Background: Although lymphoma is one of the most common malignancies in dogs, remission rates and survival times remain stagnant. Treatment with a multi-agent chemotherapy protocol induces remission for less than one year and the majority of patients relapse. Fewer than 25% of dogs live longer than two years with the currently available treatments. Targeted immunotherapy using checkpoint molecule blockade of PD-1 and PD-L1 shows promise for various types of human cancer, including relapsed/refractory lymphoma; however, little is known regarding the role of these checkpoint molecules in canine lymphoma.
Objectives: To determine the patterns of expression of mRNAs encoding PD-1 and its ligands PD-L1 and PD-L2 in lymphoma and reactive lymphoid hyperplasia controls.
Methods: Retrospective: formalin-fixed paraffin-embedded (FFPE) tissue from dogs with untreated lymphoma (n=10) and reactive lymphoid hyperplasia (n=10). Prospective: fine-needle aspirates (FNAs) from dogs with untreated lymphoma (n=10) and reactive lymphoid hyperplasia (n=10). Total RNA was extracted, and expression of PD-1, PD-L1, and PD-L2 was measured using qRT-PCR analysis of random-primed cDNA. Checkpoint molecule expression levels were determined using the $2^{\wedge\Delta\Delta CT}$ method. Lymphoma immunophenotype was assessed using immunohistochemical analysis of CD3 and CD79a (FFPE) and review of patient medical records (FNA). Data analysis included Wilcoxon ranksum tests, Dunn's procedure of multiple comparisons, Kruskal-Wallis tests, and regression within an ANOVA. Significance at $P < 0.05$.
Results: PD-1, PD-L1, and PD-L2 expression (normalized internally to 18S rRNA) was lower in lymphoma compared to reactive lymphoid hyperplasia (FFPE); the difference was significant for PD-1 and PD-L2. PD-1 and PD-L2 expression was lower in lymphoma compared to reactive lymphoid hyperplasia (FNA); the difference was significant for PD-1. PD-1, PD-L1, and PD-L2 expression was lower in B cell lymphoma compared to reactive lymphoid hyperplasia (FFPE); this difference was significant for PD-1 and PD-L2. PD-1 and PD-L2 expression was lower in B cell lymphoma compared to reactive lymphoid hyperplasia (FNA); the difference was significant for PD-1. The higher relative abundance of PD-L1 vs PD-1 and PD-L2 vs PD-1 was significantly different between lymphoma and reactive lymphoid hyperplasia (FFPE and FNA).
Conclusions: In this study, checkpoint molecule expression was not upregulated in canine lymphoma relative to canine reactive lymphoid hyperplasia, suggesting a limited application of PD-1 and PD-L1 blockade in canine lymphoma. The ligand:receptor relative abundance imbalances reflect the lower PD-1 expression relative to PD-L1 and PD-L2 in lymphoma. Although these results do not suggest that checkpoint inhibitors would be useful for treatment, they give insight into the mechanisms of unchecked lymphocyte proliferation in canine lymphoma.

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General Abstract

Lymphoma, a cancer of the white blood cells in the body, is one of the most common malignancies in dogs. Although treatment with a multi-agent chemotherapy protocol results in high remission rates, the remission duration is usually less than one year, with the majority of patients relapsing. In an effort to improve remission rates and survival times, scientists have been working to develop therapeutic interventions that target specific points in the development and replication cycle of a cancer cell. One such strategy, targeting checkpoint molecules programed death (PD)-1 and PD-L1, has shown promise for several different types of human cancers, including lymphoma.

PD-1 is a receptor on T cells, which together with its ligands, PD-L1 and PD-L2, decreases lymphocyte function when activated. This is a protective mechanism, acting to inhibit sustained harmful inflammation in a normal healthy dog. Some cancers have taken advantage of this pathway, increasing expression of PD-L1 or L-L2 in order to evade detection by the immune system. To date, little is known regarding the role and expression of these immune checkpoint molecules in dogs with lymphoma. We sought to evaluate if PD-1, PD-L1 and PD-L2 expression is significantly increased in canine lymphoma compared to reactive lymphoid hyperplasia controls.

Tissue samples were collected from two sources. Cytology samples of lymphoma and reactive lymphoid hyperplasia were collected by fine needle aspiration from clinical patients. Formalin fixed paraffin embedded tissue samples of lymphoma and reactive lymphoid hyperplasia were collected from the archived tissue bank. Using a molecular analysis technique called quantitative reverse transcription PCR (qRT-PCR) we measured the amount of messenger RNA (mRNA) encoding PD-1 and its ligands PD-L1 and PD-L2 in lymphoma and in reactive lymphoid hyperplasia controls.

In our results we did not observe an upregulation in the expression of checkpoint molecules in canine lymphoma relative to canine reactive lymphoid hyperplasia. This suggests there may be a limited therapeutic application for PD-1 and PD-L1/PD-L2 blockade in canine lymphoma. Although these results do not suggest that checkpoint inhibitors would be useful for treatment, they give insight into the mechanisms of unchecked lymphocyte proliferation in canine lymphoma.

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Chapter 1: Introduction

Canine lymphoma is the most common hematopoietic malignancy; however, despite a variety of management strategies, veterinary medical oncologists are commonly presented with patients that relapse or respond poorly to the currently available therapies. Ultimately, medical therapy will fail to control the unchecked neoplastic lymphocyte proliferations in virtually all cases. Unfortunately, since lymphoma is incurable, the vast majority of canine patients will eventually relapse given sufficient time. Surgery and radiation therapy offer local treatment strategies; however, these are of limited value given the systemic nature of lymphoma.

Current treatment strategies for canine lymphoma are designed to enhance quality of life and minimize treatment-related complications; however, improvements in long-term survival times and duration of remission have remained stagnant. There is an unmet need for additional treatment strategies that have the potential to improve drug resistant relapse, provide new options for rescue chemotherapy, extend duration of remission, and improve long-term survival times.

Programmed death (PD)-1 is a receptor on T cells, which, when bound with its ligands, PD-L1 or PD-L2, expressed primarily on antigen presenting cells, serves to downregulate the immune response by decreasing effector lymphocyte function. While this can be a protective mechanism in normal health acting to inhibit disorders such as autoimmunity, overexpression of PD-L1 can result in T cell exhaustion, thereby reducing immune clearance and enhancing survival of the neoplastic population. Overexpression of PD-L1 and PD-L2 has been described in people with various types of lymphoma, and overexpression of PD-L1 is associated with unfavorable outcomes. Promising effects of targeted immunotherapy using checkpoint blockade of PD-1 and PD-L1 in people with relapsed and refractory lymphoma creates an opportunity to investigate

analogous canine cancers. Currently, there is limited information regarding the roles and expression of these checkpoint molecules in canine lymphoma. In addition, studies evaluating expression of PD-1 and PD-L1 in dogs are conflicting both in detection modalities and results.

The clinical usefulness of PD-1/PD-L1/PD-L2 mRNA expression by qPCR in detecting canine patients whose lymphomas are likely to respond to therapies targeting the PD-1/PD-L1 pathway is unknown. If found, identification of significant differences in expression will provide valuable information regarding the PD-1/PD-L1/PD-L2 axis in canine lymphoma. In addition, identifying significant differences in expression could constitute the basis for further work leading to the adoption of PD-L1/PD-L2 as diagnostic assay markers with potential as future targets of immunotherapy.

The proposed study investigated the patterns of expression of mRNAs encoding PD-1 and its ligands PD-L1 and PD-L2 in lymphoma and in reactive lymphoid hyperplasia controls. We hypothesized that PD-L1 and PD-L2 are significantly upregulated in lymphoma, suppressing the immune response and allowing unchecked neoplastic lymphocytes to evade detection by the immune system. In this study, we sought to test the hypothesis by determining the patterns of expression of mRNAs encoding PD-1 and its ligands PD-L1 and PD-L2 using formalin-fixed paraffin embedded tissue from dogs with lymphoma and reactive lymphoid hyperplasia.

Chapter 2: Literature Review

I. Lymphoma

A. Epidemiology

The canine non-Hodgkin lymphomas represent a diverse array of clinically distinct and morphologically unique forms of lymphoid neoplasia. They are characterized by the neoplastic transformation and proliferation of B and T lymphocytes at differing developmental stages. Lymphoma is the most common spontaneous hematopoietic malignancy in dogs, as well as the most commonly managed neoplasm among veterinary medical oncologists. It represents 83% of all hematopoietic neoplasia (Withrow 2007). In addition to being the most common hematopoietic malignancy in dogs, lymphoma is also one of the three most commonly diagnosed neoplasms in dogs, second to mammary neoplasia (Merlo 2008).

In dogs, lymphoma represents approximately 7-24% of neoplasia, with an annual incidence between 13-114 cases per 100,000 dogs (Withrow 2007, Dorn 1967, Teske 1994, Dobson 2002). Some studies have reported incidence rates of up to 24 and 33 per 100,000 dogs. (Dorn 1970, Teske 1994). An age-adjusted incidence of at-risk dogs was reported to be 107 per 100,000 dogs (Dobson 2002). Although the exact incidence may be difficult to determine, it appears to be increasing similar to the increasing incidence of non-Hodgkin lymphoma in people, though reasons are unclear (Seelig 2016). Reports in the human literature report an incidence that has nearly doubled over the last thirty years. (Howlader 2012, Chiu 2012).

Nevertheless, in the United States, with approximately seventy-five million pet dogs and an estimated incidence ranging from 21.7 to 107 cases per 100,000 dogs at risk, the number of newly diagnosed cases of canine lymphoma is likely approximately 16,000-80,000. (Richards 2015, Dorn 1970, Dobson 2002). Additional supporting evidence for the rising incidence of canine lymphoma is an epidemiologic study that was performed using the Veterinary Medical Data Base Program; this study found that rates of canine lymphoma patients presenting to veterinary teaching hospitals more than doubled in incidence between 1964-2002. (Villamil 2009).

Any age and any breed of dog, either male or female, can be affected and diagnosed with lymphoma. Widely cited reports have shown that particular dog breeds have a statistically significant increased risk for developing lymphoma when compared to the average risk for all dog breeds (Dorn 1967, Teske 1994). Support for existing heritable risk factors includes familial clustering that was observed in bullmastiffs, rottweilers, and Scottish terrier lines (Onions 1984, Teske 1994).

Medium-sized to larger dog breeds also tend to be overrepresented but increases in growth hormone are not a known factor in the development of lymphoma (Edwards 2003, Villamil 2009, Lantinga Van Leeuwen 2000). Breeds shown to have an increased incidence of lymphoma include Bulldogs, Bullmastiffs, Boxers, Golden Retrievers, Flat-Coated retrievers, Bernese Mountain dogs, and Rottweilers, whereas breeds with lower relative risk include Poodles, German Shorthaired Pointers, Bichon Frise, West Highland White terriers, and German Shepherds (Bienzle 2011, Edwards

2003, Modiano 2005). The lifetime risk of Golden Retrievers in developing lymphoma is ~1:8, compared with a lifetime risk in people of ~1:50 (Modiano 2005). The predisposition for breed-specific susceptibility to lymphoma and genetic basis for breed-related increased risk is unclear (Modiano 2005, Bienzle 2011).

In addition, some dog breeds have a predisposition for developing specific B-cell or T-cell immunophenotypes of lymphoma, contributing to support of a genetic component. For instance, while T-cell lymphoma accounts for 25-35% of canine lymphoma, up to 82% of lymphoma in boxers comprises T-cell lymphoma with the majority being TCRαβ, CD4 + T cells (Lurie 2004, Lurie 2008, Modiano 2005). A boxer has a ~4-fold higher risk to develop lymphoma than the average risk of any dog (Modiano 2005). The lifetime risk of a Golden Retriever in developing lymphoma is ~1:8, compared to a lifetime risk in people of ~1:50 (Modiano 2005). The Spitz breeds (e.g., Siberian Husky, Alaskan Malamute, and Chinese Shar-Pei) and most Asian lap-dog breeds (e.g., Shih Tzu and Lhasa Apso) tend to more commonly develop T-cell lymphoma, whereas breeds which almost always develop B-cell lymphoma include Bassett Hounds and Cocker Spaniels (Modiano 2005). This same study found that Golden Retrievers develop T-cell and B-cell lymphoma with relatively equal frequency. Doberman Pinchers have a small though significant excess of B-cell origin lymphoma, and mixed breed dogs develop B-cell lymphoma and T-cell lymphoma with a similar frequency to purebred dogs (approximately 70% B-cell and 30% T-cell) (Modiano 2005).

Middle-aged to older dogs are primarily affected, with the incidence rate increasing with age from 1.5 cases per 100,000 dogs for dogs less than one year old, to 84 cases per 100,000 dogs for those greater than ten years old (Dorn 1967). Golden Retrievers, Bullmastiffs, and Bulldogs are breeds that develop lymphoma at an earlier age, though as larger breeds, they have shorter lifespans. (Edwards 2003). Although there is variability due to breed, most dogs are diagnosed with lymphoma between six and nine years old (Edwards 2003, Modiano 2005, Pastor 2009). While a gender predisposition is not apparent, intact female dogs may have a reduced risk, according to an epidemiologic study using the Veterinary Medical data Base Program (Villamil 2009). Early (at less than one year of age) spaying or neutering of Golden Retrievers and Vizslas reportedly increases the risk of developing lymphoma; however, this is not the case for Labrador Retrievers (Torres de la Riva 2013, Zink 2014, Hart 2014).

B. Etiology and Molecular Biology

Canine lymphoma arises sporadically and spontaneously. While multiple factors contribute to the heterogeneity of canine lymphoma, such as patient genetics, immune status, phenotypic variation, anatomic location, and subtypes, a single precise etiology has yet to be identified. Although canine lymphoma does not have a definitive cause, several potential associations have been investigated, including etiologic agents, environmental pollutants, toxin exposure, and genetic aberrations.

Canine lymphoma secondary to transmissible viral etiologies has not been definitively demonstrated. Viral infections contribute to the pathogenesis of

lymphoma in people. These include human immunodeficiency virus, hepatitis C virus, Epstein-Barr virus, Kaposki sarcoma herpes virus, and human T-lymphotropic virus (Richards 2015). Although the significance in lymphomagenesis remains to be determined, a recent study identified the presence of an Epstein-Barr virus-like gamma herpesvirus in five cases of B-cell lymphoma in dogs (Huang 2012).

Numerous associations with environmental and/or toxin exposure exist in people with lymphoma, and several studies in veterinary medicine support this evidence. This is unsurprising since pet dogs share people's environments. For example, in a French study, environmental contaminants were associated with an increased risk of developing canine lymphoma (Pastor 2009). They found a correlation between the frequency of diagnosing canine lymphoma and the location of waste incinerators, polluted sites, and radioactive storage. Other environmental contaminants associated with potential increased risk for canine lymphoma include household chemicals and certain lawn herbicides (Gavazza 2001, Hayes 1995).

Studies in veterinary medicine are limited regarding the molecular biology of lymphomagenesis. However, a variety of genetic aberrations have been identified in canine lymphoma and characterization of these abnormalities remains an active area of research and ongoing investigation. It has been noted that chromosomal gains are more common than chromosomal losses and multiple regions of chromosomes are affected in canine lymphoma (Thomas 2003). Genetic aberrations in thirty-two out of thirty-eight canine autosomes are affected in canine lymphoma, with a gain on

chromosomes thirteen and thirty-one and loss on chromosome fourteen being most common (Thomas 2003). In an epidemiologic study, Golden Retrievers were highlighted as having a chromosome 14 deletion that was uniquely identified in B-cell lymphoma. (Modiano 2005). The loss of chromosome eleven commonly occurs in T-cell lymphoma (Thomas 2003).

In addition, there has been investigation into alterations in proto-oncogenes and tumor suppressor genes in canine lymphoma. It has been noted that expression of the proto-oncogene, c-kit, is generally low in canine lymphoma, but was shown to be increased in some aggressive T-cell lymphomas (Giantin 2013). Furthermore, N-ras oncogene mutations are not common in canine lymphoma (Mayr 2002). Mutations in the p53 tumor suppressor gene are also rarely noted in canine lymphoma (Tomiyasu 2010). Constitutive expression of the Myc oncogene was observed in canine diffuse large B-cell lymphoma, highlighting a potential shared oncogene translocation with Burkitt lymphoma in people (Breen 2008). TFPI-2 is a tumor suppressor gene which becomes inactivated during tumor progression in people; reduced expression of the TFPI-2 was frequently detected in dogs with diffuse large B-cell lymphoma (Ferraresso 2014).

Aside from chromosomal imbalances, defective DNA repair pathways are also associated with an increased risk of lymphomagenesis. Risk of non-Hodgkin lymphoma in people increases with increased polymorphisms in DNA repair and oxidative stress genes (Wang 2006). It was found that Golden Retrievers with

lymphoma not only have increased sensitivity (odds ratio = 21.2) to chromosome damage compared with healthy Golden Retrievers and healthy mixed breed dogs, but they also demonstrate decreased DNA repair capability; this pilot study suggests an heritable component to an impaired DNA damage response in association with development of canine lymphoma (Thamm 2013).

C. Common Types of Lymphoma

Various histologic classification schemes exist to characterize the heterogenous lymphomas, which is beyond the scope of this work. In general, canine lymphoma classification schemes utilize similar criteria to those for human non-Hodgkin lymphomas, and are modified for canine tumor architecture and morphology. The World Health Organization (WHO) classification system for human lymphoma is the most widely applied criteria used by veterinary pathologists to classify canine lymphoma. Important features in the classification of lymphoma for this system are nodular or diffuse growth pattern, cell distribution of non-neoplastic follicles, nuclear morphology and size, mitotic count, and immunophenotype of T or B cell origin. (Valli 2011).

Classification is based on six categories that include the majority (79.5% of 300 total cases) of five canine lymphoma subtypes (Valli 2011). The six categories for the most commonly diagnosed canine lymphoma entities are: diffuse large B-cell lymphoma (145 cases), peripheral T-cell lymphoma not otherwise specified (42 cases), nodal T zone lymphoma (38 cases), T lymphoblastic lymphoma (12 cases),

marginal zone lymphoma (11 cases), and disease other than lymphoma (20). (Valli 2011). The last category comprises lymphoid hyperplasia (N=11), atypical follicular hyperplasia, (N=9), and benign follicular hyperplasia (N=2), all of which are typically asymptomatic, the latter of which must be differentiated from indolent lymphomas (e.g., marginal zone lymphoma, T zone lymphoma, follicular lymphoma). (Valli 2011, Valli 2006).

Each lymphoma type has unique anatomic and histomorphologic features, as well as individual immunophenotypic characteristics, highlighting the heterogeneity of the canine lymphomas. Lymphoma is characterized anatomically as either multicentric, alimentary, mediastinal, or extra nodal. The majority of dogs with lymphoma have multicentric lymphoma affecting one or more peripheral lymph nodes (Withrow 2007, Ponce 2010, Vezzali 2010). B cell lymphoma is more common than T cell lymphoma, and T cell lymphoma subtypes generally have a more aggressive clinical course (Avery 2014). High-grade tumors with diffuse architecture comprise roughly 85% of canine lymphomas, whereas approximately 10% of canine lymphomas comprise low-grade tumors with diffuse architecture consisting of neoplastic small lymphocytes (Teske 1994, Fournel-Fleury 1997, Bienzle 2011).

B-cell lymphoma comprises two most commonly recognized WHO histologic forms, which include diffuse large B cell lymphoma and marginal zone lymphoma of B cell origin (Valli 2013, Valli 2011). The most common form of canine lymphoma is the diffuse large B-cell lymphoma (DLBCL) subset; this is also the most common form

of non-Hodgkin lymphoma in adult human patients (Swerdlow 2008, Aresu 2015, Fournel-Fleury 1997, Valli 2013, Daisuko 2014, Seeling 2016, Vezzali 2010). The T cell lymphomas can be subclassified into two major forms: aggressive peripheral T cell lymphoma and T zone lymphoma (Avery 2014, Lurie 2008). T cell phenotypes that are associated with aggressive clinical disease include the peripheral T cell lymphoma not otherwise specified and T cell lymphoblastic lymphoma (Avery 2014).

In dogs with diffuse large B cell lymphoma, there is a diffuse and generalized effacement of lymph node architecture by a population of neoplastic large lymphocytes having round nuclei with a high mitotic rate (Valli 2011). Marginal zone lymphoma consists of low grade, neoplastic medium sized B cells with intermediate sized nuclei, which are characteristically arranged around fading germinal centers. (Valli 2011). Peripheral T cell lymphoma consists of diffuse and generalized effacement of lymph node architecture in multiple lymph nodes by a variable to high grade population of mixed pleomorphic cell morphologies often having cleaved to oval nuclei. (Valli 2011). T cell lymphoblastic lymphoma consists of high grade, neoplastic T cells with intermediate sized nuclei and evenly dispersed chromatin obscuring nucleoli; there is focal perinodal colonization and a thin capsule. (Valli 2011). T zone lymphoma is considered low grade and expands the paracortical lymph node architecture; it consists of a uniform population of neoplastic small T lymphocytes with round nuclei and extended pale cytoplasm. (Valli 2011).

The prognostic significance of each entity is noted in a widely cited study which evaluated subtypes in association with specific treatment and survival data (Valli 2013).

D. Diagnostic Approach

The diagnosis of lymphoma in veterinary medicine can be made via cytologic assessment, flow cytometry, PCR (polymerase chain reaction) for antigen receptor rearrangement, or histologic analysis. Of these tools, cytologic assessment using fine needle aspiration is the most commonly used technique in the diagnosis of lymphoma because it is rapid, safe, minimally invasive, relatively inexpensive, and effective. (Cohen 2003, Sozmen 2005, Amores-Fuster 2015). It is a reliable sensitive technique to diagnose lymphoma because the more common forms of canine lymphoma are of a diffuse nature and larger lymphocyte size; the cytologic picture of the less common subtypes are less well-known. (Rout 2017, Sapierzynski 2016).

In the majority of dogs, lymphoma manifests as a generalized or localized lymphadenopathy, with less frequent occurrences in extra nodal sites (Ponce 2010). Lymphadenopathy is a frequently encountered entity in both human and veterinary medicine with many different underlying causes that range from benign reactive processes to neoplastic lymphoproliferative disorders to metastatic disease. (Sapierzynski 2009, Slack 2016, Medhi 2015). Most often the cytologic diagnosis of enlarged lymph node(s) in dogs is lymphoma or reactive hyperplasia (Amores Fuster 2015, Sapierzynski 2009).

In general, a fine needle aspirate from a lymph node with diffuse lymphoma reveals a monomorphic population of large lymphocytes, whereas in a reactive lymph node, a mixed population of cell types is present. Differentiation of a reactive lymph node relies on identifying a mixed cell population comprising a heterogenous lymphoid population of primarily small lymphocytes, intermediate lymphocytes, and less than 50% large lymphocytes, usually less than 20%, along with the presence of plasma cells and other inflammatory cell types (Burkhard 2013, Messick 2008, Raskin 2015). General cytologic features of lymphoma include greater than 50% of the cells comprising a homogeneous monomorphic population of large or atypical lymphocytes, and this is often greater than 80-90% of the cell population; in addition, plasma cells and other inflammatory cell types are rarely observed. (Burkhard 2013, Messick 2008, Sapierzynski 2010, Flournel-Fleury 1994).

Several of the major subtypes of canine lymphoma have characteristic cytologic features, allowing for a relatively straightforward diagnosis (Zandvliet 2016, Sozmen 2005, Rout 2017). Cytomorphologic analysis relies on these distinguishing cytologic features, such as cell size along with nuclear and cytoplasmic features. For example, in dogs with diffuse large B cell lymphoma, lymphocytes are often one to one and half times the size of a neutrophil with uniformly large round nuclei having dispersed fine immature chromatin, and one or more large round, prominent, nucleoli. (Zandvliet 2016, Rout 2017, Ponce 2004). Cytoplasm is scant, deeply basophilic, and occasionally contains colorless punctate vacuoles, and mitotic figures are common.

(Zandvliet 2016, Rout 2017, Ponce 2004). In addition, this type of lymphoma is composed of medium sized CD21 cells using flow cytometry (Rout 2017).

Another common subtype of canine lymphoma with distinguishing cytologic features is multicentric T cell lymphoma. This is a CD4 phenotype and has an aggressive clinical course, increased incidence of hypercalcemia, and mediastinal masses (Avery 2014, Lurie 2008, Rout 2017). In a study evaluating fifteen biopsies of CD4 T cell lymphoma, ten were classified histologically as peripheral T cell lymphoma not otherwise specified and five were classified as lymphoblastic T cell lymphoma. (Avery 2014). Cytologically, the lymphocytes are of intermediate to large size with a round, indented, or cleaved nucleus having finely dispersed chromatin, and inapparent or inconsistent nucleoli, together with moderate amounts of pale cytoplasm. (Zandvliet 2016, Rout 2017).

The third subtype of canine lymphoma with commonly recognized cytologic features is T zone lymphoma. A unique immunophenotype is associated with T zone lymphoma, allowing for a diagnosis via flow cytometry rather than biopsy. These T cells were found to lack expression of the CD45 pan leukocyte antigen, and may express CD4, CD8, or neither. (Seelig 2014). In fine needle aspirates, T zone cells are small to intermediate sized lymphocytes with abundant clear cytoplasm that shows an asymmetric extension to form a mirror handle structure; nuclei are small and round to medium in size with dense chromatin. (Mizutani 2016, Rout 2017).

Depending on the morphologic characteristics identified by cytology, and the limitations of cytologic analysis, ancillary diagnostics such as flow cytometry and PCR for antigenic rearrangement can be used to further refine the diagnosis, provide a more definitive diagnosis, and/or obtain important prognostic information (Zandvliet 2016, Sapierzynski 2016, Aniolek 2014, Bienzle 2011). Cytologic diagnosis of small cell lymphomas, indolent lymphomas, and atypical lymphoid proliferations is challenging, as is differentiating early lymphoma from reactive hyperplasia (Burkhard 2013, Zandvliet 2016).

Immunophenotyping by flow cytometry permits the objective examination of the types of lymphocytes present in a single cell suspension by characterizing their cell size, cytoplasmic complexity, and the types of proteins they express. (Avery 2011, Comazzi 2011, Rout 2017). Flow cytometry is typically employed once a diagnosis of lymphoma is established; certain patterns of expression, phenotypes, or specific markers aid in lymphoma classification as well as having prognostic utility. (Avery 2011, Comazzi 2011, Rigetti 2010). It should be noted that just as there are limitations to using cytology in isolation, flow cytometric analysis should not replace a cytopathologic or histopathologic diagnosis (Rigetti 2010).

PCR for antigenic receptor rearrangement should be reserved for cases in which a sample is not available for immunostaining or flow cytometry and can also be used on air-dried fine needle aspirate preparations (Thalheim 2013). Likewise, when there is diagnostic uncertainty, this test is useful when morphologic and immunophenotypic

features are unable to distinguish a reactive polyclonal from a neoplastic monoclonal lymphoid proliferation, particularly in indolent lymphoma, or in cases of early lymphoma that develop within reactive hyperplasia (Beinzle 2011, Burkhard 2013). This test detects clonally rearranged T or B cell receptor genes; however, lymphocyte clonality does not always indicate neoplasia and may be seen in cases of tick-borne diseases such as ehrlichiosis. In dogs, this assay detects 75-80% of confirmed lymphoma cases, so while a positive test result supports the diagnosis of lymphoma, a negative result does not rule out lymphoma (Burkhard 2013, Bienzle 2011).

While in most cases of canine lymphoma cytology is sufficient for establishing an initial diagnosis of lymphoma, histologic analysis is employed for a more definitive classification and grading of lymphoma subtypes, particularly follicular lymphomas and small cell lymphomas. (Valli 2011, Bienzle 2011). Incisional or excisional lymph node or other tissue biopsy with subsequent histopathology are used in veterinary medicine when cytologic assessment yields inconclusive results (Messick 2008). Immunohistochemical analysis is performed after histopathologic assessment to assign B or T cell lineage, and commonly uses CD79a and CD3 antibodies. Formalin fixed paraffin embedded tissue availability also provides a source of DNA/RNA for additional analysis, such as PCR for antigenic receptor rearrangement, gene expression analysis, and other immunohistochemical studies. Disadvantages to surgical biopsy include the need for general anesthesia, financial constraints, and practical limitations (Bienzle 2011).

E. Current Treatments and Outcomes

Regardless of etiology or diagnostic modality, the gold standard for first line treatment of dogs with high-grade multicentric lymphoma is conventional chemotherapy (Withrow 2007, Zandvliet 2016). While chemotherapy is the cornerstone of lymphoma treatment given lymphoma's systemic nature, additional therapeutic options beyond traditional chemotherapy include best supportive care, bone marrow transplantation, novel cytotoxic drugs, and immunotherapy. (Thamm 2019, Saba 2017, Bienzle 2011, Marconato 2011). Without treatment, high-grade multicentric lymphoma is usually fatal within a matter of weeks (Withrow 2007). Palliative supportive care using corticosteroids, which are lympholytic, result in survival times of one to two months (Withrow 2007).

The cytotoxic chemotherapy most commonly used for first line treatment of canine lymphoma is a multi-agent drug therapy since it provides the highest response rate, longest disease control and response duration, and survival times. (Withrow 2007, Chun 2009, Zandvleit 2016). A multi-agent chemotherapy protocol is a combination of cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP protocol), with or without L-asparaginase (L-CHOP). (Withrow 2007, Zandvliet 2016). Complete response rates of 85-95%, median duration of response of six to ten months, and median survival times of approximately twelve months are observed with CHOP based protocols (Withrow 2007, Thamm 2019, Curran 2015). CHOP-based protocols involve approximately six months of alternating chemotherapy followed by rechecks to assess remission (Bienzle 2011, Thamm 2019, Zandvliet

2016, Withrow 2007); however, there are newer shorter protocols with response rates and survival times similar to CHOP (Curran 2015). Unfortunately, all but 5% of canine patients on a multi-agent protocol will relapse, with fewer than 25% having survival times beyond two years. (Thamm 2019, Withrow 2007).

There are a variety of single-agent protocols with the most common being single-agent doxorubicin. (Withrow 2007). Approximately 50-75% of dogs will have a complete response rate, and median survival times are reported to range from six to eight months. (Withrow 2007). This is similar to studies highlighting a seven-month median disease control period and a median survival of nine months (Chun 2009), or a 65-85% response rate with a median response duration of one hundred to one hundred seventy days (Lori 2010, Mutsaers 2002, Valerius 1997, Postorino 1989). Lastly, it should be noted that rescue protocols are used when patients fail to respond to a first line treatment or come out of remission; however, these are limited and generally offer a two to three-month response duration and more toxicity. (Zandvliet 2016).

Bone marrow transplantation in conjunction with chemotherapy has been successfully utilized in people with relapsed non-Hodgkin lymphoma, and is offered to select canine patients at North Carolina State University College of Veterinary Medicine (Marconato 2010). In a study that evaluated a large group of canine patients treated with chemotherapy followed by autologous bone marrow transplantation, median survival times of one hundred and forty-five days were observed. (Frimberger 2006).

Although initial response rates are high with a multi-agent drug protocol, relapse is common, as are potential treatment-related complications (Withrow 2007). Relapse is thought to be a result of drug resistance, which is multifactorial. (Withrow 2007, Zandvliet 2014). Rabacfosadine was conditionally approved in 2017 by the Food and Drug Administration for treatment of canine lymphoma. (Thamm 2019). It is a prodrug with a selective cytotoxicity for neoplastic lymphoid cells, that in addition to treatment of naïve multicentric lymphoma, is approved for relapsed B cell lymphoma. (Saba 2017). Alternating rabacfosadine/doxorubicin was reported to have an overall response rate of 84% and median progression free interval of nearly two hundred days, similar to conventional multi-agent protocols, albeit with fewer office visits. (Thamm et al 2017).

Immunotherapy using anti-CD20 monoclonal antibody therapy has improved outcomes in people with B cell lymphoma, particularly in people with diffuse large B cell lymphoma, and without toxicity increases. (Coiffier 2002, Pfreundschuh 2006, Lim 2014). Although an anti-canine CD20 monoclonal antibody was developed and received USDA licensure in 2014, it was withdrawn from the market since unpublished studies did not demonstrate improved outcomes. (Thamm 2019).

Recent advances in immunotherapy targeting the immune checkpoint molecules PD-1 and PD-L1 have shown promise in various types of human cancer, including Hodgkin and non-Hodgkin lymphoma, particularly in the treatment of relapsed lymphoma;

however, much less is known regarding the role of these molecules in veterinary species. The checkpoint molecules will be discussed in chapter two.

The use of chimeric antigen receptor-engineered T cells (CAR-T cells) is another recent advancement in people with B cell lymphoma. (Thamm 2019). A CD19 directed CAR-T cell therapy in people with relapsed or refractory diffuse large B cell lymphoma demonstrated a significant 50% overall response rate, as well as identified 95% of those with a complete response at three months also being sustained at six months. (Schuster 2017). Clinical studies in dogs using CD20 specific CAR-T cell-based therapy have yet to be published; however, the development of methodologies to generate canine CAR-T cells is an active area of investigation. (Panjwani 2016).

II. Checkpoint Molecules

A. Role in Normal Immunity

The immune system's ability to distinguish self from non-self is a complex network of regulatory signaling pathways. Together, both positive and negative signaling pathways function to main immunologic self-tolerance while at the same, defend a host against pathogens and diseases such as cancer. (Freeman 2000, Hartley 2016). The PD (programmed cell death) pathway, comprising the PD-1 receptor, together with its ligands PD-L1/PD-L2, is one of the important checkpoint molecule pathways that plays a critical role in delivering negative immunoregulatory signals through T cells. (Freeman 2000, Hartley 2016). The PD-1/PD-L1/PD-L2 pathway is part of the B7-CD28 family, which consists of inhibitory pathways that attenuate T cell

responses. (Keir 2007). These pathways provide a mechanism for an inhibitory signal that regulates T cell activation through fine-tuning T cell proliferation and production of effector cytokines. (Keir 2007, Alegre 2001).

Antigen recognition and subsequent T cell activation provides effector functions to T cells, and this well-known model of T cell activation is a two-signal theory. The first stimulatory signal in T cell receptor engagement consists of the T cell receptor complex engaging with a major histocompatibility (MHC) peptide complex. (Alegre 2001). In addition to the T cell receptor-MHC peptide complex is a second signal; this involves the simultaneous recognition of CD80/CD86 (B7-1/B7-2) on an antigen presenting cell by the costimulatory molecule CD28, expressed on both naïve and primed T cells. (Alegre 2001). Together, these two signals lead to normal T cell activation, proliferation, differentiation, and cytokine production; however, without CD28 co-stimulation, T cells undergo apoptosis or anergy and become incapable of cytokine production. (Alegre 2001).

In addition to positive co-stimulatory molecules, activated T cells possess a balance of negative inhibitory receptors, including cytotoxic T lymphocyte antigen 4 (CTLA-4) and PD-1. CTLA-4 and PD-1 are structurally similar but have distinct roles. (Buchbinder 2016). In contrast to CTLA-4, instead of stopping potentially autoreactive T cells during initial phases of naïve T cell activation in lymph nodes, PD-1 works at later stages in peripheral tissues to regulate previously activated T cells. (Buchbinder 2016).

In people and rodents, the PD-1 receptor is a type I transmembrane protein whose transcription is induced primarily in activated CD4+ and CD8+ T cells, as well as B cells, natural killer (NK) cells, and some myeloid cells. (Freeman 2000, Nguyen 2015). PD-1 has two known ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC) that are also type I transmembrane proteins, and these molecules have complex expression patterns. (Dong 1999, Freeman 2000, Latchman 2001, Nguyen 2015). PD-L1 expression is induced by interferon-γ (IFN-γ) and is more broadly expressed than PD-L2. (Chiku 2016). PD-L1 is constitutively expressed on antigen presenting cells, macrophages, dendritic cells, T cells, and B cells, although it can also have low levels of expression on a wide variety of non-hematopoietic cells. (Nguyen 2015). PD-L2 expression is induced by interleukins 4 and 13 (IL-4 and IL-13), and its expression pattern is more restricted to dendritic cells and macrophages compared to PD-L1. (Chiku 2016, Nguyen 2015). It has been suggested that the T_H1 response is preferentially regulated by PD-L1 expression and PD-L2 expression regulates that T_H2 response. (Loke 2003).

PD-1 is expressed on T cells only after T cell activation where it functions to downregulate the magnitude of the effector phase of T cell differentiation. (Keir 2008). Engagement of the PD-1 receptor, expressed on activated T cells, together with PD-L1, results in the inhibition of T cell receptor mediated lymphocyte proliferation and cytokine secretion. (Freeman 2000). Specifically, the binding of PD-1 to its ligands diminishes T cell proliferation, reduces T cell survival, and decreases

the production of IFN- γ, tumor necrosis factor-α (TNF- α), and IL-2. (Keir 2008). PD-1 expression decreases once the antigen (either microbial antigen or tumor antigen) is cleared. Harmful sustained inflammation is therefore prevented through the PD-1/PD-L1/PD-L2 pathway by maintaining T cell homeostasis.

It was shown that the outcome of the PD-1/PD-L1 interaction is dependent upon the strength of T cell receptor signaling and the strength of the CD28 signaling. (Freeman 2000). The inhibitory effects of the PD-1/PD-L1 interaction at the T cell activation stage can be evaded by increasing levels of either T cell receptor or CD28 signaling. (Freeman 2000).

Despite its importance in modulating an immune response, the exact mechanisms underlying PD-1 mediated inhibition of T cell function remain to be fully characterized. (Hui 2017). It was previously demonstrated that an inhibitory tyrosine-containing sequence in the PD-1 molecule utilizes phosphatases which deactivate T lymphocytes or induce T lymphocyte apoptosis; however, additional mechanisms were thought to be involved. (Freeman 2000, Pardoll 2012). In addition to T cell receptor signal attenuation by recruiting phosphatases, PD-1 can regulate gene expression through various cell cycle signaling pathways such as PI3K and RAS. (Sui 2015). A more recently proposed additional mechanism for the inhibition is that PD-1 suppresses T cell function by inactivating CD28 signaling. (Hui 2017). Those results revealed that PD-1 favors dephosphorylation of the T cell costimulatory receptor, CD28, over dephosphorylation of the T cell receptor. (Hui 2017). In

addition, a study by Kamphorst et. al determined that the effectiveness of PD-1 targeted therapy is dependent on CD28-costimulation of PD-1 positive exhausted CD8+ proliferating T cells. (Kamphorst 2017).

The expression of PD-1 and PD-L1 has been investigated in veterinary species mostly using cross-reactive human or bovine antibodies. It was demonstrated in dogs with visceral leishmaniasis that PD-1 signaling through its ligands, PD-L1 and PD-L2, induces apoptosis of T lymphocytes. (Chiku 2016). Both porcine and feline PD-1 are similar to human PD-1 in structure and sequence. (Jeon 2007, Folkl 2010). IFN-γ treated T cells in cattle with bovine leukemia virus resulted in PD-1 upregulation. (Ikebuchi 2013). Recently, it was demonstrated in peripheral blood from healthy dogs that 5-10% of CD4+ T cells and 20-25% of CD8+ T cells expressed PD-1 using monoclonal antibodies specific for canine PD-1; in people only 5% of CD8+ T cells from peripheral blood express PD-1, suggesting a biological difference between species. (Coy 2017).

B. Role in Neoplasia

In addition to limiting the initiation, amplitude, and duration of an immune response to pathogens, thus shielding tissues from immune mediated injury, the PD-1/PD-L1/PD-L2 pathway also inhibits immune responses to tumors. (LaFleur 2018, Sui 2015). Protective host strategies that facilitate tumor suppression are diverse and distinct; different stages of tumor progression confer different types of immune responses. (Ostroumov 2018). The critically important immune cells during the

malignant state of tumor development are CD4+ and CD8+ effector T cells, which mediate elimination of tumor cells through tumor-specific antigens. (Ostroumov 2018).

Cell-mediated antitumor immunity is primarily mediated through cytotoxic CD8+ T cells upon recognition of tumor antigen by a T cell receptor; this interaction allows the CD8+ T cell to directly kill tumor cells via synaptic exocytosis of cytotoxic perforins and granzymes. (Durgeau 2018, Tsukumo 2018). Likewise, CD8+ T cells can indirectly enhance antitumor ability via cytokine secretion of IFN-γ and TNF. (Durgeau 2018, Tsukumo 2018). Supporting this mechanism of an antitumor response is the well-known correlation between the number of tumor infiltrating CD8+ T cells and the control of tumor progression. (Durgeau 2018). The activity of tumor antigens specific for CD8+ T cells can be diminished when tumor cells express PD-1, thereby strengthening tumor growth. (Liu 2013).

Likewise, PD-1 blockade was noted to augment tumor antigen specific T cell responses and impede tumor growth. (Sui 2015). Therefore, effector CD8+ T cell functions are impaired in an immunosuppressive environment. Inhibiting this antitumor immunity via PD-1/PD-L1/PD-L2 regulation, specifically through checkpoint blockade, promotes tumor progression. A key mechanism that plays a role in a dysfunctional T cell response against tumors is T cell exhaustion, which was identified initially in chronic infection, and later highlighted as part of the tumor microenvironment. (Wherry 2011, Dyck 2017). Exhausted T cells are characterized

by loss of IL-2 production, a diminished capacity for proliferation, decreased cytotoxicity, and reduced production of inflammatory cytokines. (Dyck 2017).

In addition, exhausted T cells are those that have experienced high levels of stimulation or a reduction in CD4+ T cell help; increased expression of PD-1 is a symbol of exhausted T cells. (Wherry 2011). The majority of tumor infiltrating lymphocytes express increased PD-1 levels and are associated with a dysfunctional impaired antitumor response. (Sui 2015). Chronic antigen exposure such as occurs with cancer, exposes T cells to persistent inflammatory signals resulting in decline of T cell function; this results in insufficient control of tumors (Wherry 2011). This state of T cell exhaustion leads to a failure to transition to quiescence, sustained upregulation of inhibitory receptors, transcription factor alterations, and failure to develop functional antigen-independent memory T cells. (Wherry 2011). Interestingly, in a review by Dyck et al, it was noted that infection-induced T cell exhaustion can be reversible, whereas tumor-induced T cell exhaustion is only reversible at early stages of malignancy and becomes irreversible at later stages. (Dyck 2017).

It has been demonstrated that the PD-L1/PD-L2 ligand can be upregulated in many different tumor types, suppressing the immune response and allowing the cancer cells to evade the immune system. Immunotherapy using checkpoint inhibitor blockade of PD-1 or its ligands has generated considerable interest in recent years given its successful outcome in various human cancers, including lymphoma, melanoma, renal

cell carcinoma, and non-small cell lung cancer. (Sui 2015). By using therapeutic antagonistic antibodies against inhibitory molecules, effector T cell function can be restored. The transformative effect of checkpoint molecule blockade therapy on the potential to improve clinical outcomes highlights the protective antitumor role of the PD-1/PD-L1/PD-L2 pathway.

C. Immune Evasion by Lymphoma

Cancer cells can employ multiple mechanisms to escape immune recognition, and this feature is recognized as a hallmark of cancer (Hallahan 2011). A review paper by Charette et. al classifies the numerous different immune escape strategies of different types of human lymphoma into two primary mechanisms. (Charette 2018). Neoplastic lymphocytes may either hide from the immune system or they can defend themselves against immune system destruction. (Charette 2018). These two types of mechanisms are reviewed in detail by Charette et. al and key features of such unchecked lymphocyte proliferation are highlighted here. (Charette 2018).

Achieving invisibility status can be achieved in multiple ways, including preventing antigens from being presented via alteration of MHC class I and MHC class II molecules, downregulating co-stimulatory molecules CD80/CD86, and preventing adhesion. (Charette 2018). Mutations in the beta2-microglobulin gene occur in about 30% of diffuse large B cell lymphomas with an irreversible loss of class I MHC molecules on neoplastic lymphocytes in 55-75% of diffuse large B cell lymphomas. (Challa-Malladi 2011). In the same study, genetic inactivation of both CD58 and

MHC-I on neoplastic lymphocytes leads to inactivation of natural killer cells, which occurs in 61% of diffuse large cell lymphoma. (Challa-Malladi 2011).

Several gene alterations are associated with epigenetic transcriptional regulation of MHC-II downregulation and loss. (Charette 2018). 20% of diffuse large B cell lymphomas have loss of MHC-II expression which is associated with worse outcomes. (Tada 2016, Rimsza 2004). The most commonly altered genes in diffuse large B cell lymphoma are histone methyltransferases (HMTs) and histone acetyltransferases (HATs); 50% of germinal center B cell diffuse large cell lymphomas and 30% of activated B cell like diffuse large cell lymphomas exhibit these alterations. (Pasqualucci 2011).

Co-stimulatory molecules CD80/CD86 are expressed on antigen presenting cells, including B cells, where binding to CD28 can promote T cell activation; CD80/86 (preferentially) bind to the inhibitory receptor CTLA-4 leading to T cell inhibition. (Charette 2018). CD80 was shown to be expressed in 90% of diffuse large B cell lymphomas. (Dakappagari 2012). The prognostic significance of CD80 and CD86 is unknown, possibly in part due to their dual specificity. (Charette 2018). Expression of intercellular adhesion molecule I is lost in only 7% of diffuse large B cell lymphomas. (Stopeck 2000).

In addition to becoming invisible and hiding from immune detection, neoplastic lymphocytes employ various mechanisms to avoid immune clearance. These

mechanisms include apoptosis resistance, expression of inhibitory ligands, and/or inducing an immunosuppressive environment. (Charette 2018). Inhibition of granzyme B is mediated via the protease inhibitor 9 (PI9), which was found to be expressed in 33% of diffuse large B cell lymphomas in one study, thereby protecting against apoptosis. (Murris 2004). Loss of the FAS (CD95) ligand, the extrinsic apoptotic pathway activator, was noted in 51% of diffuse large B cell lymphoma in another study; expression of FAS on lymphoma cells was associated with improved survival. (Chatzitolios 2010). A BCL-2 mutation is one of the most common mutated genes in non-Hodgkin lymphoma and is present in 37% of diffuse large B cell lymphomas; a mutation in this anti-apoptotic protein leads to inhibition of apoptosis. (Shuetz 2012).

Another lymphoma immune escape mechanism involves immune checkpoint molecules. The checkpoint molecules PD-L1 and PD-L2 are overexpressed not only in neoplastic lymphocytes, but also in the tumor microenvironment. (Laurent 2015). PD-1 ligands engage the PD-1 receptor causing inhibition of both T cell activation and the antitumor response. Diffuse large B cell lymphomas of the more aggressive germinal center B cell subtype often express PD-L1, while PD-L2 is primarily expressed in Hodgkin lymphoma and primary mediastinal large B cell lymphoma. (Kiyasu 2015, Roemer 2016). Another important finding in the Kiyasu study was that PD-L1 expression in diffuse large B cell lymphoma is associated with poor survival. (Kiyasu 2015).

Finally, lymphoma can evade immune detection through inducing an immunosuppressive environment. (Charette 2018). The most notable components in diffuse large B cell lymphomas are the expression of galactin-3, which protects tumor cells against death, and indoleamine (IDO) upregulation, which suppresses cytotoxic T cells and is associated with more aggressive disease (Hoyer 2004, Liu 2014).

D. Expression in Lymphoma

Checkpoint molecule blockade therapy in people with lymphoid malignancy has only recently emerged; however, this treatment strategy has shown promising clinical efficacy, particularly in people with relapsed Hodgkin lymphoma and non-Hodgkin lymphoma. (Galanina 2017).

The underlying mechanisms that lead to unchecked neoplastic lymphocyte proliferation have not been fully elucidated in all cases; however, common mechanisms contributing to PD-L1/PD-L2 overexpression in hematologic malignancy include genetic alteration of chromosome 9p24, which encodes PD-L1 and PD-L2, and Epstein-Barr virus (EBV) infection. (Shi 2014, Galanina 2017, Charette 2018). In 19% of diffuse large B cell lymphoma, there is a genetic alteration in 9p24. (Georgiou 2016). Epstein-Barr virus infection induces PD-L1 expression through an EBV oncoprotein, latent membrane protein 1, in mediastinal large B cell lymphomas. (Green 2012).

PD-L1 is expressed both on neoplastic lymphocytes in diffuse large B cell lymphoma, and on tumor infiltrating cells of the microenvironment, primarily macrophages. (Andorsky 2011, Chen 2013). This finding was consistent with a retrospective study by Kiyasu et. al, which also demonstrated the expression of PD-L1 on tumor cells in newly diagnosed diffuse large B cell lymphoma; in addition, this study showed that PD-L1 expression is associated with poor overall survival. (Kiyasu 2015). In many other studies, a prognostic value for PD-L1 has been assessed in non-Hodgkin lymphomas; however, the results are controversial not only due to the heterogeneity of lymphoma subtypes evaluated, but because of different prognostic outcomes. (Zhao 2018). A meta-analysis across nine studies analyzed the relationship between PD-L1 expression and prognosis in non-Hodgkin lymphomas and reduced heterogeneity by grouping subtypes. (Zhao 2018). The analysis established that high PD-L1 expression in diffuse large cell B cell lymphomas correlated with a poor prognosis. (Zhao 2018).

Patients with diffuse large B cell lymphoma who had a low number of tumor infiltrating lymphocytes and concurrent PD-L1 positivity had a poorer prognosis. (Kiyasu 2015). In contrast, the presence of high numbers of PD-1 positive tumor infiltrating lymphocytes is associated with better overall survival, as demonstrated in a retrospective analysis of diffuse large B cell lymphoma cases. (Ahearne 2014). Several other studies have demonstrated similar findings and shown that a high number of PD-1 positive tumor infiltrating lymphocytes is associated with better progression-free survival and overall survival. (Kwon 2016, Fang 2017).

In a study by Shi, it was demonstrated that positive PD-L2 IHC expression was detected in 72% of primary mediastinal B cell lymphoma, an aggressive large cell lymphoma. (Shi 2014). In the same study, only 3% of diffuse large B cell lymphomas were positive; in addition, PD-L2 was shown to be restricted to tumor cells and was not identified in macrophages in the tumor microenvironment. (Shi 2014). The results of these findings suggested that PD-L2 IHC may be used to distinguish primary mediastinal B cell lymphoma from diffuse large B cells lymphoma. In another study evaluating PD-L2 IHC expression, positive PD-L2 expression was detected in 41% of Hodgkin lymphomas, 78% of primary mediastinal large B cell lymphomas, and 7% of anaplastic large cell lymphomas (Panjwani 2018).

Recently, a preliminary retrospective analysis of PD-L1 IHC expression was performed to evaluate the PD-1/PD-L1/PD-L2 axis as a therapeutic target for checkpoint molecule immunotherapy in dogs. (Makaewa 2014). PD-L1 was detected in tumor tissues with several types of neoplasia, including 69.2% of melanomas (9/13), 66.7% of mastocytomas (4/6), and 70% of renal cell carcinomas (7/10). (Makaewa 2014). Diffuse large B cell lymphoma was not evaluated. In addition, it was shown that PD-L1 expression on the same various canine tumor cell lines can be induced by treatment with IFN-γ. (Makaewa 2014).

Using flow cytometry, Hartley et. al demonstrated basal PD-L1 expression on all fourteen canine tumor cell lines evaluated with the lowest expression detected on the

T cell lymphoma cell line. (Hartley 2016). This study also suggested PD-L1 variability in tumors of hematopoietic origin. (Hartley 2016). In this same study, PD-L1 was significantly upregulated on all tumor cell lines, including lymphoma, as well as tumor infiltrating macrophages, following IFN-γ treatment. (Hartley 2016).

In a more recent study by this group, expression of PD-1 and PD-L1 was analyzed using flow cytometry from canine lymph node aspirates. (Hartley 2018). Expression was compared between dogs with untreated lymphoma (N=40; 34 B cell, 6 T cell) and healthy dogs (N=17). (Hartley 2018). PD-1 expression by malignant lymphocytes was not increased when compared to normal T and B cells from healthy dog lymph nodes. (Hartley 2018). The expression of PD-L1 was significantly increased in malignant B cells relative to normal B cells from healthy lymph nodes; however, PD-L1 expression was not increased in malignant T cells relative to normal T cells. (Hartley 2018). These authors also describe a significant upregulation of dual positive PD-1/PD-l tumor infiltrating T cells in B cell lymphoma, and upregulation of dual positive PD-1/PD-L1 tumor infiltrating B cells in T cell lymphoma; the significance was not clear though may suggest concurrent immune activation with checkpoint molecule upregulation. (Hartley 2018). In addition, chemotherapy resistant lymphoma cell lines exhibited upregulated PD-1 and PD-L1; however, there was no in vivo difference in expression. (Hartley 2018).

A larger study by Maekawa demonstrated PD-L1 IHC expression in a variety of tumor types, with the highest rates of expression in 80% of mammary

adenocarcinomas (4/5), 70% of melanomas (7/10), and 70% of osteosarcomas (7/10). (Maekawa 2016). Diffuse large B cell lymphoma was one of seven other types of tumors which did not express PD-L1 (0/5). (Maekawa 2016). Flow cytometry demonstrated that PD-1 was highly expressed on CD8+ and CD4+ tumor infiltrating lymphocytes from surgically excised oral melanoma cases (N=6) when compared to peripheral blood lymphocytes from healthy dogs. (Maekawa 2016).

Canine cell lines from five tumor types, including nine various lymphoma cell lines, were evaluated for PD-L1 expression using flow cytometry and western blotting. (Shosu 2016). Flow cytometric analysis detected PD-1 in two of the lymphoma cell lines and PD-L1 in two of the other lymphoma cell lines. (Shosu 2016). Treatment with IFN-γ did not induce cell surface expression of PD-L1 in any of the lymphoma cell lines. (Shosu 2016). Western blotting detected PD-L1 protein in all nine lymphoma cell lines even though most did not have cell surface expression, demonstrating expression within the cells as well as cross-reactivity to human PD-L1; the cell culture process was suspected to have affected the loss of surface expression. (Shosu 2016). PD-L1 expression was detected by IHC analysis in all fifteen lymphoma tissue specimens, including four high-grade B cell lymphomas, five high-grade T cell lymphomas, and three each low-grade B/T cell lymphomas. (Shosu 2016). In addition, this study is the first to report PD-L1 expression in normal canine tissue specimens. (Shosu 2016). Prior to this study, PD-L1 expression in tumor specimens had only been reported by Makaewa et. al.

Kumar et al. analyzed PD-L1 expression various ways in naturally occurring canine lymphoma. (Kumar 2017). They observed positive PD-L1 mRNA expression in B cell lymphoma cell lines, as well as PD-L1 IHC positivity in formalin-fixed paraffin embedded tissues (N=3), as well as positive PD-L1 mRNA expression in peripheral blood mononuclear cells (PBMCs) from dogs with diffuse large B cell lymphoma. (Kumar 2017).

There was no significant difference in PD-L1/PD-L2 mRNA expression between lymph node aspirate cells from dogs with untreated high-grade B cell lymphoma (N=18) and age matched healthy control dogs (N=9). (Tagawa 2018). In contrast, a flow cytometric analysis demonstrated significantly higher PD-1 expression in both peripheral blood T cells and lymph node T cells from dogs with high-grade B cell lymphoma when compared to PD-1 expression in similar specimens from the control group. (Tagawa 2018). The significance of any association to antitumor immunity or clinical outcomes is unclear. (Tagawa 2018).

Two recent studies investigated the prognostic significance of checkpoint molecules in canine lymphoma. (Aresu 2018, Ambrosius 2018). In a recent gene expression profiling study in fifty dogs with diffuse large B cell lymphoma, the lymph node biopsies from the twenty-five dogs with a poor outcome were strongly positive for PD-L1 by IHC. (Aresu 2018). Those same cases with a poor outcome also had higher PD-1 expression. (Aresu 2018). Another study describes no prognostic relevance for PD-L1 in dogs with diffuse large B cell lymphoma. (Ambrosius 2018). This study

did, however, demonstrate that PD-L1 mRNA expression in half of the lymph node biopsies (N=21) with diffuse large B cell lymphoma (total = 42) had a fold change >1 (i.e., expression greater than the mean), suggestive of PD-L1 overexpression. (Ambrosius 2018). PD-L1 expression was however, noted to be variable in the lymphoma dogs. (Ambrosius 2018).

A very recent retrospective analysis evaluated the prognostic relevance of non-neoplastic lymphocytes in dogs with diffuse large B cell lymphoma. (Martini 2019). The flow cytometry data from fifty-nine treatment naïve dogs with a histopathologic diagnosis of diffuse large B cell lymphoma demonstrated that higher percentages of non-neoplastic T cells correlated with a decreased likelihood of tumor progression, possibly due to limited spread of neoplastic lymphocytes in lymph nodes. (Martini 2019).

Chapter 3: Retrospective analysis of checkpoint molecule mRNA expression in canine lymphoma and canine reactive lymphoid hyperplasia

I. Introduction

Canine lymphoma is the most common hematopoietic malignancy; however, despite a variety of treatment strategies, the majority of canine patients will eventually relapse. Improvements in long-term survival times and duration of remission have remained stagnant. There is an unmet need for additional treatment strategies that have the potential to improve drug resistant relapse, extend duration of remission, and improve long-term survival times.

Programmed death (PD)-1 is a receptor on T cells, which, when bound with its ligand, PD-L1 or PD-L2, expressed primarily on antigen presenting cells, serves to downregulate the immune response. However, many cancer types express PD-L1 and are able to evade immune detection. In human medicine, immunohistochemical analysis is commonly used to identify tumor PD-L1 expression; however, PD-L1 IHC expression is not a consistent predictor of positive response to therapy. Overexpression of PD-L1/PD-L2 has been described in people with lymphoma and overexpression of PD-L1 has been associated with unfavorable outcomes. Promising effects of targeted immunotherapy using checkpoint blockade of PD-1 and PD-L1 in people with relapsed and refractory lymphoma creates an opportunity to investigate analogous canine cancers. There is limited information regarding the roles and expression of these checkpoint molecules in canine lymphoma. Studies evaluating expression of PD-1 and PD-L1 in dogs are conflicting both in detection modalities

and results. The usefulness of qRT-PCR for determining PD-1/PD-L1/PD-L2 expression in identifying canine patients whose lymphomas may respond to therapies targeting the PD-1/PD-L1 pathway is unclear.

The proposed study investigated the patterns of expression of mRNAs encoding PD-1 and its ligands PD-L1 and PD-L2 in lymphoma and in reactive lymphoid hyperplasia controls. We hypothesized that PD-L1 and PD-L2 are significantly upregulated in lymphoma, suppressing the immune response and allowing unchecked neoplastic lymphocytes to evade detection by the immune system. In this study, we sought to test the hypothesis by measuring expression of PD-1, PD-L1, and PD-L2 using qRT-PCR analysis on formalin-fixed paraffin embedded tissue from dogs with untreated lymphoma and dogs with reactive lymphoid hyperplasia.

II. Materials and Methods

A. Study population

Biopsy samples from client-owned dogs with lymphoma or reactive lymphoid hyperplasia were selected for inclusion in this retrospective study. Specimens were selected from archived formalin-fixed paraffin-embedded tissues that were submitted to the Virginia-Maryland College of Veterinary Medicine, Veterinary Teaching Hospital for routine histopathologic diagnosis. The Veterinary Teaching Hospital medical record database was electronically searched from 2016 through 2018 to identify twenty dogs that had a histologic diagnosis of lymphoma (n=10) or reactive lymphoid hyperplasia (n=10). All histologic diagnoses were made by board-certified veterinary pathologists and pathology

residents. In addition, biopsy samples were evaluated by routine processing and hematoxylin and eosin staining.

B. RNA extraction and cDNA synthesis

Total RNA was extracted from archived formalin-fixed paraffin embedded specimens using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Two freshly cut 10 µm scrolls were obtained from each paraffin block. Prior to purification of nucleic acids, deparaffinization using melting was performed. Proteinase K followed by DNase I was used for RNA purification from FFPE tissue sections and removal of genomic DNA. The extracted RNA was resuspended/eluted in 15 µL of nuclease-free water and stored at -80° Celsius until reverse transcription was performed. RNA extraction was repeated if the RNA concentration was less than 20 ng/uL (2 lymphoma, 4 reactive lymphoid hyperplasia). The RNA concentration was measured both immediately following RNA extraction and prior to reverse transcription. A NanoDrop 2000 (Thermo Fisher Scientific) was used to measure the RNA concentration both immediately following RNA extraction and prior to cDNA synthesis. Absorbance ratios of $A_{260/280}$ were used to determine RNA purity. cDNA was synthesized according to manufacturer instructions from 220 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fischer Scientific) using MultiScribe™ Reverse Transcriptase master mixes containing random primers. Each cDNA reaction was performed in a 20 µL total volume, containing 10µL of 2X master

mix (2.0 μL 10X RT Buffer, 0.8 μL 25X dNTP, 2.0 μL 10X RT Random Primers, 1.0 μL Reverse Transcriptase, and 4.2 μL nuclease-free water) and 10μL of RNA (RNA was diluted to obtain the same amount of RNA per cDNA reactions). A parallel set of non-reverse transcriptase reactions was not performed at this step in an attempt to conserve RNA for use across all experimental procedures, including RNA concentration measurements x 2, reverse transcription reactions, RNA-only triplicate qPCR reactions, and loss during reagent transfers. cDNA was stored at -20° Celsius until qRT-PCR was performed.

C. Quantification of PD-1, PD-L1, and PD-L2

Real time quantitative RT-PCR was performed to quantify mRNA expression for PD-1, PD-L1, and PD-L2. qRT-PCR was performed using primer sets and probes for each target gene. Forward and reverse primers and probes (Appendix table 1) were previously generated and validated for PD-L1 (BH-796, BH-797, BHTP-42) and PD-L2 (BH-798, BH-799, BHTP-43); primer/probe stock was prepared prior to PCR amplification by combining the primers and probe at a final concentration of 18μM per primer and 5μM for the probe. TaqMan® Gene Expression Assays were used for 18S and PD-1 (Thermo Fisher Scientific). (Appendix table 1). The TaqMan® probes contain a FAM reporter dye linked to the 5' end of the probe, a MGB (minor groove binder) at the 3' end of the probe, and a NFQ (nonfluorescent quencher) also at the 3' end of the probe. Each real time qRT-PCR reaction was performed in a 20μL total volume, containing 1.0 μL TaqMan Gene Expression Assay Mix or primer/probe stock (PD-L1 and PD-L2),

10.0 μL TaqMan® Fast Universal PCR Master Mix, No AmpErase UNG, 8.0 μL nuclease-free water, and 1.0 μL cDNA (or nuclease-free water for the no-template control, or diluted out RNA to 20 μL for the no-RT RNA-only control) using a 7500 Fast Real-Time PCR System (Applied Biosystems). The initial incubation for 20 seconds at 95° Celsius was followed by 40 cycles, each consisting of 3 seconds at 95° Celsius (denaturation) and 30 seconds at 60° Celsius (annealing/extension). All samples were run in triplicate. The $2^{-\Delta\Delta CT}$ method was used to assess relative gene expression of PD-1, PD-L1, and PD-L2 (Livak) and the results were normalized to the 18S housekeeping gene (Appendix table 2). The reactive lymphoid hyperplasia group was chosen as the untreated control group/calibrator.

D. Immunohistochemistry

Biopsy samples from dogs with lymphoma (n=10) and reactive lymphoid hyperplasia (n=2) were assessed for CD3 or CD79a expression. Unstained formalin-fixed paraffin embedded tissues were routinely sectioned at 5 μm and processed on an automatic Ventana® Benchmark XT in accordance with previously validated staining protocols. Positive control tissues consisted of canine lymph node and colon for each cell marker. One negative control slide consisted of tissue cut from a randomly selected lymphoma patient tissue, with the primary antibody application omitted.

For the immunodetection of CD79a, a kit from Biocare Medical was used (Biocare Medical Clone HM47/A9). The kit consists of a primary monoclonal mouse anti-human CD79a antibody and a secondary alkaline phosphatase red labeled goat anti-mouse antibody (Ventana UltraView Universal Alkaline Phosphatase Red Detection Kit). For the immunodetection of CD3 labeling, a kit from Dako was used (Dako Clone A0452). The kit consists of a primary polyclonal rabbit anti-human antibody and a secondary alkaline phosphatase red labeled goat anti-rabbit antibody (Ventana UltraView Universal Alkaline Phosphatase Red Detection Kit). Visualization of antibody binding was obtained with Fast Red chromogen incubation and hematoxylin counterstaining.

E. Statistical analysis

All data analysis was performed using a commercial statistical software program (SAS Version 9.4, Cary, NC). Normal probability plots were used to assess normality. Normal probability plots showed that continuous data (relative expression levels, ΔCTs, and 2^-ΔCTs) were skewed, thus continuous data are summarized as medians (range). Statistical significance was set at $P < 0.05$.

Checkpoint molecule relative expression levels (2^-ΔΔCT) were analyzed using nonparametric methods. Comparison of expression between diagnosis groups (lymphoma, reactive) was performed using the Wilcoxon rank sum test. Within the diagnosis group, phenotype comparisons between B cell lymphoma, T cell

lymphoma, and reactive lymph node were determined using the Kruskal-Wallis test, followed by Dunn's procedure for two-way comparisons.

Relative abundance between checkpoint molecules (PD-L1 vs PD-1, PD-L2 vs PD-1, PD-L1 vs PD-L2) was determined using ΔCTs followed by transformation to 2^-ΔCTs (Appendix table 4) to stabilize the statistical model. The 2^-ΔCTs were then analyzed using nonparametric methods. Comparison of relative expression abundance between diagnosis groups (lymphoma, reactive) was performed using the Wilcoxon rank sum test. Within the diagnosis group, phenotype comparisons between B cell lymphoma, T cell lymphoma, and reactive lymph node were determined using the Kruskal-Wallis test, followed by Dunn's procedure for two-way comparisons.

Because scatter plots showed a linear relationship between checkpoint molecules, a regression analysis was performed to evaluate the relationship between PD-1 and PD-L1, PD-1 and PD-L2, and PD-L1 and PD-L2. Regression analysis within an ANOVA (analysis of covariance) framework was performed on ΔCTs; ΔCTs were used to stabilize the statistical model.

III. Results

A. Patient characteristics

Ages for dogs with lymphoma ranged from 5-13 years (1 intact male, 4 neutered males, 5 spayed females.) Ages for dogs with lymphoid hyperplasia ranged from 4 months to 12 years (6 neutered males, 4 spayed females).

The specimen site/source for the dogs with lymphoma were all peripheral lymph nodes except one mesenteric lymph node. Nine out of ten dogs had large cell lymphoma and one was diagnosed with small cell lymphoma. The specimen site/source for dogs with lymphoid hyperplasia were peripheral lymph nodes (N=5), ileocolic lymph nodes (N=1), retropharyngeal lymph nodes (N=1), mesenteric lymph nodes (N=2), and inguinal lymph nodes (N=1).

B. mRNA quantification by real-time PCR analysis

mRNA expression of PD-1 and PD-L2 was detected in all of the lymphoma and reactive lymphoid hyperplasia samples. mRNA expression of PD-L1 was detected in 9 out of 10 reactive lymphoid hyperplasia samples and all of the lymphoma samples. Undetectable levels of PD-L1 transcript were observed in one reactive lymphoid hyperplasia sample (Appendix table 2).

PD-1 expression was significantly lower ($P=0.009$) in dogs with lymphoma relative to dogs with reactive lymphoid hyperplasia (table 1a, figure 1). PD-1 expression was also significantly lower($P=0.0020$) in B cell lymphoma relative to reactive lymphoid hyperplasia (table 1c, figure 1).

Comparison of PD-L1 expression between dogs with lymphoma and dogs with reactive lymphoid hyperplasia showed no significant difference in expression (P=0.1288); however, expression was lower in dogs with lymphoma relative to those with reactive lymphoid hyperplasia (table 1a, figure 2). Comparison of phenotypes showed no statistically significant differences in PD-L1 expression (P=0.2534, table 1a, figure 2).

Comparison of PD-L2 expression between dogs with lymphoma and dogs with reactive lymphoid hyperplasia showed significantly decreased PD-L2 in dogs with lymphoma (P=0.0238) relative to those with reactive lymphoid hyperplasia (table 1a, figure 3). There was significantly decreased PD-L2 expression in B cell lymphoma relative to reactive lymph node (P=0.0081, table 1b, figure 3).

The relative abundance of immune checkpoint molecule expression levels was compared within each sample between PD-L1 vs PD-1, PD-L2 vs PD-1, and PD-L1 vs PD-L2 (table 2a). The abundance of PD-L1 relative to PD-1 expression was significantly different between lymphoma and reactive lymphoid hyperplasia (P=0.0192), including B cell lymphoma and reactive lymphoid hyperplasia (P=0.0032, table 2a, table 2b, figure 4). The relative abundance of PD-L1 vs PD-1 when expressed as a mean ± std dev in the reactive lymph node tissue was 0.23 ± 0.34 and was more than twice as high (1.32 ± 1.33) in the lymphoma tissue.

The abundance of PD-L2 relative to PD-1 was also significantly different between lymphoma and reactive lymphoid hyperplasia ($P=0.0046$), including B cell lymphoma and reactive lymphoid hyperplasia ($P=0.0017$, table 2a, table 2c, figure 5). The relative abundance of PD-L2 vs PD-1 when expressed as a mean ± std dev in the reactive lymph node tissue was 0.41 ± 0.23 and was more than twice as high (1.58 ± 1.48) in the lymphoma tissue.

The abundance of PD-L1 relative to PD-L2 was not statistically significant between lymphoma and reactive lymphoid hyperplasia; however, there was a significant difference between B cell lymphoma and T cell lymphoma ($P=0.0189$, table 2a, figure 6). The relative abundance of PD-L1 vs PD-L2 when expressed as a mean ± std dev in the reactive lymph node tissue was 0.84 ± 1.20 and was higher (0.90 ± 0.83) in lymphoma.

Scatter plots demonstrate individual cases plotted between diagnosis groups to compare simultaneous target expression relative to reactive lymph node normalized to 18S rRNA (figure 7, figure 8, figure 9). There is a linear relationship between checkpoint molecules such that an increase in one is associated with an increase in the other, for both lymphoma and reactive lymphoid hyperplasia (figure 7, figure 8, figure 9).

Regression analysis of PD-1 ΔCT vs PD-L1 ΔCT determined a statistically significant but equal slope for lymphoma (0.9256, $P<0.0001$) and reactive

lymphoid hyperplasia (0.7430, P=0.0017). For ΔCT, in lymphoma, as PD-L1 increased by one unit, PD-1 increased by 0.9256; in reactive lymphoid hyperplasia, as PD-L1 increased by one unit, PD-1 increased by 0.7430. However, the difference was not significant (P=0.4826, figure 10).

Regression analysis of PD-1 ΔCT vs PD-L2 ΔCT determined statistically significant but equal slopes for lymphoma (0.9598, P<0.0001) and reactive lymphoid hyperplasia (1.0526, P<0.0001). For ΔCT, in lymphoma, as PD-L2 increased by one unit, PD-1 increased by 0.9598; in reactive lymphoid hyperplasia, as PD-L2 increased by one unit, PD-1 increased by 1.0526. However, the difference was not significant (P=0.6477, figure 11).

In contrast, PD-L2 ΔCT vs PD-L1 ΔCT regression analysis (figure 12) showed a statistically significant unequal slope between lymphoma (0.9743, P<0.0001) and reactive lymphoid hyperplasia (0.5412, P=0.0034). There is a significantly steeper slope of the regression line for lymphoma than for reactive lymphoid hyperplasia (figure 12). For ΔCT, in lymphoma, as PD-L1 increased by one unit, PD-L2 increased by 0.9743; in reactive lymphoid hyperplasia, as PD-L1 increased by one unit, PD-L2 increased by 0.5412 (figure 12). This difference was significant (P=0.0497, figure 12).

C. Immunohistochemical evaluation

Of the ten dogs that had a histologic diagnosis of lymphoma, three were previously identified as B-cell lymphoma using CD79a IHC. The remaining 7/10 were subcategorized as B or T-cell lymphoma using CD79 and CD3 labeling by a board-certified anatomic pathologist. In 5 samples, most neoplastic cells diffusely exhibit cytoplasmic immunoreactivity for CD79a, supporting a diagnosis of B cell lymphoma. In 2 samples, the neoplastic cells exhibit diffuse strong cytoplasmic immunoreactivity for CD3, consistent with lymphoma of T cell origin (image 1). The positive CD3 control and negative control were examined and deemed appropriate (image 2, image 3). The CD79a control showed unexplained diffuse nonspecific staining throughout lymphoid tissue, adipose tissue, muscle, and endothelium, and therefore, revalidation of the assay may be necessary in the future.

The two out of ten reactive lymphoid hyperplasia samples that were chosen for IHC evaluation showed 18S normalized expression for PD-1, PD-L1/PD-L2 that fell close to the means for each group. These samples exhibited mixed CD79a and CD3 staining, consisting of a CD3-rich paracortex and CD79a-rich germinal center as expected.

IV. Discussion: see Chapter 5: Discussion, Conclusions, and Further Research.

V. Images, Tables, and Figures

Image 1: Lymph node section, lymphoma. Diffuse strong cytoplasmic reactivity of neoplastic T cells with CD3. Immunolabeling with anti-CD3. 4x objective.

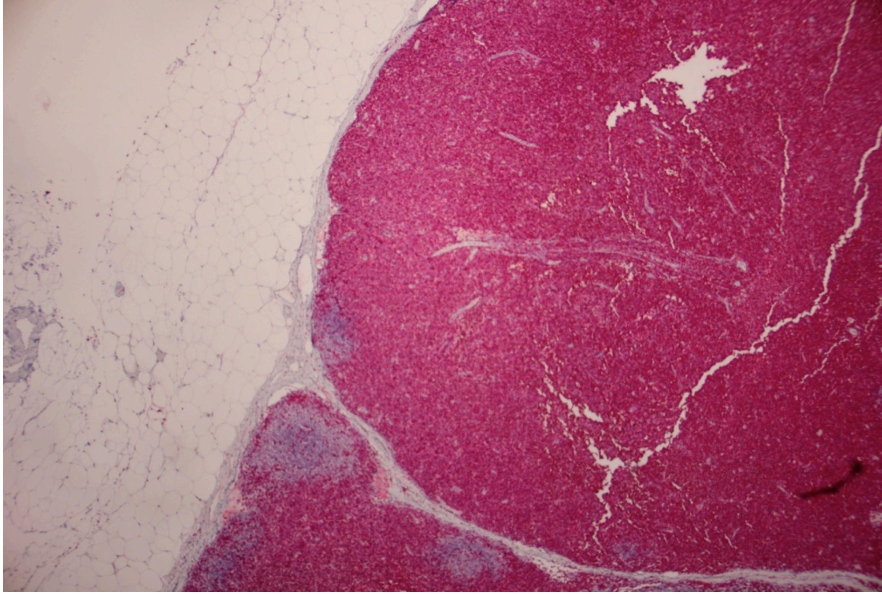


Image 2: CD3 positive control section of canine lymph node tissue. CD3 rich paracortex and medulla and minimal immunoreactivity in follicular germinal centers. 4x objective.

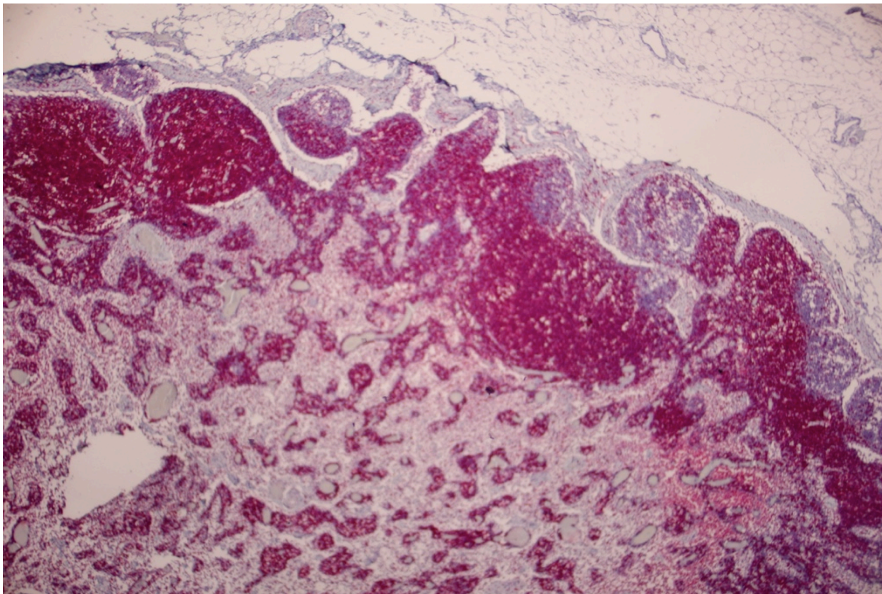


Image 3: CD3 negative control section of canine lymph node tissue. Primary antibody application omitted. 4x objective.

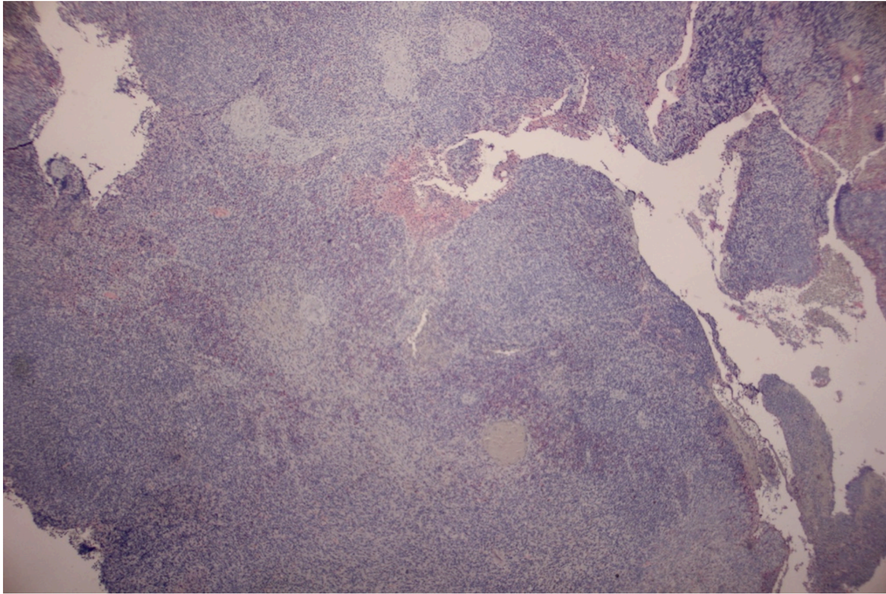


Table 1a: **Summary of statistical results for FFPE Expression**

Outcome	Predictor	Category	n	Median (Range)	P Value
PDL1 expression	Diagnosis	Lymphoma	10	0.2 (0.0-4.3)	0.1288
		Reactive	9	0.5 (0.1-24.7)	
	Phenotype	B cell	8	0.2 (0.0-3.6)	0.2534
		Reactive	9	0.5 (0.1-24.7)	
		T cell	2	2.2 (0.0-4.3)	
PDL2 expression	Diagnosis	Lymphoma	10	0.1 (0.0-12.4)	**0.0238**
		Reactive	10	1.1 (0.1-12.5)	
	Phenotype	B cell	8	0.1 (0.0-0.7)	**0.0302**
		Reactive	10	1.1 (0.1-12.5)	
		T cell	2	6.2 (0.0-12.4)	
PD1 expression	Diagnosis	Lymphoma	10	0.0 (0.0-3.8)	**0.009**
		Reactive	10	1.6 (0.0-6.8)	
	Phenotype	B cell	8	0.0 (0.0-0.2)	**0.0086**
		Reactive	10	1.6 (0.0-6.8)	
		T cell	2	1.9 (0.0-3.8)	

Median expressed as 2^-ΔΔCT

Table 1b: **PDL2 expression by phenotype: P value for Dunn's procedure of two-way comparisons**

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.00815**
2	B cell	T cell	0.37780
3	Reactive	T cell	0.47145

Table 1c: **PD1 expression by phenotype: P value for Dunn's procedure of two-way comparisons**

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.00205**
2	B cell	T cell	0.36351
3	Reactive	T cell	0.33698

Table 2a: Summary of statistical results for FFPE Expression of Relative Abundance

Outcome	Predictor	Category	n	Median (Range)	P Value
L1_PD_Exp	Diagnosis	Lymphoma	10	0.8 (0.1-3.6)	**0.0192**
		Reactive	9	0.1 (0.1-1.1)	
	Phenotype	B cell	8	1.0 (0.2-3.6)	**0.0113**
		Reactive	9	0.1 (0.1-1.1)	
		T cell	2	0.2 (0.1-0.2)	
L2_PD_Exp	Diagnosis	Lymphoma	10	1.1 (0.3-5.5)	**0.0046**
		Reactive	10	0.3 (0.1-0.7)	
	Phenotype	B cell	8	1.3 (0.3-5.5)	**0.005**
		Reactive	10	0.3 (0.1-0.7)	
		T cell	2	0.9 (0.7-1.1)	
L1_L2_Exp	Diagnosis	Lymphoma	10	0.6 (0.1-2.8)	0.3023
		Reactive	9	0.3 (0.1-3.8)	
	Phenotype	B cell	8	0.6 (0.3-2.8)	**0.0346**
		Reactive	9	0.3 (0.1-3.8)	
		T cell	2	0.2 (0.1-0.3)	

Median expressed as $2^{-\Delta CT}$

Table 2b: L1_PD expression by phenotype: P value for Dunn's procedure of two-way comparisons

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.00322**
2	B cell	T cell	0.14400
3	Reactive	T cell	0.72363

Table 2c: L2_PD expression by phenotype: P value for Dunn's procedure of two-way comparisons

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.00171**
2	B cell	T cell	0.83070
3	Reactive	T cell	0.08874

Table 2d: L1_L2 expression by phenotype: P value for Dunn's procedure of two-way comparisons

Obs	Group 1	Group 2	P value
1	B cell	Reactive	0.07096
2	B cell	T cell	**0.01897**
3	Reactive	T cell	0.21120

Figure 1: qRT-PCR relative gene expression level of PD-1 in FFPE tissue. Lymphoma (n=10) vs reactive (n=10) (P=0.009). B cell lymphoma (n=8) vs reactive (n=10) (P=0.0020).

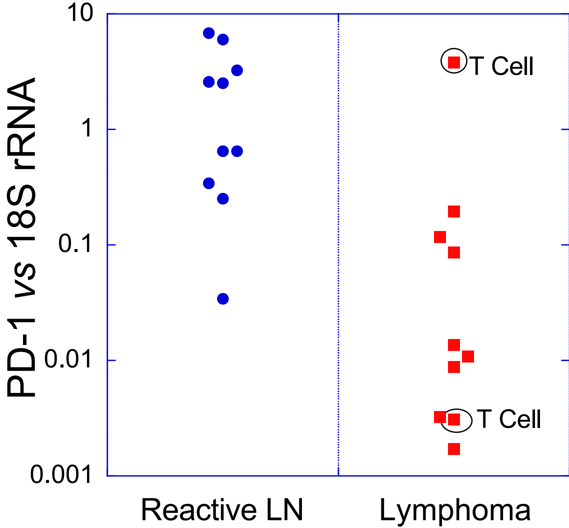


Figure 2: qRT-PCR relative gene expression level of PD-L1 in FFPE tissue. Lymphoma (n=10) vs reactive (n=9) (P=0.1288). B cell (n=8) vs T cell (n=2) vs reactive (n=9) (P=0.2534).

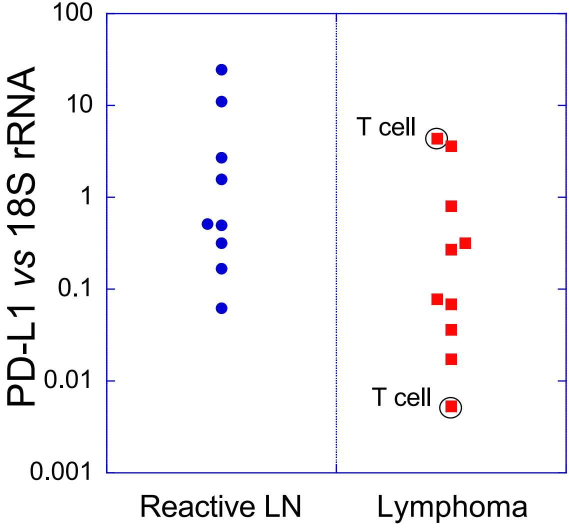


Figure 3: qRT-PCR relative gene expression level of PD-L2 in FFPE tissue. Lymphoma (n=10) vs reactive (n=10) (P=0.0238). B cell lymphoma (n=8) vs reactive (n=10) (P=0.0081).

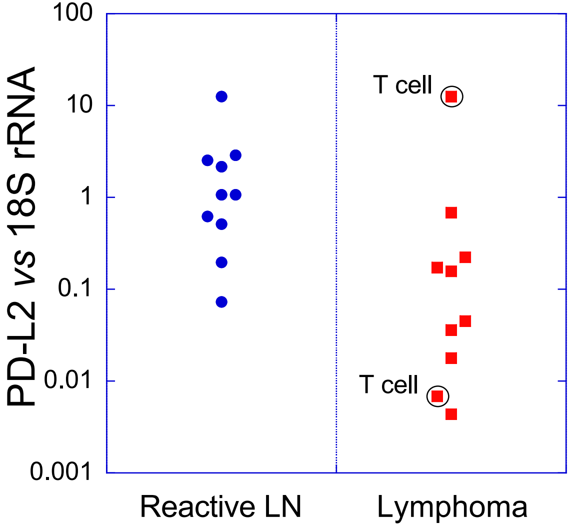


Figure 4: Abundance of PD-L1 relative to PD-1 in FFPE tissue. Lymphoma (n=10) vs reactive (n=9) (P=0.0192). B cell lymphoma (n=8) vs reactive (n=9) (P=0.0032).

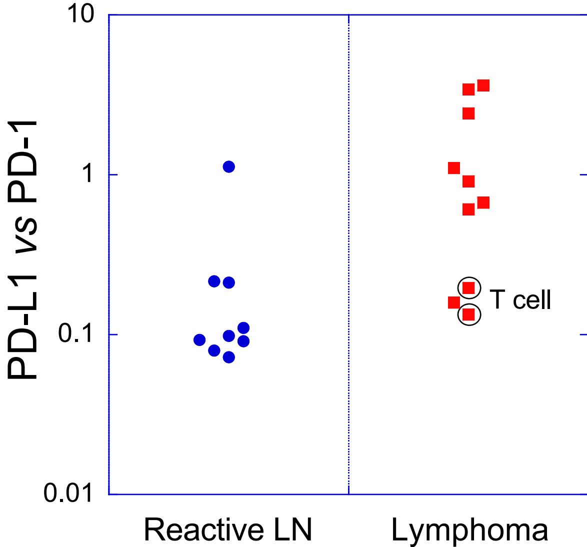


Figure 5: Abundance of PD-L2 relative to PD-1 in FFPE tissue. Lymphoma(n=10) vs reactive (n=10) (P=0.0046). B cell lymphoma (n=8) vs reactive(n=10) (P=0.0017).

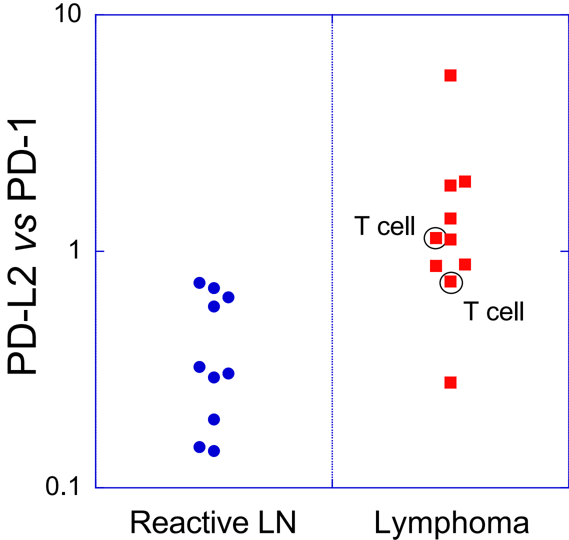


Figure 6: Abundance of PD-L1 relative to PD-L2 in FFPE tissue. Lymphoma (n=10) vs reactive (n=9) (P=0.3023). B cell (n=8) vs T cell lymphoma (n=2) (P=0.0189).

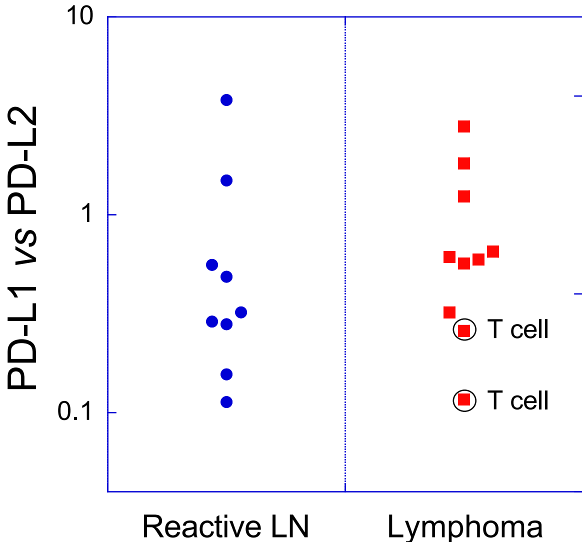


Figure 7: FFPE tissue. Scatterplot of simultaneous PD-L1 and PD-1 expression in lymphoma and in reactive lymphoid hyperplasia. Expression is normalized to 18S and is relative to reactive lymph node.

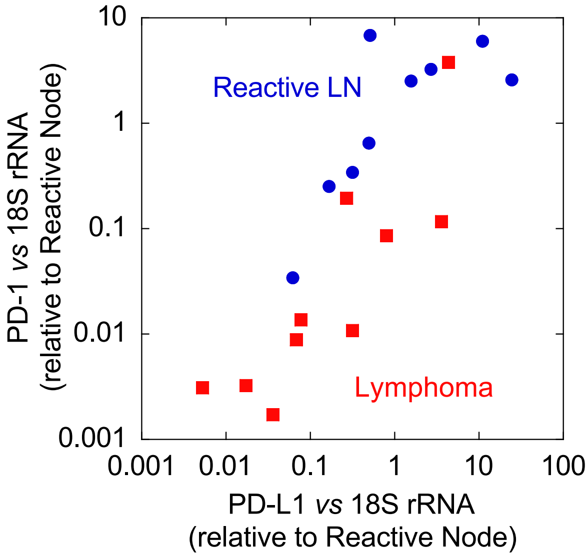


Figure 8: FFPE tissue. Scatterplot of simultaneous PD-L1 and PD-L2 expression in lymphoma and in reactive lymphoid hyperplasia. Expression is normalized to 18S and is relative to reactive lymph node.

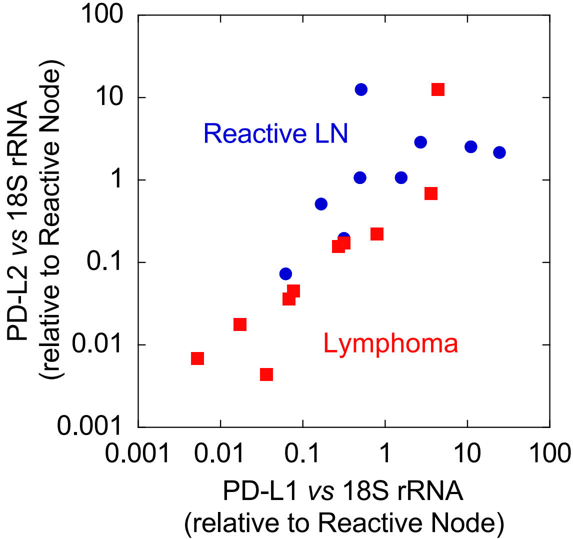


Figure 9: FFPE tissue. Scatterplot of simultaneous PD-L2 and PD-1 expression in lymphoma and in reactive lymphoid hyperplasia. Expression is normalized to 18S and is relative to reactive lymph node.

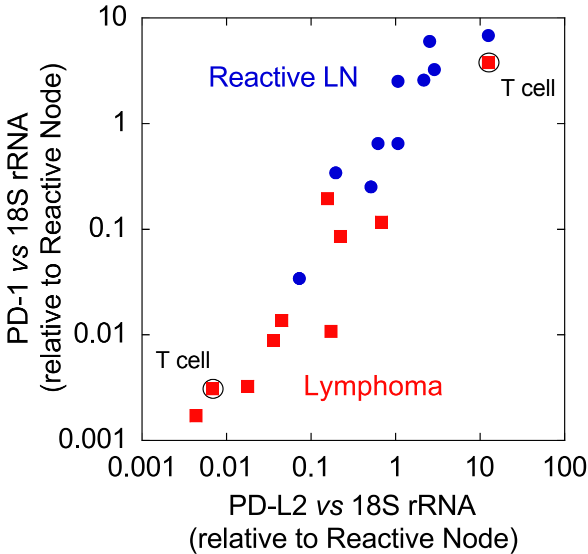


Figure 10: FFPE tissue. Scatterplot and fitted regression line of PD-1 vs PD-L1 for reactive lymph node versus lymphoma using ΔCT. Slopes are not significantly different (P=0.4826).

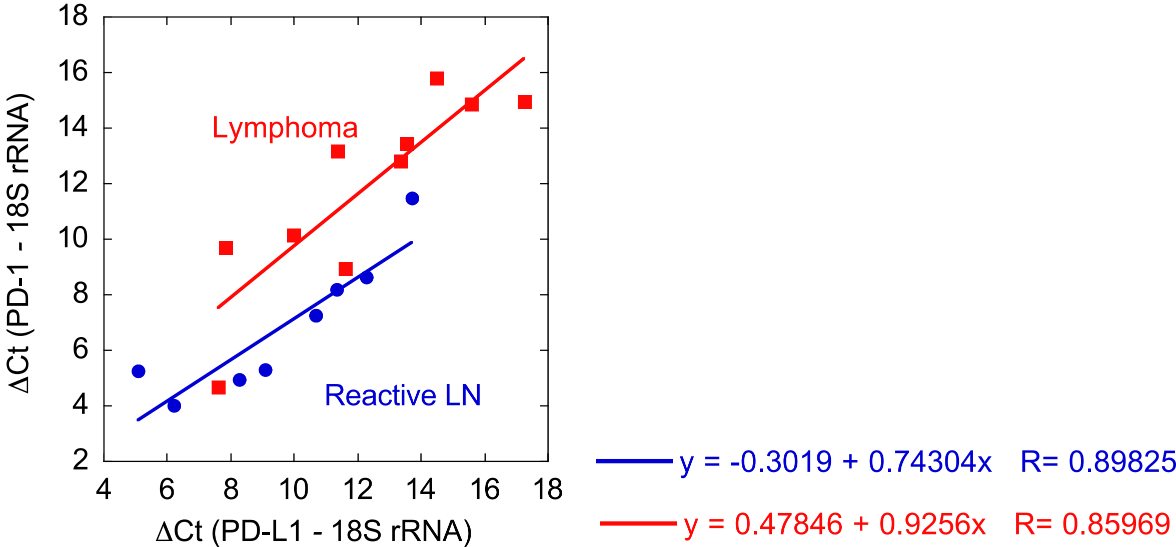


Figure 11: FFPE tissue. Scatterplot and fitted regression line of PD-1 vs PD-L2 for reactive lymph node versus lymphoma using ΔCT. Slopes are not significantly different (P=0.6477).

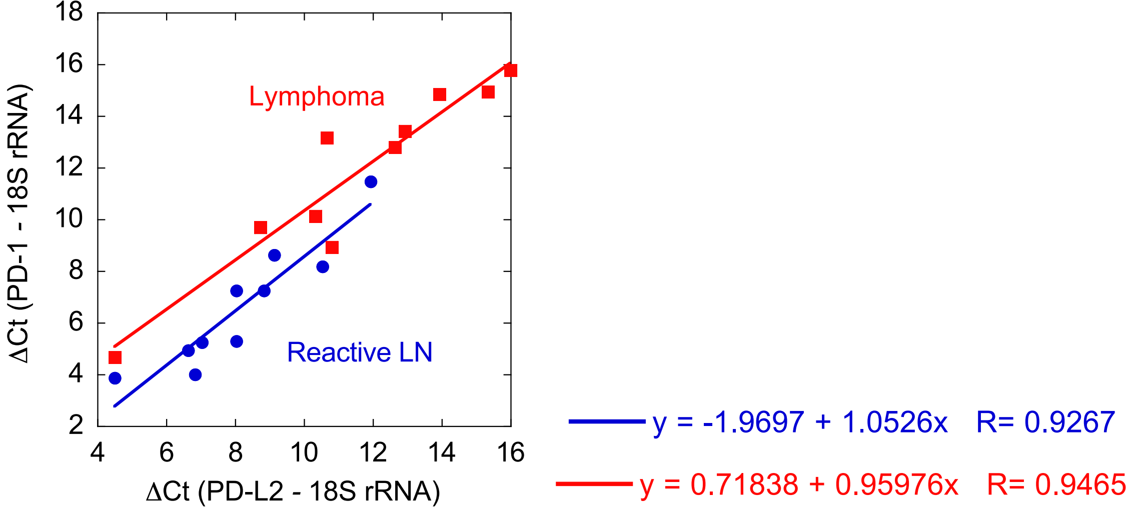
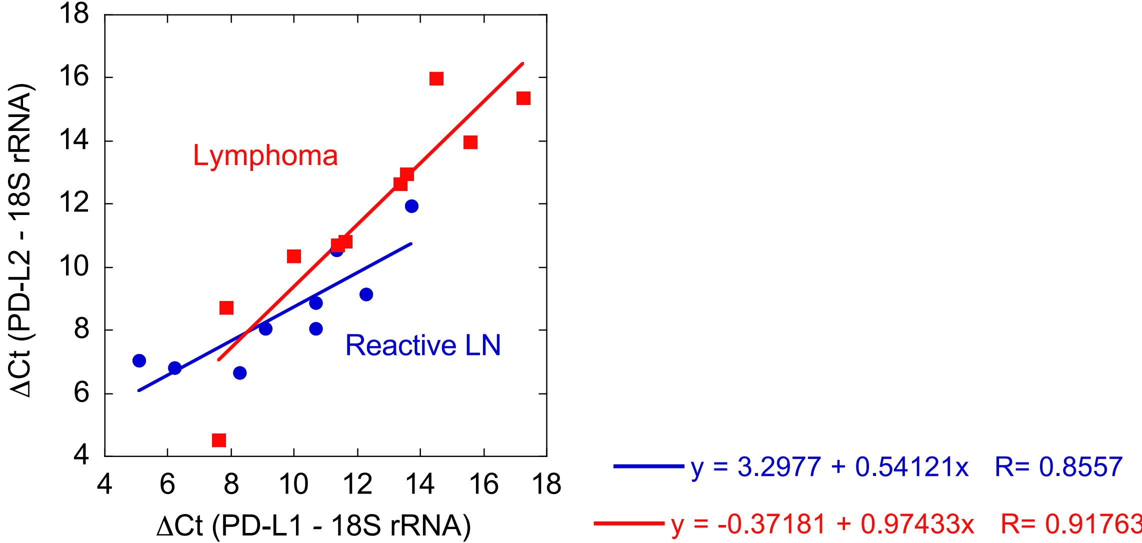


Figure 12: FFPE tissue. Scatterplot and fitted regression line of PD-L2 vs PD-L1 for reactive lymph node versus lymphoma using ΔCT. Slopes are significantly different (P=0.0497).



Chapter 4: Prospective analysis of checkpoint molecule mRNA expression in canine lymphoma and canine reactive lymphoid hyperplasia

I. Introduction

Canine lymphoma is the most common hematopoietic malignancy; however, despite a variety of treatment strategies, the majority of canine patients will eventually relapse. Improvements in long-term survival times and duration of remission have remained stagnant. There is an unmet need for additional treatment strategies that have the potential to improve drug resistant relapse, extend duration of remission, and improve long-term survival times.

Programmed death (PD)-1 is a receptor on T cells, which, when bound with its ligand, PD-L1 or PD-L2, expressed primarily on antigen presenting cells, serves to downregulate the immune response. However, many cancer types express PD-L1 and are able to evade immune detection. In human medicine, immunohistochemical analysis is commonly used to identify tumor PD-L1 expression; however, PD-L1 IHC expression is not a consistent predictor of positive response to therapy. Overexpression of PD-L1/PD-L2 has been described in people with lymphoma and overexpression of PD-L1 has been associated with unfavorable outcomes. Promising effects of targeted immunotherapy using checkpoint blockade of PD-1 and PD-L1 in people with relapsed and refractory lymphoma creates an opportunity to investigate analogous canine cancers. There is limited information regarding the roles and expression of these checkpoint molecules in canine lymphoma. Studies evaluating expression of PD-1 and PD-L1 in dogs are conflicting both in detection modalities and results. The usefulness of

qRT-PCR for determining PD-1/PD-L1/PD-L2 expression in identifying canine patients whose lymphomas may respond to therapies targeting the PD-1/PD-L1 pathway is unknown.

The proposed study investigated the patterns of expression of mRNAs encoding PD-1 and its ligands PD-L1 and PD-L2 in lymphoma and in reactive lymphoid hyperplasia controls. We hypothesized that PD-L1 and PD-L2 are significantly upregulated in lymphoma, suppressing the immune response and allowing unchecked neoplastic lymphocytes to evade detection by the immune system. In this study, we sought to test the hypothesis by measuring expression of PD-1, PD-L1, and PD-L2 using qRT-PCR analysis on fine-needle aspirates from dogs with untreated lymphoma and dogs with reactive lymphoid hyperplasia.

II. Materials and Methods

A. Study population

Fine-needle aspirates (FNAs) were obtained from the lymph nodes of twenty adult client-owned dogs presenting to the Virginia Tech Veterinary Teaching Hospital for evaluation of peripheral lymphadenopathy. Ten patients diagnosed with untreated lymphoma and ten patients diagnosed with reactive lymphoid hyperplasia were included/enrolled in this prospective study. Exclusion criteria included dogs with cytologic evidence of metastatic disease in the examined lymph node aspirate, or dogs that received medication known to potentially alter checkpoint molecule expression (glucocorticoids, chemotherapeutics).

FNA samples were collected by veterinary hospital faculty and/or veterinary students, interns, and residents, and were routinely processed and stained with Wright-Giemsa. All cytologic diagnoses were made by board-certified veterinary pathologists and pathology residents. FNA samples were collected primarily between April 2018 and February 2019 with a single sample collected in July 2017. This study was approved by the Virginia Tech Institutional Animal Care and Use Committee and by the Veterinary Teaching Hospital Board.

B. RNA extraction and cDNA synthesis

Lymph node aspirate samples were thawed in TRIzol LS ™ from -80° Celsius and total RNA was extracted from lymph node aspirates using TRIzol™ LS reagent (Thermo Fischer Scientific) according to the manufacturer instructions. The extracted RNA was resuspended/eluted in 25 μL of nuclease-free water and stored at -80° Celsius until reverse transcription was performed. RNA extraction was repeated if the RNA concentration was less than 20 ng/uL (2 lymphoma aspirates, 3 reactive lymph node aspirates). The RNA concentration was measured both immediately following RNA extraction and prior to reverse transcription. A NanoDrop 2000 (Thermo Scientific) was used to measure the RNA concentration both immediately following RNA extraction and prior to cDNA synthesis. Absorbance ratios of $A_{260/280}$ were used to determine RNA purity. cDNA was synthesized according to manufacturer instructions from 220 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit

(Thermo Fischer Scientific) using MultiScribe™ Reverse Transcriptase master mixes containing random primers. Each cDNA reaction was performed in a 20 µL total volume, containing 10µL of 2X master mix (2.0 µL 10X RT Buffer, 0.8 µL 25X dNTP, 2.0 µL 10X RT Random Primers, 1.0 µL Reverse Transcriptase, and 4.2 µL nuclease-free water) and 10µL of RNA (RNA was diluted to obtain the same amount of RNA per cDNA reactions). A parallel set of non-reverse transcriptase reactions was not performed at this step in an attempt to conserve RNA for use across all experimental procedures, including RNA concentration measurements x 2, reverse transcription reactions, RNA-only triplicate qPCR reactions, and loss during reagent transfers. cDNA was stored at -20° Celsius until qRT-PCR was performed.

C. Quantification of PD-1, PD-L1, and PD-L2

Real time quantitative RT-PCR was performed to quantify mRNA expression for 18S, PD-1, PD-L1, and PD-L2. qRT-PCR was performed using primer sets and probes for each target gene. Forward and reverse primers and probes (Appendix table 1) were previously generated and validated for PD-L1 (BH-796, BH-797, BHTP-42) and PD-L2 (BH-798, BH-799, BHTP-43); primer/probe stock was prepared prior to PCR amplification by combining the primers and probe at a final concentration of 18µM per primer and 5µM for the probe. TaqMan® Gene Expression Assays were used for 18S and PD-1 (Thermo Fisher Scientific, Appendix table 1). The TaqMan® probes contain a FAM reporter dye linked to the 5' end of the probe, a MGB (minor groove binder) at the 3' end of the probe,

and a NFQ (nonfluorescent quencher) also at the 3' end of the probe. Each real time qRT-PCR reaction was performed in a 20μL total volume, containing 1.0 μL TaqMan Gene Expression Assay Mix or primer/probe stock (PD-L1 and PD-L2), 10.0 μL TaqMan® Fast Universal PCR Master Mix, No AmpErase UNG, 8.0 μL nuclease-free water, and 1.0 μL cDNA (or nuclease-free water for the no-template control, or diluted out RNA to 20 μL for the no-RT RNA-only control) using a 7500 Fast Real-Time PCR System (Applied Biosystems). The initial incubation for 20 seconds at 95° Celsius was followed by 40 cycles, each consisting of 3 seconds at 95° Celsius (denaturation) and 30 seconds at 60° Celsius (annealing/extension). All samples were run in triplicate. The $2^{-\Delta\Delta CT}$ method was used to assess relative gene expression of PD-1, PD-L1, and PD-L2 (Livak) and the results were normalized to the 18S housekeeping gene (Appendix table 3). The reactive lymphoid hyperplasia group was chosen as the untreated control group/calibrator.

D. Statistical analysis

All data analysis was performed using a commercial statistical software program (SAS Version 9.4, Cary, NC). Normal probability plots were used to assess normality. Normal probability plots showed that continuous data (relative expression levels, ΔCTs, and 2^-ΔCTs) were skewed, thus continuous data are summarized as medians (range). Statistical significance was set at $P < 0.05$.

Checkpoint molecule relative expression levels ($2^{-\Delta\Delta CT}$) were analyzed using nonparametric methods. Comparison of expression between diagnosis groups (lymphoma, reactive) was performed using the Wilcoxon rank sum test. Within the diagnosis group, phenotype comparisons between B cell lymphoma, T cell lymphoma, and reactive lymph node were determined using the Kruskal-Wallis test, followed by Dunn's procedure for two-way comparisons.

Relative abundance between checkpoint molecules (PD-L1 vs PD-1, PD-L2 vs PD-1, PD-L1 vs PD-L2) was determined using ΔCTs followed by transformation to $2^{-\Delta CT}$s (Appendix table 5) to stabilize the statistical model. The $2^{-\Delta CT}$s were then analyzed using nonparametric methods. Comparison of relative expression abundance between diagnosis groups (lymphoma, reactive) was performed using the Wilcoxon rank sum test. Within the diagnosis group, phenotype comparisons between B cell lymphoma, T cell lymphoma, and reactive lymph node were determined using the Kruskal-Wallis test, followed by Dunn's procedure for two-way comparisons.

Because scatter plots did not show an obvious linear relationship between checkpoint molecules, a regression analysis within an ANOVA (analysis of covariance) was not performed to evaluate the relationship between PD-1 and PD-L1, PD-1 and PD-L2, and PD-L1 and PD-L2.

III. Results

A. Patient characteristics

The inclusion criteria were expanded in June 2018 to include non-peripheral lymph nodes in an attempt to increase sample numbers. All dogs except one had peripheral lymphadenopathy; one dog had portal lymphadenopathy and was included in the study. Ages for dogs with lymphoma ranged from 4-12 years (2 intact males, 2 neutered males, 6 spayed females.) Ages for dogs with reactive lymphoid hyperplasia ranged from 2-14 years (2 intact males, 3 neutered males, 4 spayed females, 1 intact female). Representative images of reactive lymphoid hyperplasia and lymphoma are depicted in image 1 and image 2, respectively.

Immunophenotype was available from 7 out of 10 dogs with lymphoma using flow cytometry. B cell lymphoma was identified in three dogs and T cell lymphoma was identified in 4 dogs. Large cell lymphoma was identified in 7 dogs and small cell lymphoma was identified in 3 dogs; this was achieved with cytologic evaluation and/or flow cytometric analysis.

B. mRNA Quantification by real-time PCR analysis

PD-1, PD-L1, and PD-L2 expression was undetectable in one dog with lymphoma and one dog with reactive lymphoid hyperplasia. Undetectable levels of PD-L2 transcript was identified in one dog with lymphoma. PD-1 and PD-L1 transcript levels were undetectable in one dog with reactive lymphoid hyperplasia.

Transcript levels of PD-L1 and PD-L2 were not detected in one dog with reactive lymphoid hyperplasia (Appendix table 3).

Seven out of ten dogs with lymphoma had a known phenotype and these were categorized as either B-cell lymphoma (N=3) or T-cell lymphoma (N=4). Transcript levels of PD-1, PD-L1, and PD-L2 were identified in all three B cell lymphoma samples. Transcript levels of PD-1 and PD-L1 were identified in all of the dogs with T cell lymphoma. Detectable levels of PD-L2 transcript were identified in all but one dog with T cell lymphoma (Appendix table 3).

Comparison of PD-1 expression between dogs with lymphoma and dogs with reactive lymphoid hyperplasia showed significantly decreased PD-1 in dogs with lymphoma (P=0.038) relative to those with reactive lymphoid hyperplasia (table 1a, figure 1). There was significantly decreased PD-1 expression in B cell lymphoma relative to reactive lymphoid hyperplasia (P=0.0035, table 1b, figure 1).

Comparison of PD-L1 expression between dogs with lymphoma and dogs with reactive lymphoid hyperplasia showed no significant difference (P=0.4702) in expression; phenotype comparisons also showed no significant differences (P=0.5235, table 1a, figure 2).

PD-L2 was less expressed (P=0.071) in dogs with lymphoma compared to dogs with reactive lymphoid hyperplasia, though this was not statistically significant; there was no statistically significant difference (P=0.2266) between phenotype comparisons. (table 1a, figure 3).

The relative abundance of PD-1, PD-L1, and PD-L2 expression levels was compared within each sample between PD-L1 vs PD-1, PD-L2 vs PD-1, and PD-L1 vs PD-L2. The abundance of PD-L1 relative to PD-1 showed no significant difference (P=0.7552) between lymphoma and reactive lymphoid hyperplasia (table 2a, figure 4); however, the phenotype comparisons approached statistical significance (P=0.0532, table 2a). The abundance of PD-L1 relative PD-1 was in fact significantly different between B cell lymphoma and reactive lymphoid hyperplasia (P=0.0270) and between B cell and T cell lymphoma (0.0304, table 2b, figure 4).

The abundance of PD-L2 relative to PD-1 was not significantly different (P=0.4001) between lymphoma and reactive lymphoid hyperplasia (table 2a, figure 5). The abundance of PD-L2 relative to PD-1 was significantly different between B cell lymphoma and reactive lymphoid hyperplasia (P=0.01675) and between B cell lymphoma and T cell lymphoma (P=0.0360, table 2c, figure 5).

The relative abundance of PD-L1 to PD-L2 was not significantly different (P=0.7766) between lymphoma and reactive lymphoid hyperplasia (table 2a,

figure 6). Phenotype comparisons also showed no statistically significant differences (P=0.4352, table 2a, figure 6).

Although significant variations in relative abundance between the reactive lymph aspirates and lymphoma aspirates were not identified, the relative abundance of transcripts was in fact different between groups when expressed as a mean ± std dev. The relative abundance of PD-L1 vs PD-1 in the reactive lymph node aspirates was 1.5 ± 2.54 and was more than twice as high (3.36 ± 5.57) in the lymphoma aspirates. The relative abundance of PD-L2 vs PD-1 in the reactive lymph node aspirates was 0.20 ± 0.23 and was more than twice as high (0.60 ± 0.82) in the lymphoma aspirates.

Because scatter plots (figure 7, figure 8, figure 9) did not show an obvious linear relationship between checkpoint molecules, a regression analysis within an ANOVA (analysis of covariance) was not performed to evaluate the relationship between PD-1 and PD-L1, PD-1 and PD-L2, and PD-L1 and PD-L2 (figures 7-9).

IV. Discussion: see Chapter 5: Discussion, Conclusions, and Further Research.

V. Images, tables, and figures

Image 1: Representative fine-needle aspirate of reactive lymphoid hyperplasia. Wright-Giemsa stain. 50x objective.

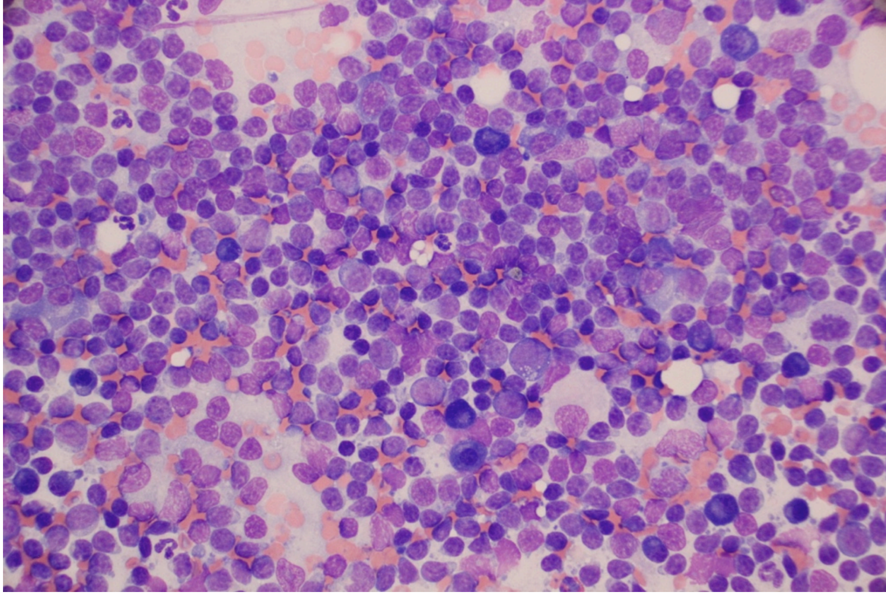


Image 2: Representative fine-needle aspirate of lymphoma. Wright-Giemsa stain. 50x objective.

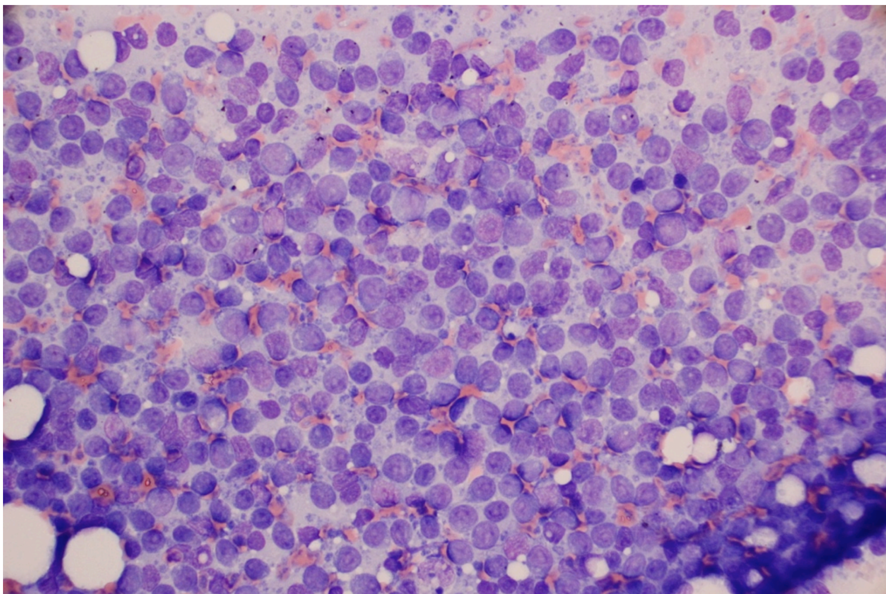


Table 1a: Summary of statistical results for FNA Expression

Outcome	Predictor	Category	n	Median (Range)	P Value
PDL1 expression	Diagnosis	Lymphoma	9	0.4 (0.1-1.5)	0.4702
		Reactive	7	0.4 (0.1-24.2)	
	Phenotype	B cell	3	0.6 (0.4-1.5)	0.5235
		Reactive	7	0.4 (0.1-24.2)	
		T cell	4	0.3 (0.1-0.9)	
PDL2 expression	Diagnosis	Lymphoma	8	0.1 (0.1-0.3)	0.071
		Reactive	8	0.3 (0.1-816.7)	
	Phenotype	B cell	3	0.1 (0.1-0.2)	0.2266
		Reactive	8	0.3 (0.1-816.7)	
		T cell	3	0.1 (0.1-0.3)	
PD1 expression	Diagnosis	Lymphoma	9	0.2 (0.0-7.7)	**0.038**
		Reactive	8	0.9 (0.5-2.9)	
	Phenotype	B cell	3	0.0 (0.0-0.0)	**0.012**
		Reactive	7	0.9 (0.5-2.9)	
		T cell	4	0.5 (0.1-0.6)	

Median expressed as $2^{-\Delta\Delta CT}$

Table 1b: PD1 expression by phenotype: P value for Dunn's procedure of two-way comparisons

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.00350**
2	B cell	T cell	0.15900
3	Reactive	T cell	0.13406

Table 2a: Summary of statistical results for FNA Expression of Relative Abundance

Outcome	Predictor	Category	n	Median (Range)	P Value
L1_PD_Exp	Diagnosis	Lymphoma	9	0.4 (0.0-15.7)	0.7552
		Reactive	7	0.3 (0.0-6.9)	
	Phenotype	B cell	3	9.4 (3.8-15.7)	**0.0532**
		Reactive	7	0.3 (0.0-6.9)	
		T cell	4	0.3 (0.1-0.5)	
L2_PD_Exp	Diagnosis	Lymphoma	8	0.1 (0.0-2.3)	0.4001
		Reactive	7	0.1 (0.0-0.6)	
	Phenotype	B cell	3	1.1 (0.9-2.3)	**0.04**
		Reactive	7	0.1 (0.0-0.6)	
		T cell	3	0.1 (0.0-0.1)	
L1_L2_Exp	Diagnosis	Lymphoma	8	3.5 (0.8-13.8)	0.7766
		Reactive	7	3.1 (0.7-10.7)	
	Phenotype	B cell	3	4.1 (4.1-13.8)	0.4352
		Reactive	7	3.1 (0.7-10.7)	
		T cell	3	2.9 (1.5-4.4)	

Median expressed as 2^-ΔCT

Table 2b: L1_PD expression by phenotype: P value for Dunn's procedure of two-way comparisons

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.02708**
2	B cell	T cell	**0.03040**
3	Reactive	T cell	0.83811

Table 2c: L2_PD expression by phenotype: P value for Dunn's procedure of two-way comparisons

Obs	Group 1	Group 2	P value
1	B cell	Reactive	**0.01675**
2	B cell	T cell	**0.03603**
3	Reactive	T cell	0.92940

Figure 1: qRT-PCR relative gene expression level of PD-1 in FNA samples. Lymphoma (n=9) vs reactive (n=8) (P=0.038). B cell lymphoma (n=3) vs reactive (n=8) (P=0.0035).

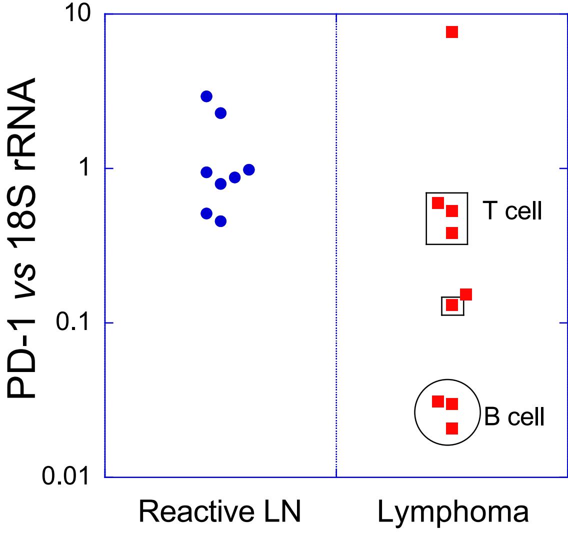


Figure 2: qRT-PCR relative gene expression level of PD-L1 in FNA samples. Lymphoma (n=9) vs reactive (n=7) (P=0.4702). B cell (n=3) vs T cell (n=4) vs reactive (n=7) (P=0.5235).

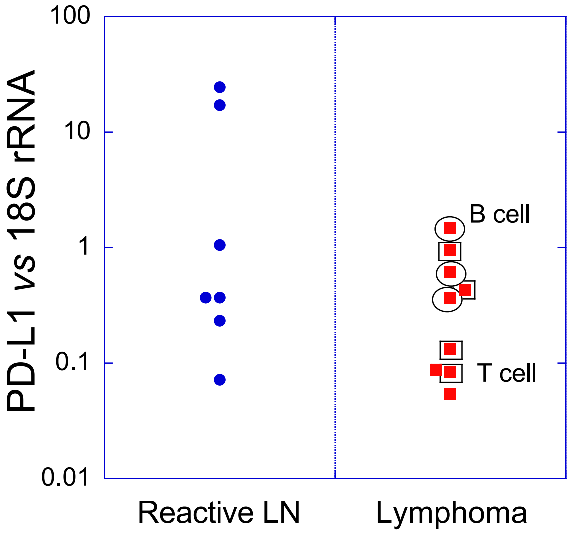


Figure 3: qRT-PCR relative gene expression level of PD-L2 in FNA samples. Lymphoma (n=8) vs reactive (n=8) (P=0.0710). B cell (n=3) vs T cell (n=3) vs reactive (n=8) (P=0.2266).

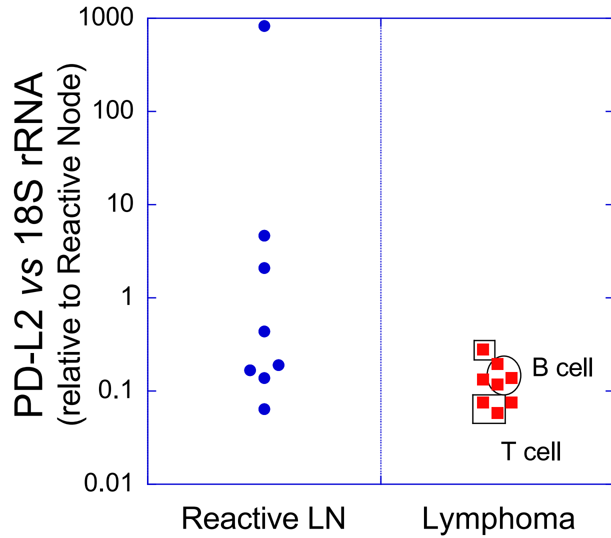


Figure 4: Abundance of PD-L1 relative to PD-1 in FNA samples. Lymphoma (n=9) vs reactive (n=7) (P=0.7552). B cell lymphoma(n=3) vs reactive (n=7) (P=0.0270). B cell (n=3) vs T cell lymphoma (n=4) (P=0.0304).

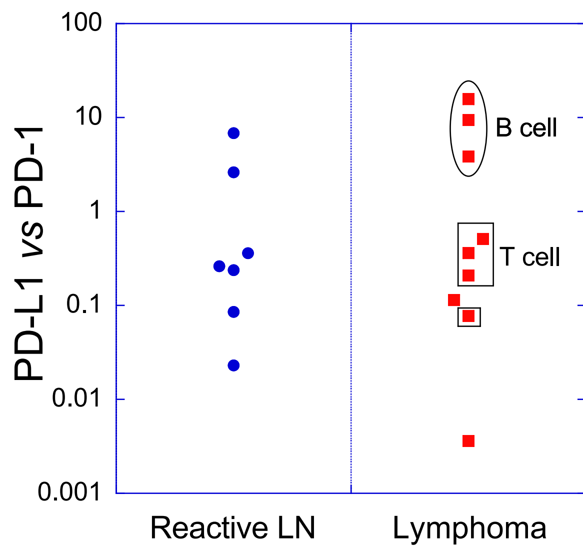


Figure 5: Abundance of PD-L2 relative to PD-1 in FNA samples. Lymphoma (n=8) vs reactive (n=7) (P=0.4001). B cell (n=3) vs reactive (n=7) (P=0.0167). B cell (n=3) vs T cell (n=3) lymphoma (P=0.0360).

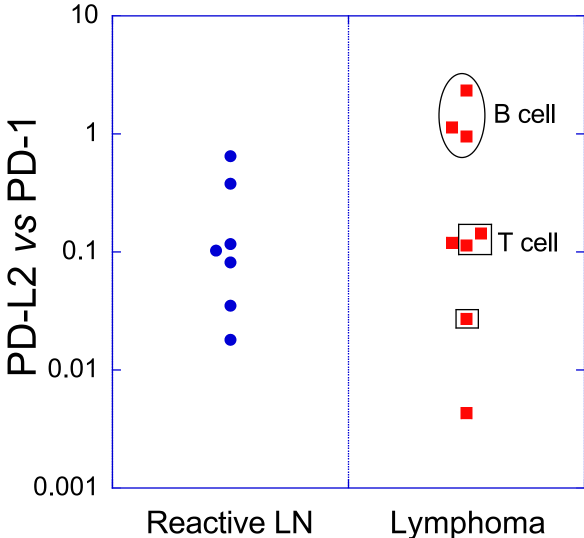


Figure 6: Abundance of PD-L1 relative to PD-L2 in FNA samples. Lymphoma (n=8) vs reactive (n=7) (P=0.7766). B cell (n=3) vs T cell (n=3) vs reactive (n=7) (P=0.4352).

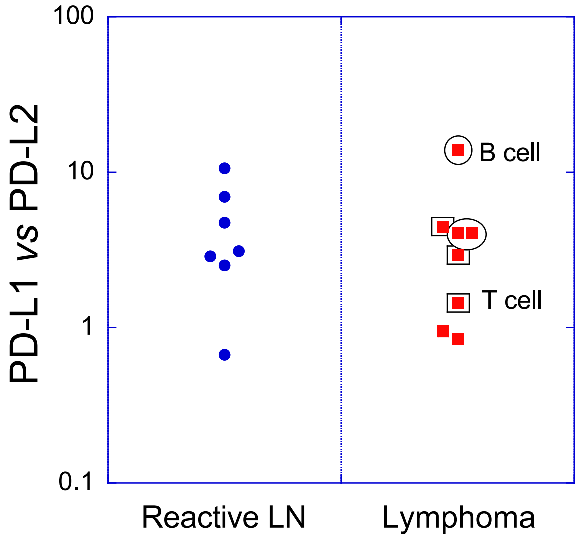


Figure 7: FNA samples. Scatterplot of simultaneous PD-L1 and PD-1 expression in lymphoma and in reactive lymphoid hyperplasia. Expression is normalized to 18S and is relative to reactive lymph node. Regression analysis was not performed due to lack of linear relationship.

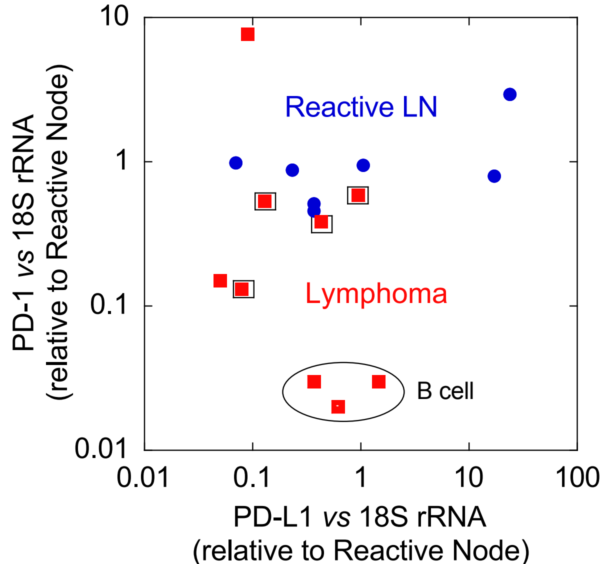


Figure 8: FNA samples. Scatterplot of simultaneous PD-L1 and PD-L2 expression in lymphoma and in reactive lymphoid hyperplasia. Expression is normalized to 18S and is relative to reactive lymph node. Regression analysis was not performed due to lack of linear relationship.

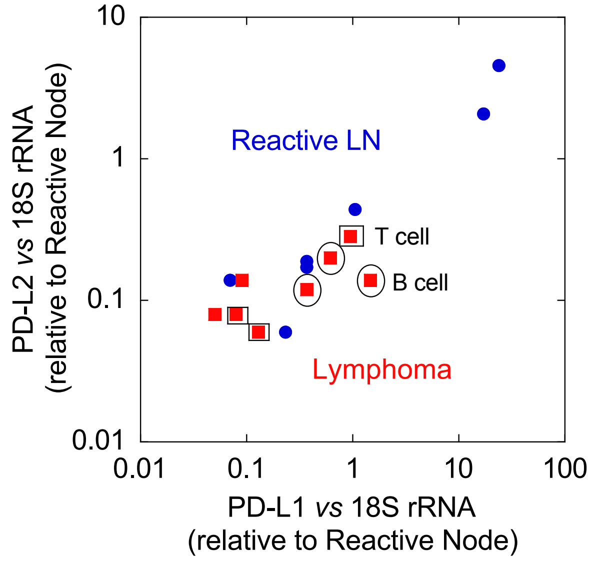
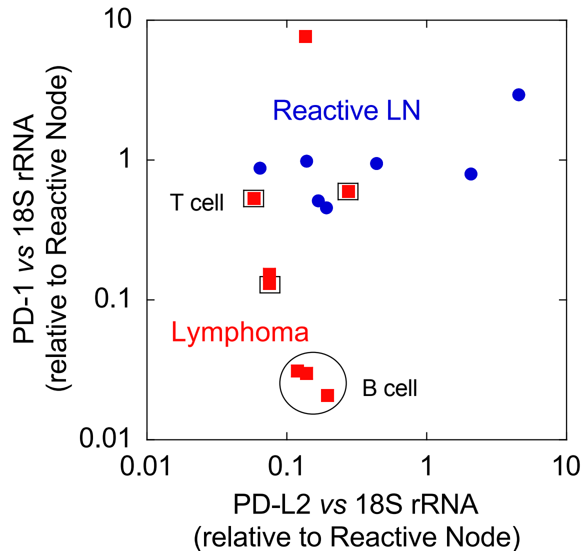


Figure 9: FNA samples. Scatterplot of simultaneous PD-L2 and PD-1 expression in lymphoma and in reactive lymphoid hyperplasia. Expression is normalized to 18S and is relative to reactive lymph node. Regression analysis was not performed due to lack of linear relationship.



Chapter 5: Discussion, Conclusions, and Further Research

Overexpression of PD-L1 and PD-L2 has been described in people with various types of lymphoma, and overexpression of PD-L1 is associated with unfavorable outcomes. Currently, there is limited and conflicting information regarding the roles and expression of these checkpoint molecules in canine lymphoma. Our hypothesis that PD-L1 and PD-L2 would be significantly increased in lymphoma relative to reactive lymphoid hyperplasia was not supported. Overall, the results of both the retrospective portion and the prospective portion of this study demonstrated that mRNA expression of PD-1, PD-L1, and PD-L2 trended lower in lymphoma compared to reactive lymphoid hyperplasia.

Reactive lymph nodes were chosen as the control group because reactive lymphadenopathy is a common clinical finding that can mimic lymphoma, and we wanted to gain a better understanding of checkpoint molecule expression in a reactive entity. Reactive lymph nodes serve as a good control model to establish comparability since PD-1, PD-L1, and PD-L2 expression can be upregulated during antigenic stimulation in a reactive lymph node. Inclusion of a control model whose baseline checkpoint molecule expression is already upregulated strengthens the comparison when assessing the effect of lymphoma on checkpoint molecule upregulation. To our knowledge, this study is unique in that it is the first of its kind evaluating checkpoint molecule mRNA expression in reactive lymph node hyperplasia; in addition, it is the first of its kind comparing checkpoint molecule mRNA expression between lymphoma and reactive lymphoid hyperplasia.

In both formalin-fixed paraffin-embedded (FFPE) tissue and fine-needle aspirates (FNAs), there was no significant difference in PD-L1 expression between dogs with lymphoma and dogs with reactive lymphoid hyperplasia. These results are similar to other published work. In a study by Tagawa et al., PD-L1 and PD-L2 mRNA expression was evaluated in lymph node aspirate cells from dogs with untreated high-grade B cell lymphoma and age-matched healthy controls; they found no significant difference in PD-L1 or PD-L2 mRNA expression between those groups. (Tagawa 2018). In a recent study by Ambrosius et al., the mean PD-L1 mRNA expression in formalin-fixed paraffin embedded tissues from dogs with diffuse large cell lymphoma was greater than the mean PD-L1 expression for healthy control lymph nodes and tonsils; however, interestingly, this was only true for 50% of the lymphoma specimens, suggesting that overexpression of PD-L1 was in fact not detected in the other 50% of lymphoma specimens.

(Ambrosius 2018). The latter findings are more similar to our study results for PD-L1 for both FFPE and FNA specimens in that PD-L1 overexpression was not demonstrated in lymphoma specimens.

Although it was not statistically significant, our study revealed a decreased median PD-L1 expression in canine lymphoma compared to reactive lymphoid hyperplasia in FFPE tissue. These results differ from those of both Ambrosius et al. (previously discussed) and Hartley et al., who demonstrated upregulated PD-L1 expression in canine lymphoma. (Ambrosius 2018, Hartley 2018). In the Hartley study, PD-L1 expression was analyzed using flow cytometry from lymph node aspirate cells from forty dogs with untreated lymphoma (36 B cell, 6 T cell) and compared with that of healthy controls; they found significantly increased PD-L1 expression in the malignant B lymphocytes relative to the healthy control B lymphocytes, though the mechanism for the PD-L1 upregulation was not investigated. (Hartley 2018). In people with diffuse large B cell lymphoma, high PD-L1 expression as detected by IHC, correlated with a poor prognosis, as determined in a meta-analysis. (Zhao 2018). Although our study did not investigate clinical outcomes, the prognostic value of PD-L1 IHC expression has been assessed in dogs with diffuse large B cell lymphoma and results are conflicting. (Aresu 2018, Ambrosius 2018). In contrast to the Hartley study, our study did not identify significant differences in PD-L1 expression in dogs with B cell lymphoma (when compared to dogs with T cell lymphoma or dogs with reactive lymphoid hyperplasia). Similarly, the Tagawa study did not identify significant differences in PD-L1 mRNA expression in dogs with B cell lymphoma compared to healthy controls.

It was observed by Makaewa et al. that none of the five canine diffuse large cell lymphoma samples exhibited PD-L1 IHC positivity. (Makaewa 2016). Yet another study observed positive PD-L1 IHC expression in all fifteen canine lymphoma samples, including high grade and low-grade lymphomas; however, using flow cytometry, this same study only detected PD-L1 expression on only two of nine canine lymphoma cell lines. (Shosu 2016). The Kumar study is in keeping with the Shosu study in that PD-L1 expression was observed in canine lymphoma (N=3) as detected by IHC; additionally, canine B cell lymphoma cell lines demonstrated positive PD-L1 mRNA expression. (Kumar 2017). In our FFPE study, positive PD-L1 expression was detected in 100% of lymphoma and 90% of reactive lymphoid hyperplasia, as well as all of the B cell lymphoma and T cell lymphoma cases. Likewise, in our FNA study, positive PD-L1 expression was detected in 70% of lymphoma and 90% of reactive lymphoid hyperplasia, as well as all of the B cell lymphoma and T cell lymphoma cases. Collectively, these findings may reflect an inherent variability of PD-L1, not only within lymphoma itself, but across different tissue specimens and detection modalities, though the reason for the conflicting expression heterogeneity is not immediately apparent.

Positive PD-L2 IHC expression in people is primarily detected in mediastinal B cell lymphoma, which is an aggressive large cell lymphoma, as well in Hodgkin's lymphoma. (Shi 2014, Panjwana 2018, Kiyasu 2015). In a study by Shi et al., only 3% of diffuse large B cell lymphomas in people were PD-L2 IHC positive. (Shi 2014). To the authors' knowledge, PD-L2 expression in canine lymphoma has only been evaluated in the Tagawa study and in our study. (Tagawa 2018). Our study is the first to show that PD-L2 is significantly decreased in canine lymphoma. In our study, PD-L2 was decreased in lymphoma relative to reactive lymphoid

hyperplasia for both the FFPE tissues and the FNA samples, although only statistically significant for FFPE tissues. In the study by Tagawa et al., both PD-L1 and PD-L2 mRNA expression was evaluated in lymph node aspirate cells from dogs with untreated high-grade B cell lymphoma (n=17) and age-matched healthy controls (n=9); they found no significant difference in PD-L1 or PD-L2 mRNA expression between those groups. (Tagawa 2018). Despite not observing significantly decreased PD-L2 in aspirates from dogs with B cell lymphoma, the Tagawa study did observe a lower median PD-L2 in B cell lymphoma compared to healthy controls; the significance of this relationship in the Tagawa study was unknown. Similarly, our study showed that expression of PD-L2 was lower in B cell lymphoma relative to reactive lymphoid hyperplasia controls, though this difference was only significant in FFPE tissue. The significance of this is unclear. Expression of PD-L2 could be higher in a reactive entity due to antigenic stimulation; in addition, a normal lymph node, though considered a control, is inherently reactive to some degree given its role in antigen presentation and processing.

Based on our results, PD-L1 and PD-L2 expression could be lower in lymphoma compared to a reactive lymph node for several reasons. Since these molecules are expressed on antigen-presenting cells, and PD-L1 and PD-L2 can be further upregulated by cytokines such as IFN-γ, it is likely and not unexpected that our reactive lymph node samples had higher numbers of cytokine (e.g., IFN-γ)-stimulated antigen presenting cells, and therefore higher expression of PD-L1 and PD-L2, despite the lack of statistical significance. In follicular hyperplasia (i.e., a reactive follicle), the increase in the number of secondary follicles is associated with an increase in the number of reactive germinal centers; and more reactive germinal centers indicates increased

antigenically stimulated lymphocytes, macrophages, and dendritic cells. Additionally, the lack of a substantial T-cell stimulated immune response in lymphoma's tumor microenvironment is also a possibility. A study by Hartley et al. using flow cytometry demonstrated significant upregulation of canine PD-L1 in lymphoma cell lines following treatment with IFN-γ, including PD-L1 upregulation on tumor-infiltrating macrophages. (Hartley 2016). The lack of cytokine-driven ligand upregulation on either neoplastic lymphocytes or in the tumor microenvironment is also a consideration.

Both retrospective and prospective analysis demonstrated lower overall PD-1 expression in dogs with lymphoma relative to those with reactive lymphoid hyperplasia; this difference was statistically significant for both FFPE tissue and FNA samples. Expression of PD-1 was also significantly lower in dogs with B cell lymphoma compared to dogs with reactive lymphoid hyperplasia in both the FFPE tissues and the FNA samples. A flow cytometric analysis demonstrated significantly higher PD-1 expression in aspirated lymph node T cells from dogs with B cell lymphoma when compared to PD-1 expression in aspirated lymph node T cells in the control group. (Tagawa 2018). Flow cytometric analysis by Hartley et al. identified no significant difference in PD-1 expression by malignant B cells from dogs with B cell lymphoma compared to normal B cells from healthy control dog lymph node aspirates. (Hartley 2018). Interestingly, the study by Hartley showed higher PD-1 expression in B cell lymphoma compared to healthy control dogs when they evaluated PD-1 expression in tumor infiltrating T cells in B cell lymphoma compared to T cells in normal lymph node. (Hartley 2018).

The results from both of those studies are suggestive of suppression of antitumor immunity in B cell lymphoma, tumor induced T cell exhaustion in B cell lymphoma, or increased T cell activation, all of which would be in contrast to what our study found. The majority of tumor infiltrating lymphocytes in people express increased PD-1 levels and are associated with a dysfunctional impaired antitumor response. (Sui 2015). The prognostic significance of PD-1 expression in people with lymphoma is variable, and in the one study in dogs with lymphoma, higher PD-1 IHC expression was associated with a poor outcome. (Aresu 2018). Certainly, the Tagawa and Hartley PD-1 expression results are more in agreement with each other and both studies used flow cytometry and similar patient population comparisons (i.e., healthy lymph node controls). The significance of our finding is unclear given that our lymphoma population was of mixed B/T cell origin, our control was a reactive lymph node, not all phenotype identities were known in our FNA population, and the sample size for our lymphoma group was smaller than those studies.

Nonetheless, it is not unexpected that PD-1 expression in our study was lower in lymphoma relative to a reactive lymph node because expression of PD-1 is induced upon T cell activation, a frequent occurrence in an antigenically stimulated reactive lymph node; these findings would suggest limited T cell activation in lymphoma, particularly in B cell lymphoma. Our findings would also suggest a limited role of PD-1 mediated suppression of antitumor immunity in canine lymphoma, particularly in canine B cell lymphoma. These results from our study may also suggest that exhausted T cells, which are those experiencing high levels of stimulation and increased PD-1 expression with diminished T cell function (Wherry 2011), are not part of the canine lymphoma immune evasion strategy. It is possible that the lower PD-1 expression in

lymphoma may be due to downregulation of the PD-1 receptor by the neoplastic lymphocyte. By either not expressing PD-1, or having less PD-1 expression, the neoplastic lymphocyte would have less ability to bind PD-L1/PD-L2, and would therefore evade immune detection. Perhaps PD-1 expression is downregulated during the process of lymphomagenesis through oncogenic signaling alterations. For example, during the transformation into a neoplastic lymphocyte, there may be repression of a transcription factor which then reduces PD-1 expression. Perhaps during neoplastic transformation, the dysregulated lymphocyte loses its antigen recognition ability and without an ability to become activated, it therefore loses the ability to express the PD-1 receptor. The molecular mechanisms for regulation of PD-1 expression in canine lymphoma warrant further investigation.

The higher relative abundance of PD-L1 to PD-1 in lymphoma compared to reactive lymphoid hyperplasia (only significant in FFPE samples), and in B cell lymphoma compared to reactive lymphoid hyperplasia, reflects decreased PD-1 expression in lymphoma. Likewise, the higher relative abundance of PD-L2 to PD-1 in lymphoma compared to reactive lymphoid hyperplasia (only significant in FFPE samples), and in B cell lymphoma compared to reactive lymphoid hyperplasia, reflects decreased PD-1 expression in lymphoma. It is unclear why the FNA samples showed a higher relative abundance of ligand to receptor when B cell lymphoma was compared to T cell lymphoma; although it may reflect the higher median PD-L1 in B cell lymphoma samples (though not statistically significant), the median PD-L2 was the same in B cell and T cell lymphoma and median PD-1 was higher in T cell samples than B cell samples. The higher relative abundance of PD-L1 to PD-L2 in FFPE tissue for B cell lymphoma

compared to T cell lymphoma may reflect the lower median PD-L2 in B cell lymphoma (not statistically significant).

In both lymphoma and reactive lymphoid hyperplasia, the linear relationship between ligand receptor pairs and ligand pairs suggests concurrent increasing T cell activation and ligand upregulation. The regression analysis for PD-L2 vs PD-L1 identified a significantly different slope between lymphoma and reactive lymphoid hyperplasia, indicating a significant difference in the PD-L2 change increment per unit change of PD-L1. In lymphoma, there is a greater difference in the PD-L2 ΔCT change increment per unit change of PD-L1 ΔCT than in reactive lymphoid hyperplasia. In lymphoma, this corresponds to a smaller PD-L2 incremental increase in expression per unit increase of PD-L1 expression. In other words, in reactive lymphoid hyperplasia, there is a larger incremental increase in PD-L2 expression than in lymphoma per unit increase in PD-L1 expression. This might indicate that in an antigenically stimulated reactive lymph node, assuming higher PD-1 induction from T cell activation, the need is greater for PD-L2 to participate in delivering a balance of inhibitory signals to control excessive cytokine production and limit tissue damage.

mRNA expression of PD-1, PD-L1 and PD-L2 was consistently detected in all of the formalin-fixed paraffin embedded lymphoma samples, and all but one of the formalin-fixed paraffin embedded reactive lymph nodes which had undetectable PD-L1. However, it was less consistently detected in the lymphoma aspirates and the reactive lymph node aspirates. It is likely that the absence of PD-1 in three of the fine-needle aspirate samples was due to inadequate cellularity from the collection method as supported by the concurrent absence of detectable PD-

L1 and PD-L2 in two of the samples (one each of lymphoma and reactive lymphoid hyperplasia) and the concurrent absence of detectable PD-L1 in one sample (reactive lymphoid hyperplasia). The concurrent lack of detectable PD-L1 and PD-L2 in one of the reactive lymphoid hyperplasia samples may also suggest inadequate cellularity of the sample. The total mean RNA concentration (89.12 ng/μL) and the total mean RNA quality (1.64) in the fine-needle aspirate samples was lower than the total mean RNA concentration (303.98 ng μL) and total mean RNA quality (1.95) in the formalin-fixed paraffin embedded tissue. This study showed that it is feasible to use fine needle-aspirate samples to extract RNA for qRT-PCR analysis of checkpoint molecules. Despite their limited amounts of tissue, they provided a generally similar mRNA expression analysis to the formalin fixed paraffin embedded tissue.

The nature of a cytologic specimen is different than the nature of a formalin-fixed paraffin embedded tissue sample, which may explain the inconsistencies between results. Cytologic specimens can inherently result in cellular variability between aspirates, whether because of lesion distribution, aspiration technique, aspiration needle size used, or blood contamination of the sample, all of which could affect gene expression measurement. A biopsy specimen retains tissue architecture and is a different representation of the abundance and types of cells that are present, whether be it malignant lymphocytes or tumor-infiltrating nonmalignant cells.

This study had several limitations. The small sample size for each analysis may limit the power to detect differences between groups, particularly in detecting differences between B and T cell lymphoma, and particularly since not all B/T cell identifies were known in the fine-needle aspirate samples. Likewise, the inconsistent expression of PD-1, PD-L1, and PD-L2 in the fine-

needle aspirate samples may also limit the power to detect differences between groups. Another drawback was that we did not select for a specific lymphoma subtype or a specific immunophenotype. A unified lymphoma subgroup analysis in a larger sample size may reduce a source of heterogeneity and produce different results. For example, there may be differences in checkpoint molecule gene expression between subgroups, which may in part account for contradictory results in the current veterinary literature. And lastly, while we did detect expression of PD-1, PD-L1, and PD-L2, the localization of PD-1, PD-L1, and PD-L2 could not be determined in our study.

Continued investigation into which cell types express each checkpoint molecule in lymphoma and its microenvironment are warranted in order to increase our understanding of the role of this complex pathway and its role in the biological behavior of lymphoma. Additional studies are warranted to confirm the extent to which checkpoint molecules are expressed in canine lymphoma. Furthermore, ongoing studies should focus on establishing consistent, reliable, validated methods to detect checkpoint molecule expression in dogs with lymphoma because results are variable depending on the detection technique and tissue specimen. This would allow for more consistent identification of patients whose lymphoma may or may not respond to targeted checkpoint therapy. Although checkpoint molecule expression was not upregulated in lymphoma in the present study, this does not necessarily imply that PD-1 or PD-L1 immunotherapy would not benefit dogs with lymphoma. In fact, in human medicine, both PD-L1 positive and PD-L1 negative cancer patients treated with PD-1 or PD-L1 inhibitors have shown significantly prolonged overall survival, so it is not yet fully known which patients may benefit. (Shen 2018). Future studies are also warranted in which checkpoint molecule expression is

correlated with clinical outcomes. In light of the role of CD28 co-stimulation in rescuing exhausted CD8+ T cells by PD-1 targeted therapy, future studies could focus on investigating the relationship between the expression of CD28 positive PD-1 positive CD8+ T cells and the response to PD-1 and PD-L1 inhibitors.

This study contributes additional information on PD-1/PD-L1/PD-L2 mRNA expression in canine lymphoma. It provides further evidence that PD-1, PD-L1, and PD-L2 are expressed in canine lymphoma. It also provides information on checkpoint molecule expression in a non-neoplastic reactive entity. Overall, checkpoint molecule expression was not upregulated in lymphoma relative to reactive lymphoid hyperplasia, suggesting a limited application of PD-1 and PD-L1 blockade in canine lymphoma. The ligand: receptor ratio imbalances reflect the lower PD-1 expression in lymphoma. Although these results do not suggest that checkpoint inhibitors would be useful for treatment, they give insight into the mechanisms of unchecked lymphocyte proliferation in canine lymphoma. These results support the possibility that the PD-1/PD-L1/PD-L2 pathway may not be a major mechanism of immune evasion by canine lymphoma.

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