Molecular basis of immunotolerance in canine neoplasia

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ABSTRACT

Melanoma is a highly malignant neoplasia with high rates of metastasis in humans and dogs. Regardless of being considered a highly immunogenic neoplasm, the function of the immune system is hampered by the expression of immune checkpoint molecules by the cancer cells. In contrast, soft tissue sarcomas are poorly immunogenic, as Tumor infiltrating Lymphocytes are lacking, or when present they are usually at the periphery of the tumor. Still, soft tissue sarcomas are considered immunosuppressed. Checkpoint molecules from the PD-axis are overexpressed in numerous human malignant neoplasia and have recently gained attention with a few reports in canine tumors. Immunotherapies against these checkpoint molecules have shown great efficacy in humans, but in order to determine translational approaches into canine patients, more research is needed. Here we determined the gene expression of Programed Death receptor-1, and its ligands PD-L1 and PD-L2 in canine tumors with two distinct immune profiles. Our results show that regardless of their immune profiles, melanoma versus soft tissue sarcoma, checkpoint molecules expression was higher in malignant tumors with a higher grade. Additionally, we evaluated the expression of these molecules in a set of patients that received histotripsy, which is a non-invasive and non-thermal ultrasound focused therapy that

induces mechanical stress to the cells, leading to liquefactive necrosis. Here we reported a focal decrease of the expression of these checkpoint molecules in tissue sections obtain at the treatment interface, compared to those taken from untreated areas of the tumor. In addition, a positive relationship was noticed between the infiltration of CD3+ T lymphocytes and the expression of these checkpoint molecules in both canine melanoma, and soft tissue sarcoma. Our findings demonstrate that immunotherapies targeting these checkpoint molecules have a great potential for efficacy in canine neoplasia, along or combined with tumor ablation therapies that increased immune cell infiltration in poorly immunogenic neoplasia.

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GENERAL AUDIENCE ABSTRACT

Melanoma is a highly malignant tumor and very resistant to therapy for humans and dogs. At the same time, this neoplasia is usually highly infiltrated by cells from the immune system. However, this immune infiltration is often inhibited by molecules expressed by the melanoma cells. In contrast, soft tissue sarcoma is considered poorly immunogenic, as they often contain low levels of immune cell infiltrates but are still considered immune suppressed. In this study, we determined the expression of molecules that inhibit the effect of T lymphocytes, specifically Programed cell death receptor-1, PD-Ligand 1, and PD-Ligand 2 for these neoplasms with distinct immune profiles. We encounter that despite their immune profiles, the expression of these molecules is higher in malignant tumors. Additionally, we evaluated the expression of these molecules in a set of patients that received histotripsy, which is a non-invasive and non-thermal focused ultrasound therapy that induces mechanical stress to the cancerigenous cells, leading to its death (necrosis). Here we reported a focal decrease of the expression of these checkpoint molecules in tissue sections obtain at the treatment interface, compared to those taken from untreated areas of the tumor. In addition, a positive relationship was noticed between the infiltration of T lymphocytes and the expression of these checkpoint molecules in both canine melanoma, and soft tissue sarcoma. Our findings demonstrate

that immunotherapies targeting these checkpoint molecules have a great potential for efficacy in canine neoplasms, along or combined with tumor ablation therapies that increased immune cell infiltration in poorly immunogenic neoplasia.

DEDICATION

This dissertation is dedicated to my family; my daughter Emma, for her endurance during these past 4 years of intense work that took away many hours from our time together, and who supported me unconditionally in a way that goes beyond expectation for a child of her short age; to my husband Kristobal, who embraced me through all difficult times and always guided me, and his infinite support as a father, a husband, a colleague, and more importantly as my best friend; to my parents and sisters for always being there despite the long distance. Lastly, to my advisors Tanya LeRoith and William Huckle, for always believing that I could become a successful professional in both my Anatomic Pathology Residency, and my Ph. D in Biomedical Sciences and Pathobiology.

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CHAPTER 1. INTRODUCTION

The era of immunotherapies. For several years, conventional therapies such as radiation and chemotherapies were the standard for the treatment of malignant neoplasms for humans and dogs, still, survival remained low especially for highly malignant neoplasia such as melanoma,(1-3) until the last decade when the introduction of immunotherapies showed great promise as a more effective treatment.(2, 4-6) Great efforts have been made for a better understanding on how the immune system interacts with the cancerous cells and the Tumor Microenvironment (TME). This led to the development of monoclonal antibodies that block certain immune checkpoint molecules highly expressed by cancer cells with the ability to inhibit the effect of the host immune system.(7) Among the most successful immunotherapies in humans are those that inhibit the interaction between Programed Death-1 (PD-1), and its ligands PD-L1 and PD-L2.(8-10) The binding of the receptor PD-1 on the surface of T lymphocytes with its ligands PD-L1 or PD-L2, induces inhibition of the T cell effect, leading to immunotolerance, promoting tumor progression, and increased rates of metastasis for some patients.(9, 11-16)

Tumor infiltrating lymphocytes characterization. The presence of TILs is associated with better outcomes, and correlation with a favorable prognosis has been demonstrated in several human studies. (17-29) Given this correlation, a system has been created for humans and dogs in order to characterize the TILs infiltration in the TME, where the distribution of TILs can be "absent" as no lymphocytes directly opposed to the tumor cells; "non-brisk" as isolated, focal and/or segmental infiltration in the tumor; and "brisk" as TILs segmentally infiltrating either at the entire base of the tumor or diffusely infiltrating within the tumor.(21, 30) In addition to the distribution of the TILs within the TME, numerous investigators have emphasized the importance of the characterization of this TILs population, reporting that tumors with high

numbers of CD8 T cells that express cytolytic enzymes and CD4 T cells lacking inhibitory checkpoint molecules are usually associated to good prognosis when compared to TILs overexpressing PD-1.(24) Density of CD3+ TILs has been associated with favorable outcomes in dogs with histiocytic sarcoma, suggesting that not only the distribution of TILs is important for the prognosis.(30) In addition to distribution and density, the nature of these TILs plays an important role in the course of the disease, as higher levels of Foxp3+ T cells are reported in high-grade gliomas, and melanoma, linked with an increased risk of death in dogs.(31-34)

*Hot versus Cold tumors***.** Highly malignant neoplasia can be infiltrated by a large number of Tumor Infiltrating Lymphocytes (TILs) which is called a "hot" tumor; or in contrast, they can be considered "cold" when devoid of such TILs, or when these TILs are restrained at the periphery of the neoplasm. Despite the presence of TILs, malignant neoplasms including melanoma can over express checkpoint molecules from the PD-axis inhibiting the T cell effect. (27-29)

Immunotherapies targeting the PD-axis. Immunotherapies directed against proteins constituting the PD-axis are effective in numerous human neoplasms, but their effect is influenced by distinct immune profiles from the specific type of neoplasia, with higher success rates for those considered inflamed or "hot" tumor, than "cold" tumors that usually lack T cell infiltration.(2, 15, 35, 36)

The expression of checkpoint molecules from the PD-axis has been reported in numerous human, and recently canine neoplasia by various researchers.(37-40) The alternative PD-1 ligand PD-L2, although less studied than PD-L1, has been observed in several human tumors even in the absence of PD-L1 (12) or associated with increased infiltration of tumor cells, and metastasis.(41, 42) Reports in human research show that the expression of PD-L2 has a strong

correlation with the expression of PD-1 and PD-L1 (40, 41) Similar findings for PD-L2 expression have been reported by a single study in canine melanoma. (43) The expression of checkpoint molecules from the PD-axis in canine neoplasia with association to poor prognosis similar to that in humans has set the foundation for the development of such immunotherapies for canine patients.

In humans, the FDA has already approved the use of anti PD-1 as Nivolumab and Pembrolizumab, and recently an anti PD-L1 therapy has been approved for the treatment of urothelial carcinoma and non-small cell lung cancer, reporting favorable outcomes.(44-49) Only recently has this therapeutic approach been extended to dogs, with a chimeric anti PD-1 antibody producing a reduction in canine melanoma tumor burden, increased survival time, and complete regression in 2 cases.(50, 51) Another pilot clinical study showed that anti PD-L1 induced an objective antitumor response in canine oral melanoma,(52) and the use of Atezolizumab and Avelumab, both human anti-PD-L1, have shown cross-reactivity in canine tissue.(53)

Canine melanoma, a malignant and highly immunogenic neoplasm. Melanoma is a highly malignant neoplasia arising from melanocytes, very resistant to conventional therapies, with a high rate of metastasis in humans and dogs.(2, 4, 54, 55) Canine melanoma can arise at different locations, with median survival time of 22 months after diagnosis for cutaneous melanoma, 5 months for oral melanoma, and <3 months for Stage 3 (metastatic) disease.(56) Regardless of being considered a highly immunogenic tumor, TILs are frequently exhausted and suppressed by the expression of checkpoint molecules from the PD-axis, failing to limit tumor growth, leading to the escape of the cancerigenous cells from the TME.(7, 40, 57-59) As mentioned above, expression of PD-1 and PD-L1 has been described previously in canine melanoma by a research group, but there are no reports in canine neoplasia on the expression of

PD-L2, the PD-1 alternative ligand and for which the affinity for its receptor is greater than PD-L1.(60)

Canine soft tissue sarcoma, as a contrasting poorly immunogenic neoplasm. Soft tissue sarcomas are a diverse group of neoplasms arising from mesenchymal cells, with difficult resection, and a high rate of recurrence, where metastasis can reach up to 30% of the cases.(61, 62) Since this neoplasm has always been considered poorly immunogenic, immunotherapies were thought likely to have little impact on tumor progression,(63-65). Recent studies have reported that TILs infiltration depends on tumor subtype, where those with high levels of Copy Number Alterations have higher immunogenicity, but are still considered immunosuppressed. (64, 66, 67)

Regardless of the immune profile of the neoplasm, new therapies under investigation aim to convert "cold" tumors into a "hot" ones by the release of Tumor Associated Antigens, increasing their recognition by the immune system not only at the primary site, but also systemically, hence achieving an abscopal response.(68-71) Such promising tumor ablation therapies include histotripsy, a noninvasive, non-thermal focused ultrasound treatment that preserves tumor antigens able to be recognized by the host immune system.(70-75) PD-L1 expression has been reported in human soft tissue sarcoma, but little is known about the expression of the PD-axis in canine soft tissue sarcomas, nor how current therapies can affect their expression.(76-80)

As human companions, dogs are exposed to the same environmental hazards that contribute to the development of cancer, and the occurrence, mechanisms of disease, and response to treatment are often parallel to those in humans.(81) However, a deeper understanding of the role of checkpoint molecules in the inhibition of the immune system is needed before the

extension of human immunotherapies to canine patients can be fully realized. Accordingly, we aim to determine the expression of checkpoint molecules from the PD-axis in two canine neoplasms with distinct immune profiles. In addition, we seek to evaluate the relationship between their expression and the presence of TILs, malignancy, tumor grading, and the association between the three different checkpoint molecules, PD-1, PD-L1, and PD-L2. We expect that the expression of these checkpoint molecules will be higher in malignant neoplasm, with a higher grade. We also anticipate a positive relationship between these checkpoint molecules and the infiltration of CD3+ T lymphocytes.

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CHAPTER II

CANINE MELANOMA: A REVIEW OF DIAGNOSTICS, COMPARATIVE MECHANISMS OF DISEASE, AND IMMUNOTOLERANCE IN THE ERA OF THE IMMUNOTHERAPIES

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Abstract

Melanomas in humans and dogs are highly malignant and resistant to therapy. Since the first development of immunotherapies, interest in how the immune system interacts within the tumor microenvironment and plays a role in tumor development, progression, or remission has increased. Of major importance are tumor-infiltrating lymphocytes (TILs) where distribution and cell frequencies correlate with survival and therapeutic outcomes. Additionally, efforts have been made to identify subsets of TILs populations that can contribute to a tumor-promoting or tumor-inhibiting environment, such as the case with T regulatory cells versus CD8 T cells. Furthermore, cancerous cells have the capacity to express certain inhibitory checkpoint molecules, including CTLA-4, PD-L1, PD-L2, that can suppress the immune system, a property associated with poor prognosis, a high rate of recurrence, and metastasis. Comparative oncology brings insights to comprehend the mechanisms of tumorigenesis and immunotolerance in humans and dogs, contributing to the development of new therapeutic agents that can modulate the immune response against the tumor. Therapies that target signaling pathways such as mTOR and MEK/ERK that are upregulated in cancer, or immunotherapies with different approaches such as CAR-T cells engineered for specific tumor-associated antigens, DNA vaccines using human tyrosinase or CGSP-4 antigen, anti-PD-1 or -PD-L1 monoclonal antibodies that intercept their binding inhibiting the suppression of the T cells, and lymphokine-activated killer cells are already in development for treating canine tumors. This review provides concise and recent information about diagnosis, mechanisms of tumor development and progression, and the current status of immunotherapies directed toward canine melanoma.

Introduction

Melanoma is the one of the most aggressive and metastatic types of cancer in humans and dogs.(1-4) In humans, melanoma accounts for 80% of skin cancers.(1, 2) Early detection is a major factor in observed outcomes: for patients with an early diagnosis, the 5-year survival rate is 90%, while this rate decreases dramatically to 10% in patients diagnosed with advanced melanoma,(5) where the median overall survival is less than a year.(6) Additionally, melanoma is highly resistant to conventional therapies, and new cases are on the increase every year. Melanoma diagnoses have increased annually worldwide by 2.6% and more than 3% in countries like the US, UK, Sweden, and Norway.(7, 8)

Chemotherapy after surgical excision was the standard treatment for many years, but overall survival remained low especially for the latest stages of melanoma.(9) In the last decade, the understanding of the mechanisms and pathways leading to melanoma and the discovery of effective immunotherapies have shown great promise as more effective treatments for this neoplasm.(3, 4, 10, 11) Mutations in the RAS and RAF family of oncogenes have been detected in a significant percentage (15% and 50%, respectively) of human melanoma patients,(1, 12) leading to the development of BRAF and MEK inhibitors as potential therapeutic agents.(5) Likewise, great progress has been made in the understanding of how the immune system communicates with tumor cells in melanoma and conversely how cancerous cells can modulate the immune response of the patient, establishing the rationale for the development of therapeutic monoclonal antibodies against immune checkpoint molecules.(6) Currently, two classes of checkpoint molecules inhibitors have been approved by the FDA, monoclonal antibodies against Programed Cell Death Protein-1 (PD-1), PD-Ligand 1 (PD-L1), and against Cytotoxic T

Lymphocyte Antigen-4 (CTLA-4),(13) and more are under development, including anti-PD-L2 monoclonal antibodies.(14)

Similarly, canine melanoma is a very aggressive neoplasm, accounting for 3-8% of all neoplastic diseases in dogs and a median overall survival of 8-12 months after diagnosis.(15, 16) The most common location for canine melanoma is the oral cavity, accounting for up to 35.8% of all malignant tumors at that site,(3) with occurrence at lower frequencies in the skin, eye, and digits.(15) In contrast to the human disease, virtually no driver mutations have been identified for canine melanomas,(17-19) although investigators are continuing to probe the MAPK signaling pathway since key proteins in this cascade are up-regulated in melanoma.(20) As yet, no effective treatments for canine melanoma have been developed that parallel the recent advances made in treating the human disease.

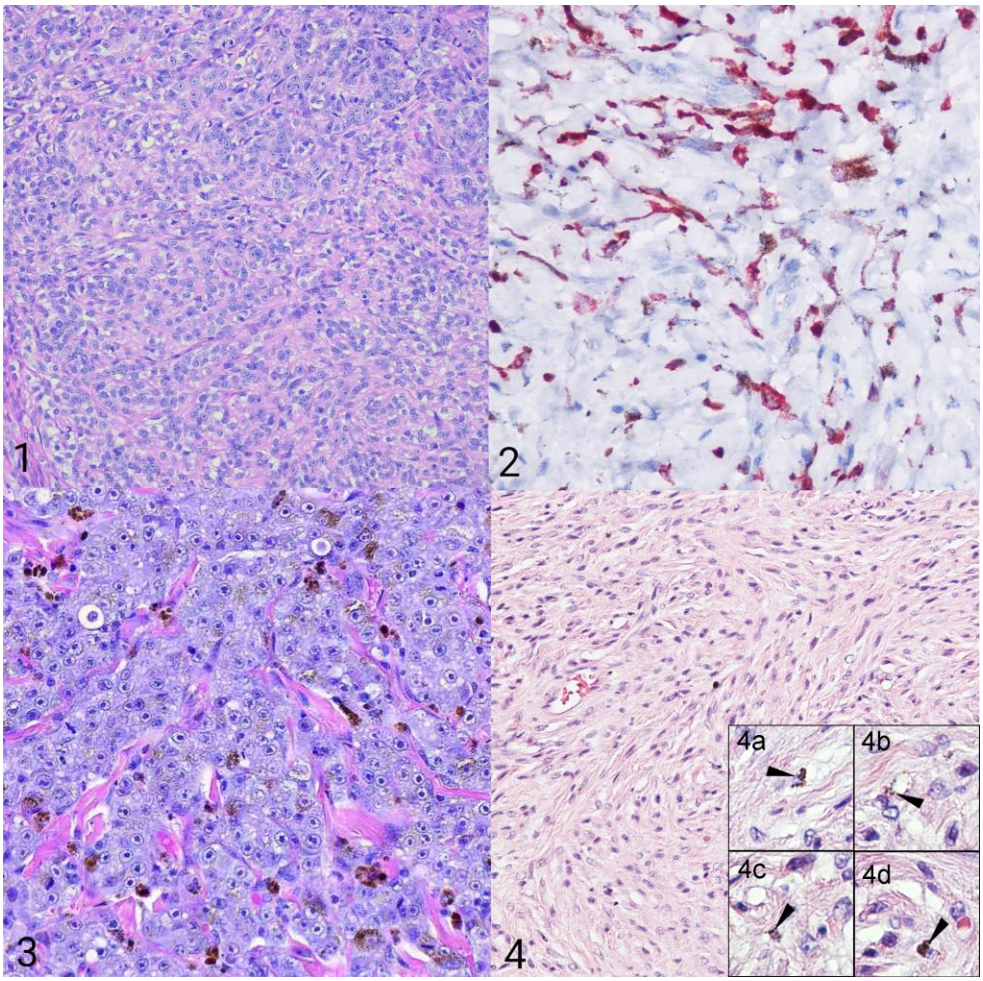
Considering that melanoma is one the most aggressive cancers in both humans and dogs, it is crucial to devise new treatments, especially for advanced cases where available options are of limited efficacy. (21, 22) After three decades using conventional modalities, the discovery of immunotherapies is transforming how this disease can be managed.(22) In this review, we will address important pathways in the pathogenesis of melanoma, along with the fundamentals of diagnosis, new immunotherapies that have been developed, and their efficacy for the treatment of malignant melanoma in humans. We endeavor to highlight the promise, as well as the pitfalls, of translating mechanistic insights and thus therapeutic opportunities between humans and dogs.

Morphology and features of canine melanoma

Origin and histologic features

Melanomas arise from melanocytes, which have an embryologic origin in melanoblasts that are derived from the neuroectoderm of the neural crest.(23) Melanoblasts migrate from the neural crest to the integumentary system and localize within the epidermis and hair follicles where they differentiate into melanocytes or remain as melanocyte stem cells.(23, 24) Melanin production by melanocytes occurs in cytoplasmic organelles called melanosomes, via a reaction catalyzed by tyrosinase through a chain of conversions that transform tyrosine to DOPA and on to melanin. The resulting granules of melanin are then transferred from melanocytes to keratinocytes by a membrane vesicle-mediated process.(25, 26) This is the usual process for pigmentation of dermal keratinocytes by melanocytes. An alternative pathway provides for production of melanocytes from a bipotential precursor cell (Schwann/melanoblast) that migrates along nerve sheaths.(24, 27) This process is regulated in part by microphthalmia-associated transcription factor (MITF) and the receptor tyrosine kinase KIT and its ligand.(15, 27, 28)

The diagnosis of melanomas can be challenging, as they can resemble other types of tumors such as carcinomas, sarcomas, lymphomas, or tumors of an osteogenic origin.(29) In addition, even though most melanomas have very characteristic melanin granules in their cytoplasm, they can also be amelanotic (Figures 1-2). In these instances, the recognition of morphologic features described by Smedley et al. and the use of immunohistochemical (IHC) markers are very useful tools.(30, 31) In addition to their intracytoplasmic melanin granules, melanomas can feature varied intratumor cell



Figures 1-4. Common canine melanoma morphologies. The panel shows the more classical melanoma morphologies. **Figure 1**. Dog oral melanoma, 20X. Amelanotic, cords of polygonal cells with no apparent intracytoplasmic melanin granules. Neoplastic cells have a large nucleus with a prominent nucleolus. **Figure 2**. Dog oral melanoma 20X. Amelanotic melanoma immunolabeled for Melan-A detected in the cytoplasm. **Figure 3**. Dog oral melanoma 20X. Epithelioid melanoma with polygonal cells arranged in cords and nests, with scattered intracytoplasmic melanin granules. **Figure 4**. Dog cutaneous melanoma 20X. Fusiform melanoma, elongated to spindloid cells arranged in streaming bundles, with occasional intracytoplasmic melanin granules (**inset 4.a-d**, 40X).

morphology, the presence of neoplastic cells at the epidermal-dermal or mucosal-submucosal junction, finely stippled to vesiculated nuclei, and often a single central and prominent nucleolus.(31) Several histological morphologies have been described for melanoma: epithelioid (Figure 3) with polygonal melanocytes arranged in cords or nests is the most frequently

diagnosed; next, fusiform (Figure 4) with spindloid cells arranged in streaming bundles; mixed with a combination of both polygonal and spindloid morphologies; small-round with cells arranged in cords; and, in cases of more undifferentiated tumors, melanomas can exhibit a neuron-like appearance.(15, 31-34) In less frequency, cellular, balloon cell, signet ring, and clear cell morphologies have been reported.(31)

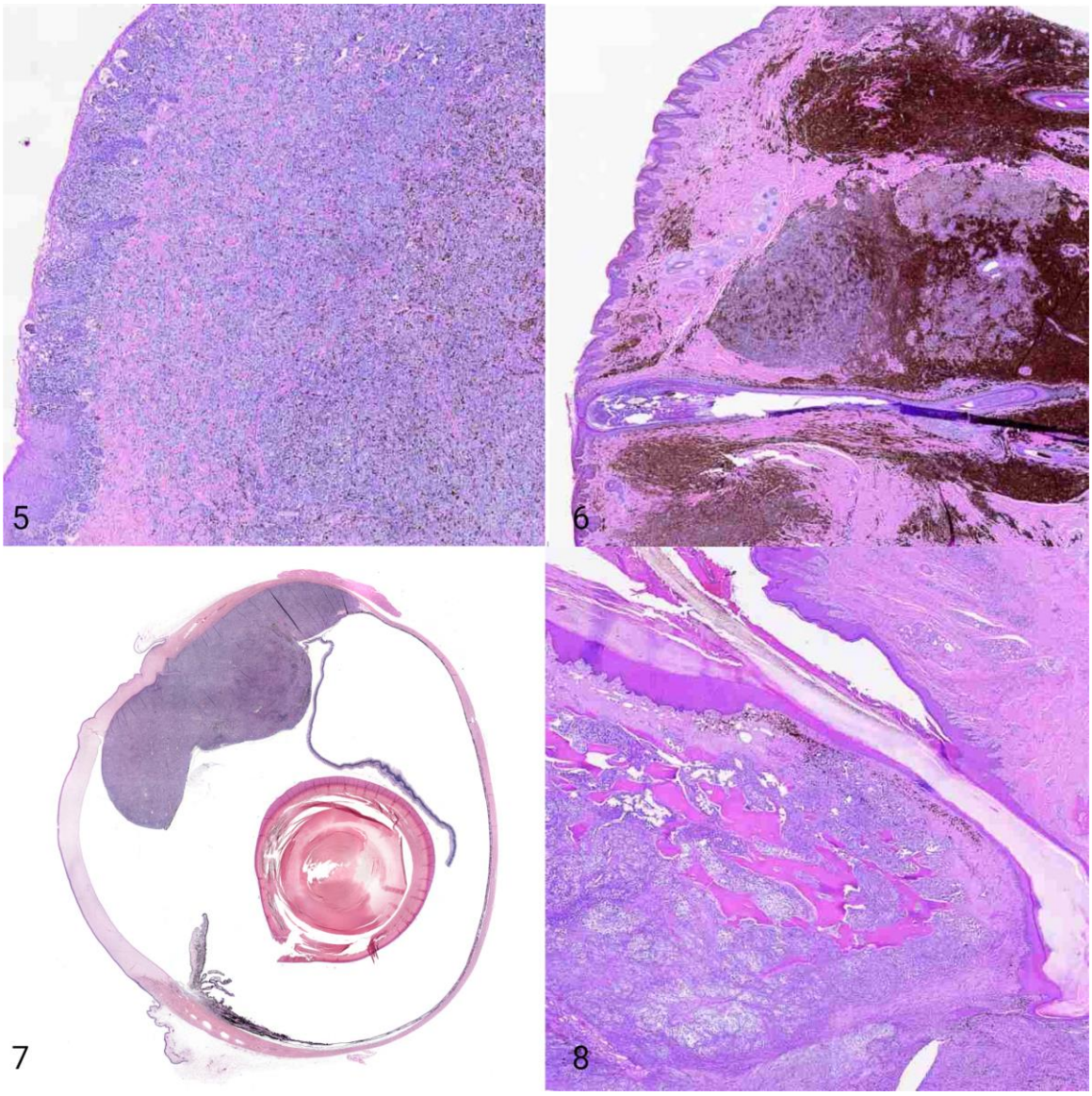
Diagnostic criteria

As melanoma can resemble multiple tumor types, IHC has become a valuable diagnostic tool. Whereas SOX10, S100, HMB-45 and Melan A are normally used as markers of human melanoma, (35, 36) common markers used to identify canine melanomas are Melan-A, PNL2, and tyrosinase related protein 1 and 2 (TRP-1 and TRP-2).(29, 30) Melan-A (Figure 2) is a protein marker specific for melanocyte differentiation, and its immunolabeling is highly sensitive and specific.(29, 37) PNL2 is a monoclonal antibody that recognizes an as-yet unidentified cytoplasmic melanocytic antigen and is described as exhibiting staining specificity similar to that of anti-Melan-A.(29) Tyrosinase, the third most-common marker, is a cytoplasmic protein Involved in melanocyte differentiation and melanin biosynthesis.(29) It has been reported that immunolabeling for these markers is reduced in tumors with spindloid or undifferentiated morphology.(29) These three markers combined can reach a sensitivity of 100%.(29, 30) Other markers such as S100 have been suggested in the past for humans and dogs, with good results in canine melanoma cell lines,(38) but its specificity is considered low by other investigators,(29, 30) similar to results for vimentin.(37) HMB-45 (Human Melanoma Black) has also been reported in canine melanoma with high variability among the few reports available, and its use is not recommended in canine neoplasms.(31)

Another antigen, more recently recognized, is chondroitin sulfate proteoglycan-4 (CSPG4), also known as high molecular weight-melanoma associated antigen (HMW-MAA), an early cell-surface progression marker associated with proliferation, migration, and invasion. This maker, first described in human melanoma but lately reported in canine melanoma, has promising potential therapeutic use as the basis for a DNA vaccine,(39-42) and has been reported to be expressed more frequently in amelanotic melanomas.(40)

Although originating most frequently at oral, cutaneous, digital, subungual, or ocular sites (Figures 5-8), melanomas can also occur in the gastrointestinal tract, nervous system, and muco-cutaneous junctions.(33) Traditionally, oral melanoma has been most closely associated with malignancy, whereas the cutaneous counterpart typically has a relatively benign course of disease, and digit or subungual with increased rate of recurrence. Ocular melanoma has been described to be locally aggressive, but with limited metastatic potential.(15, 18, 31, 33, 34, 43, 44) However, it must be emphasized that location alone should not be used to determine prognosis.(44)

The main features used for prognostic determination were nuclear atypia, growth fraction, and mitotic index, as reported by Smedley et al. and supported by the most recent melanoma consensus, revised in 2020 by the Veterinary Cancer Society/American College Veterinary Pathology Oncology-Pathology Working Group.(44) Nuclear atypia was found to be highly correlated with outcome, especially for pigmented melanomas with epithelioid morphology. The authors suggest a threshold of >30% of cells with



Figures 5-8. The panel shows the different locations of canine melanoma. **Figure 5.** Oral melanoma (10X). Small nests of neoplastic melanocytes infiltrate the mucosal epithelium, and large sheets of neoplastic cells obscure the propria mucosa. Brown intracytoplasmic granules consistent with melanin are throughout the neoplasm. **Figure 6.** Cutaneous melanoma (10X), haired skin. Large nests of neoplastic melanocytes infiltrate the dermis. Numerous melanomacrophages infiltrate the periphery of the neoplasm. **Figure 7.** Ocular melanoma (montage of scan at 10X). A large, multilobulated neoplasm extends from and occupies the back of the eye. **Figure 8.** Digital melanoma (10X). Sheets of neoplastic melanocytes obscure the dermis and infiltrate the bone.

atypical nuclei from a total of 200 cells observed as indictive of a poor prognosis for oral and lip neoplasms and > 20% of cells with atypical nuclei for cutaneous neoplasms.(31)

Growth fraction (or Ki67 index) is measured by using IHC for the Ki67 protein (Ki67p). Detection of Ki67p is indicative of mitotic activity of intrinsic cell populations, as it is only present in actively proliferating cells (*i.e.*, in G_1-M phases of the cell cycle but not G_0) and is commonly used as a marker for aggressiveness of the neoplasms.(45) For oral melanomas, Ki67p is determined by calculating the average number of positively labeled melanocytes over a total of five areas of 1 mm^2 optical grid at 400x magnification. In cutaneous melanomas, the Ki67 index is determined by estimating the percentage of positive labeled melanocytes per 500 counted cells. Ki67p is significantly different for benign and malignant melanocytic neoplasms, with increased numbers of immunolabeled cells found in oral melanomas and associated with a poor prognosis and reduced survival.(31)

Regarding the mitotic index, briefly, it is well-established that oral and lip melanocytic neoplasms with $\geq$ 4 mitoses per 2.37mm^2 observed microscopic field have an increased risk of death within one year of diagnosis, and, for cutaneous and digital melanocytic neoplasms, $\geq$ 3 mitoses in 2.37mm^2 are statistically correlated with low 2-year survival rate.(31) In addition, the VCS/ACVP consensus group has suggested addition of tumor thickness to the prognosis indicators for cutaneous melanomas.(44) A tumor thickness of >0.95 cm is associated with poor prognosis and increased risk of recurrence and developing metastasis.(46) The authors also reported that tumor thickness is greater in melanoma than melanocytoma.

Genetic characterization of melanoma

Genomic contributors to tumorigenesis in human melanoma

Melanoma is considered one of the cancers with the highest degree of mutations,(47, 48) and four major subtypes based on the genetic mutation burden have been described in humans. The first three groups encompass activating driver mutations in B-Raf proto-oncogene serine/threonine kinase (BRAF), the GDP/GTP binding protein RAS (RAS), and the tumor suppressor Neurofibromatosis Factor 1 (NF1), respectively, while the fourth group ("Triple Wild Type") lacks any of these three alterations, although it may have a low frequency of other mutations such as those observed in KIT.(12) The detection of these of mutations in patients informs the therapeutic plan and gives insight into their prognosis. Some patients with mutated BRAF could benefit from targeted therapies such as BRAF and MEK inhibitors, and, while these inhibitors might not be effective for some other patients, they could still benefit from other approaches including immunotherapies. (22, 49)

The BRAF mutation is a somatic missense point mutation that results in a single substitution of glutamic acid for valine at amino acid 600 (V600E) in human BRAF, conferring a constitutively active state to the BRAF protein kinase. This translates into a continuous state of survival of the cell, increasing proliferation and promotion of metastasis.(50) The BRAF mutation has been described in 40-50% of all human melanoma cases(34, 47, 49) and is more commonly seen in younger patients.(12) Additionally, in patient with nevi that have the BRAF mutation, a second hit could induce malignancy by an inactivation mutation in the phosphatase and tensin protein (PTEN), since this protein has a role as a negative regulator of cell growth and survival signaling pathways.(47, 51)

Mutation of RAS is the second most common genetic alteration in human melanoma, present in 15-25% of all cases.(47, 49) This mutation alters the RAS protein and maintains

protein activation, which continuously stimulates the mitogen/extracellular signal-regulated kinase (MEK/ERK) pathway and in some cases can also lead to the activation of the Phosphatidylinositol-3-kinase (PI3K) signaling pathway, driving cell survival and proliferation.(49) Mutation in Neurofibromatosis Factor 1 gene (NF1) is the third most common mutation in melanoma and induces loss of the regulatory effect of NF1 on the RAS protein.(48, 49) Consequently, RAS is continuously activated, and the cell enters into a proliferative state. This mutation is more frequently detected in older patients and is associated with better response to checkpoint molecule blockade therapies(12) as described below.

The fourth group is the Triple Wild Type, lacking BRAF, RAS and NF1 mutations. Even though melanomas in this group do not have any of the three mutations described above, they can have other mutations like GNAQ (G signaling protein) and c-KIT (CD117, a tyrosine kinase receptor for stem cell growth factor) in a lower frequency (less than 7%); these proteins are associated with intracellular signaling and can result in uncontrolled cell proliferation.(12, 52) Triple Wild Type melanomas are mostly from non-cutaneous origin, such as mucosal melanoma and, while less common, are very aggressive and associated with a poor prognosis.(34, 48, 53) Other mutations found in human mucosal melanomas include activating mutations in SF3B1, loss of CDKN2A, PTEN, and SPRED1.(54) PTEN mutation has been reported in canine mucosal melanoma, although its role in disease severity or progression has not been established.(55)

Activation of the signaling pathway mTOR (mammalian target of rapamycin), a serine/threonine kinase, has been strongly associated with the development of different neoplasms, including melanoma.(34, 56-58) This complex is activated by the PI3K/AKT pathway and regulates cell growth. Its overexpression can lead to aberrant proliferation and increased migration, conferring on the neoplastic cells more invasive capabilities.(59-62) There

are two main components in this complex: mTORC1, which regulates numerous processes of protein synthesis that promote cell growth and can also inhibit certain catabolic process such autophagy contributing to the malignancy of the neoplasm,(60, 63, 64); and mTORC2, associated with control of cellular structure, regulation of the cytoskeleton, and cell survival.(60, 61, 63)

Another factor that has been reported commonly in cancer in multiple species is the variability of Somatically-acquired Copy Number Alterations (SCNAs), which consist of deletion or amplification of DNA fragments encoding regulators of cell proliferation.(65) Recent studies report similar increases in SCNAs in human and canine mucosal melanoma.(55, 66) In canine mucosal melanoma, increased aberrations by gain of chromosome 13 and 17, and loss of chromosome 2 and 22 were reported using comparative genomic hybridization in situ, and copy number assessments using fluorescence in situ hybridization revealed gain of c-MYC and loss of CDKN2A, whereas in humans there was gain of 1q, 6p, 8q, 7, and loss of 6q and 10.(66, 67)

Lessons for canine melanoma?

The comparative oncology approach of identifying a mutation burden in canine melanoma similar to that in human disease has met with only limited success, as canine melanomas do not appear to have any of the mutations described above in high frequency. Although the BRAF mutation was found in 3 out of 54 canine melanomas studied,(68) it is far less common than reported in the human disease, where it occurs in 40-50% of cases.(17-19, 69) Additionally, NRAS and NF-1 mutations in canine melanoma have also been reported, but at a very low frequency.(70) Although no predominant mutation has been described for canine melanoma to date, the search continues for a driver mutation that could be involved in the

development of melanoma in dogs,(20) while other investigators have proposed the use of the dog as an animal model to study Triple Wild Type and mucosal melanoma in humans.(18, 32, 34) This parallel approach could bring new perspectives regarding risk factors and experimental paradigms from which both species will benefit. For instance, activation of the mTOR signaling pathway has been also explored in canine melanoma as well as in the human disease, and studies in canine melanoma cell lines have demonstrated activation of this pathway along with the inhibitory efficacy of Rapamycin and other drugs.(71-73) Clearly there is much to be learned about the genetic drivers of melanoma in dogs.

Tumor Infiltrating Lymphocytes and Immune Checkpoint Molecules – A New Frontier for Canine Melanoma Therapy

Tumor/lymphocyte interactions in the human melanoma microenvironment

Melanoma is considered an immunogenic tumor, and in most of the cases the tumors have a high degree of lymphocytic infiltration. However, most melanomas continue to grow, suggesting that tumor infiltrating immune cells fail to control and modulate tumor invasion.(16, 74, 75) Furthermore, the tumor cells may suppress tumor infiltrating lymphocytes (TILs) and thereby escape immune surveillance. Nevertheless, the presence of TILs is associated with better responses to targeted and immune-directed therapies.(76-78) Additionally, it has been demonstrated that the presence of TILs typically correlates with a favorable prognosis in melanoma(79-84) and other human cancers.(85-88)

In view of this association, a system to characterize TILs infiltration in human melanoma was established by Clark(83) and has been adapted by other investigators for application to canine melanoma and other neoplasms. Briefly, the TIL distribution is classified as absent, brisk, or non-brisk. The “absent” category is described as no lymphocytes directly opposed to the

tumor cells; the “non-brisk” category as isolated, focal and/or segmental infiltration in the tumor; and the “brisk” category as TILs segmentally infiltrating either at the entire base of the tumor or diffusely infiltrating within the tumor.(83) Focal TILs that are in areas of fibrosis or remain in perivascular spaces but do not penetrate the tumor are also considered under the absent subclassification.(81) Other studies propose a combined system that considers the distribution and density of these TILs.(89) These authors also reported that dense TIL infiltration with a brisk distribution was strongly associated with better prognosis in contrast to the non-brisk and absent distribution.(83)

TILs comprise a heterogenous population of different lymphocytes bearing the co-receptors for the T cell receptor CD4 or CD8, including CD4 T regulatory cells (Tregs), CD4 T helper cells, CD4 memory cells, and CD8 effector cells.(90) A majority of studies that aim to classify the subset of TILs in the tumor microenvironment use IHC markers for surface proteins, such as CD20 and CD79a for B cells, CD3 for T cells, CD4 and CD8 for T cells, and FOXP3 for regulatory T cells.(91, 92)

Human melanoma patients with increased TIL abundance in the primary tumor have improved survival times compared to those with low TIL infiltration,(79) better progression-free survival,(84) and longer disease-free survival,(89) while low TIL infiltration is associated with tumor recurrence and distant metastasis.(87) (89) However, other investigators suggest that TIL infiltration alone is not predictive of outcome for non-small cell lung cancer and suggest that the subset of these TILs is more important,(86) as also described in patients with metastatic colorectal cancers.(87) These authors emphasize the importance of characterizing TILs and report that high numbers of CD8 T cells expressing cytolytic enzymes and CD4 T cells lacking inhibitory receptors were associated with a good prognosis when compared with TILs expressing

PD-1.(86) Interestingly, the association between high Ki67 expression levels and high TILs levels has been described in triple-negative breast cancer, suggesting that the antitumor immune response is increased in aggressive tumors.(93) Based on the findings described above, profiling of TILs in the tumor microenvironment provides a useful framework for understanding the contribution of different subsets of lymphocytes to formation of a tumor-promoting or tumor-inhibiting environment, associated with CD8 T cells or T regulatory cells, respectively.

Extension of tumor/lymphocyte insights to canine melanoma

Several studies in canine tumors have reported a relationship similar to that described above in human melanoma, associating density of TILs positive for the T cell co-receptor CD3 with favorable outcome in histiocytic sarcoma following resection.(94) Another study in canine gliomas determined the nature of the infiltration, differentiating between CD3 T cells and those positive for the Forkhead box protein P3 (FOXP3), a regulator of Treg cell gene expression, finding similar results with generally low numbers of Treg cells, although higher FOXP3 densities were found in high grade tumors.(95) Higher levels of Treg cells in peripheral blood and tumor-draining lymph nodes have been reported in dogs with melanomas when compared to healthy controls,(96) and has been linked to an increased risk of death.(97, 98) As explained in another study, subsets of TILs have an important role on tumor progression and outcomes, as CD8 T cells are key determinants of antitumor immunity, and CD4 T cells can either support this immune response or suppress it, as the case for Treg cells.(99) These authors evaluated TILs in canine oral melanoma, taking into consideration both TIL density and distribution using the classification proposed by Clark et al (described above), reporting that higher CD4 and CD8 TIL levels were found in less aggressive stages and also among primary tumors when compared to

recurrent tumors. Additionally, patients with a brisk and non-brisk CD8 T cell infiltration had higher overall survival rates when compared to those with absent infiltration, and no impact on survival was appreciated from variable T reg and CD4 T cell infiltration.(99)

Thus, the relationship between TILs and cancerous cells in the tumor microenvironment is an important consideration for the development of therapies targeting the regulatory molecules of the immune system. Tumor infiltration is not limited to tumor growth suppression, since it can also be associated with tumor growth and immunosuppression.(100) Importantly, it has been reported that TILs are associated with better responses to targeted and immunotherapies in humans,(76-78) especially for melanoma. (101)

Immune Checkpoint Molecules: the molecular basis of immunosuppressive tumor/lymphocyte interactions

In patients with cancer, lymphocytes can experience chronic exhaustion and express PD-1 and CTLA-4 co-inhibitory receptors. At the same time, cancerous cells can overexpress PD-1 ligand (PD-L1) and CTLA-4 and thus inhibit an antitumor response by the immune system. PD-L1, PD-L2 and CTLA-4 are highly expressed across different cancer types.(102, 103) Cancer cells can also recruit immune-suppressor cells like T regulatory lymphocytes, myeloid derived suppressor cells (MDSC), and type 2 macrophages, releasing immunosuppressive cytokines like IL-10, thereby allowing for tumor growth and proliferation, as has been described in several studies. (100, 104-106)

Interestingly, expression of PD-1's alternative ligand PD-L2 has been detected in several human tumors, and some studies have reported an overexpression of PD-L2 in absence of PD-L1.(14) Other studies have found PD-L2 to be highly correlated with PD-1 and PD-L1 expression (103, 105) or with cancer infiltration and metastasis.(105, 107) The degree to which

tumor-expressed PD-L2 can substitute for PD-L1 as an immunosuppressing agent is not well established, but an association between resistance to anti-PD-L1 immunotherapies and PD-L2 overexpression suggests an important role for PD-L2 in immune escape.(108)

After decades of conventional treatment for advanced melanoma with limited success, surgical excision, chemotherapy and radiation are now being complemented with immunotherapies.(109) After the discovery of the roles of checkpoint molecules such as PD-1, its ligand PD-L1, and CTLA-4, efforts have been redirected toward developing antibodies to inhibit their activity and release the patient's own immune system to fight cancer. (22, 109)

Current targeted and immunotherapies for melanoma in humans

In humans, patients with BRAF mutations are candidates for targeted therapies such as BRAF and MEK inhibitors. These therapies have been shown to inhibit tumor growth by decreasing the kinase activity in the MEK/ERK signaling pathway.(49, 110, 111) Clinical trials have demonstrated that combined therapies are more effective than MEK or BRAF inhibition alone, with higher response rate and longer progression free survival.(49) Vemurafenid and Dabrafenid or Trametinib, BRAF-mutant inhibitors approved by the FDA for the treatment of BRAF-V600-mutated-melanoma, have reported remission in 90% of patients.(22) However, the clinical benefit is limited by the development of therapy resistance due to mRNA splice variants of BRAF and re-activation of alternate signaling pathways.(22, 112, 113) There are several clinical trials using MEK and BRAF inhibitors in combination with immunotherapies that show improving response rates and duration of results, especially for patients unresponsive to BRAF/MEK inhibitor therapy; response rates up to 70% are reported.(111, 112, 114)

Another strategy studied for the treatment of melanoma is the activation of anti-cancer immunity, by either stimulating dendritic cells or amplifying T cell activation. For several years, attempts were made using vaccines containing melanoma antigens without success due to the immunostatic effect of the tumor microenvironment or the poor immunogenicity of the antigens, inducing either high toxicity or insufficient responses.(22, 109) Similar results were seen with agonistic antibodies for dendritic cells and cytotoxic lymphocytes (OX40 and CX3CL respectively).(109) However, studies measuring the expression of OX40 in TILs have detected an upregulation of OX40 on T regulatory lymphocytes,(115) and anti-OX40 antibodies have been proposed as adjuvants alongside immunotherapeutics. (116)

Nowadays, immunotherapy research in multiple species is focused on the development of checkpoint molecule inhibitors and their efficacy in different types of cancers, and dramatic advances have been made in treatment of human disease. A variety of antibodies inhibiting the suppressive effect that cancer cells have over TILs have been developed during the last decades. Immunotherapies that have shown good promise are mononuclear antibodies against CTLA-4, PD-1 and PD-L1.(22, 117-120) Antibodies against checkpoint molecules approved by the FDA in the treatment of metastatic melanoma are Ipilimumab (anti CTLA-4), Nivolumab and Pembrolizumab (anti PD-1).(22) In addition, an anti PD-L1 antibody (Atezolizumab) has been approved for urothelial carcinoma and non-small cell lung cancer (NSCLC).(121) These drugs have an advantage over therapies that target tumors driven by BRAF, thus making them available for patients with other types of mutations or lacking the most frequent mutations. An advantage of the newly-developed Atezolizumab is that, in addition to binding free PD-L1, it also displaces the ligand from PD-1 and appears to be more effective than other anti-PD-L1 antibody drugs currently in clinical trials.(121, 122) Additionally, immunotherapies targeting

PD-1 and/or PD-L1 have shown fewer side effects than those targeting CTLA-4.(123) A meta-analysis of 19217 human patients on immune checkpoint inhibitors showed toxicity-related death rates less than 0.4% for anti-PD-1 and anti-PD-L1, 1.08% for anti CTLA-4 and 1.23% for combined therapies.(124)

Current immunotherapies in dog cancer

Exploration of the utility of immunotherapies in canine cancer therapy is in its very early days, with much of the published work having been conducted *in vitro*. For example, combined treatment with trametinib and sapanisertib in order to inhibit both MEK and mTOR pathways (Figure 9) has been reported to induce apoptosis and reduce cell survival in canine melanoma cell lines,(72) and one study reported that inhibition of the mTOR pathway reduced invasion and angiogenesis in hemangiosarcoma cell lines.(125) However, combining these drugs with immunotherapies or targeting both pathways resulted in toxicities in studies done in cell lines and xenografts.(72, 73)

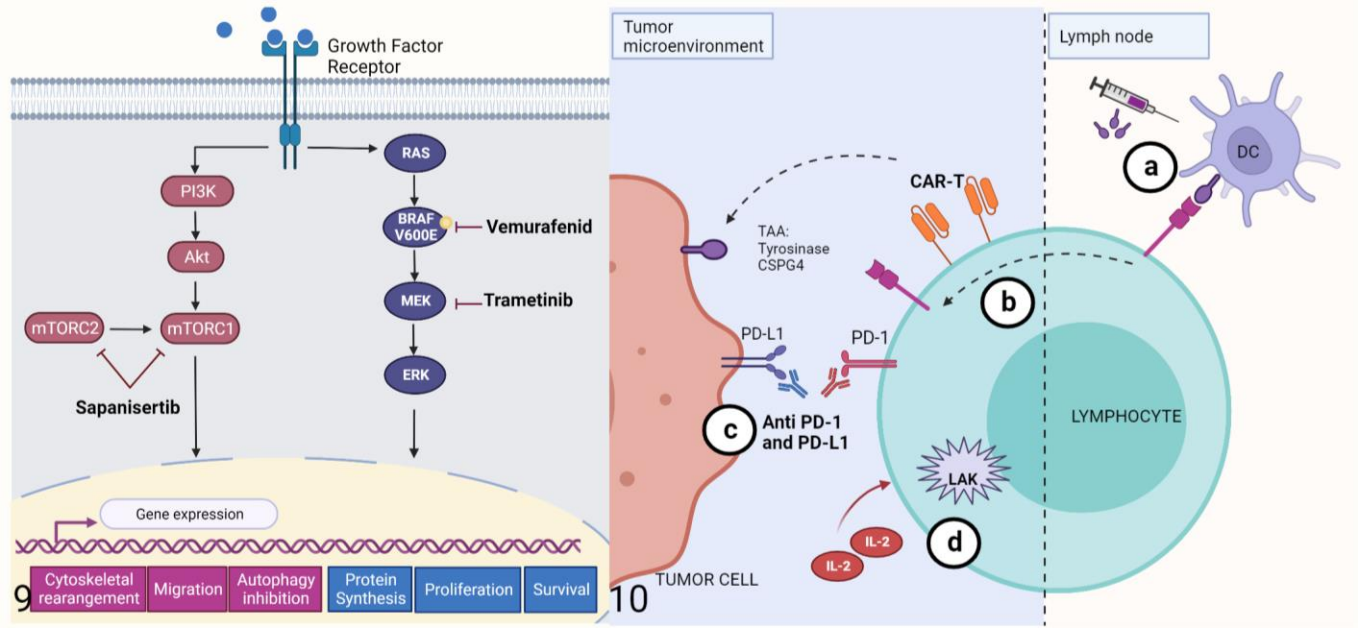
Availability of immunotherapeutic agents is limited in the treatment of canine cancers. The less comprehensive characterization and understanding of the immune response to tumors in this species is a major obstacle, due in part to limited reagent availability.(11) However, recent studies have explored vaccines, adoptive cell transfer, and anti PD-1 and PD-L1 therapy.(21, 126, 127) Currently, Oncept, a xenogenic human tyrosinase DNA vaccine, is the sole vaccine approved by the USDA for the treatment of canine melanoma (Figure 10).(21, 128, 129) Although one study reported improvements in survival times,(130) it has been established that the vaccine alone is not enough to maintain anti-tumor efficacy, since the tumor microenvironment can suppress the immune response, and, once lymphocytes have been

activated, they can express immunosuppressive checkpoint molecules.(11, 21, 128, 129) Since the discovery of CSPG4 antigen in canine melanomas, efforts have been made to develop a DNA vaccine targeting this tumor associated antigen (Figure 10), seeking to induce a safe immune response with longer overall disease-free survival when used as an adjuvant with other therapies and surgery.(39-42, 131)

Additionally, adoptive cell transfer has been explored for the treatment in canine cancers, using lymphokine-activated killer (LAK) cells and CAR-T cells. These therapies have been evaluated for canine osteosarcoma (in vitro), B cell lymphoma, and melanoma (in vitro and in vivo),(11, 100, 128, 132) resulting in high toxicity for osteosarcoma(100) but modest antitumor activity for B cell lymphoma and melanoma.(132, 133) Although LAK therapy demonstrated an immune-enhancing effect, it is not sufficient as monotherapy and thus should be evaluated as an adjuvant for other immunotherapies.(128, 132) Similarly, CAR-T cells present several disadvantages, such the high cost and time required to engineer the cells, the risk of mutations in the receptor, antigen selection for the engineering of the receptor, and the fact that CAR-T cells are transient and not sustainable over long periods of time.(100, 129)

Increased levels of CTLA-4 have also been reported in canine melanocytic tumors and associated with an immune-suppressed tumor microenvironment leading to immune escape and worsened prognosis.(98) Great efforts have been made towards the development of a canine anti-CTLA4 monoclonal antibody, with promising results in mouse models.(134) Another group has developed a chimeric heavy-chain antibody targeting cells expressing CTLA-4, such as Tregs and CD4 helper T cells, and reported an induction of the cytokine IFN-γ, whose pleiotropic actions include anti-tumor activity.(135)

Lastly, one group has made great progress in the detection of overexpression of PD-1 from TILs and PD-L1 from malignant tumors in several canine cancers, such melanoma, osteosarcoma, hemangiosarcoma, and others, in the interest of establishing a rationale for application of anti-PD therapeutics in dogs.(136, 137) Anti PD-1 chimeric antibody has been developed by a research group which reported reduction of tumor burden, objective partial response in 26% of the treated dogs with a Stage IV oral malignant melanoma, and a trend of increase of survival times, with maintained complete regression for more than 1 year in 2 cases. (127, 138) In a pilot clinical study, these investigators demonstrated that anti-PD-L1 therapy can induce an objective antitumor response for oral melanoma.(126) Furthermore, the expression of these checkpoint molecules has also been reported by our laboratory as increased in melanoma when compared with its benign counterpart melanocytoma and is associated with the density of CD3+ TILs.(139) Recently, a group tested the cross-reactivity of different FDA-approved human immune checkpoint inhibitors (ICI) in canine tissue, showing two anti PD-L1 (Atezolizumab and Avelumab) with cross-reactivity, and Atezolizumab with the most robust T-cell cytokine production *in vitro*.(140) A schematic representation of the immunotherapies in canine cancers is shown in Figure 10. These studies establish the basis for future research on the development of checkpoint molecule inhibitors as a treatment for melanoma and other cancers in dogs, and it is expected that in the future, these therapies could be an effective and affordable option for the veterinary practice.



Figures 9 and 10. Summary of small molecule- and antibody-based approaches to melanoma treatment. **Figure 9**. Targeted therapies that aim to suppress central mechanisms of cellular proliferation and metastasis by inhibition of the mTOR (Sapanisertib), and MERK/ERK (Vemurafenid, Trametinib) pathways. The mTOR pathway activates gene expression that leads to cytoskeletal rearrangement, migration, and inhibition of autophagy. The RAS/BRAF/MEK/ERK pathway activation leads to increased protein synthesis, and promotes cell proliferation and survival. **Figure 10**. Immunotherapies described to date in canine tumors. a) Immunogens encoded by DNA vaccines, including tyrosinase (Oncept) or CSPG4, are first recognized by dendritic cells (DC) which later present antigen fragments to T cells at the lymph node; these T cell are able to target the antigen at the tumor microenvironment; b) CAR-T cells engineered for specific tumor-associated antigens (TAAs) and cloned in vitro before being reincorporated into the patient; c) Monoclonal antibodies recognizing PD-L1 blocking its binding with PD-1 receptor; and d) Lymphokine-activated killer cells activated in vitro with IL 2.

Conclusion

Comparative oncology between humans and dogs will set the foundations for a better understanding of common factors that are associated with the development of melanoma in humans. Additionally, it will provide insight into which factors are associated with the development of melanoma in dogs, and by what mechanism. As dogs spontaneously develop melanoma, are exposed to the same environmental hazards as humans, and have similar physiology to humans, they are excellent candidates for future development of immunotherapies. The evident lack of effective therapies for melanoma in dogs increases the urgency of the search for an effective treatment. Human benefit from immunotherapies has been documented in numerous studies, and it would be remarkable to continue exploring this option for treating dog cancers.

Lastly, study of the frequencies of occurrence, the spatial and temporal distribution, and functional categorization of TILs has great therapeutic potential. As new insights are gathered from recent studies, TILs will provide a representation of patients' immune system and the tumor microenvironment, offering a promising tool for determining therapeutic approaches.

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CHAPTER III
PD-1, PD-L1 AND PD-L2 GENE EXPRESSION AND TUMOR INFILTRATING LYMPHOCYTES IN CANINE MELANOMA

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Abstract

Melanoma in humans and dogs is considered highly immunogenic; however, the function of tumor-infiltrating lymphocytes (TILs) is often suppressed in the tumor microenvironment. In humans, current immunotherapies target checkpoint molecules, (such as PD-L1, expressed by tumor cells), inhibiting their suppressive effect over TILs. The role of PD-L2, an alternative PD-1 ligand also overexpressed in malignant tumors and in patients with anti-PD-L1 resistance, remains poorly understood. In the current study, we evaluated the expression of checkpoint molecule mRNAs in canine melanoma and TILs. Analysis of checkpoint molecule gene expression was performed by RT-qPCR using total RNA isolated from formalin-fixed and paraffin-embedded melanomas (n=22) and melanocytomas (n=9) from the Virginia Tech Animal Laboratory Services archives. Analysis of checkpoint molecule expression revealed significantly higher levels of *PDCD1* (*PD-1*) and *CD274* (*PD-L1*) mRNAs and an upward trend in *PDCD1LG2* (*PD-L2*) mRNA in melanomas relative to melanocytomas. Immunohistochemistry revealed markedly increased numbers of $CD3^+$ T cells in the highest *PD-1*-expressing subgroup of melanomas compared to the lowest *PD-1* expressors, whereas densities of $IBA1^+$ cells (macrophages) were similar in both groups. $CD79a^+$ cell numbers were low for both groups. As in human melanoma, overexpression of the PD-1/PD-L1/PD-L2 axis is a common feature of canine melanoma. High expression of *PD-1* and *PD-L1* correlates with increased numbers of $CD3^+$ cells. Additionally, the high level of $IBA1^+$ cells in melanomas with low *PD-1* expression and low $CD3^+$ cells levels suggest that the expression of checkpoint molecules is modulated by interactions between T cells and cancer cells rather than histiocytes.

Introduction

In humans, melanoma is considered the deadliest type of skin cancer. More than 600,000 patients in the United States were recorded as living with melanoma in 2017.[25] Mutations in the *RAF* and *RAS* families of oncogenes are detected in high frequency in human melanomas.[29,5] Similarly, canine melanoma is also very aggressive, with median survival time of 22 months after diagnosis for cutaneous melanoma, 5 months for oral melanoma, and <3 months for Stage 3 (metastatic) disease.[24] However, in contrast with humans where point mutations in the *BRAF* family are associated with 50% of the cases, this mutation appears to be rare in canine melanoma[37],[23] (Stevenson, Huckle, LeRoith, unpublished data), and few convincing driver mutations have been described for canine melanomas.[10,13,33] Because of this, the dog has been proposed as an attractive animal model to study the oncogenic mechanisms in human triple wild-type (lacking any of the known driver mutations in *BRAF*, *RAS*, or *NF1*) and mucosal melanomas, which comprise almost 40% of the total human melanoma cases.[13,15,34,43]

While increases in melanoma diagnoses occur worldwide every year,[2] great progress has been made in the understanding of the mechanisms of the disease, leading to the development of new therapies for human melanoma and other cancers.[12,30] Although melanoma is considered an immunogenic tumor, the function of tumor-infiltrating lymphocytes (TILs) is often suppressed in the tumor microenvironment (TME), failing to control and modulate tumor invasion and leading to escape from immune surveillance.[26,11] Lymphocytes in patients with cancer often experience exhaustion resulting in overexpression of PD-1 co-inhibitory receptor (encoded by *PDCD1*); moreover, cancer cells frequently overexpress a PD-1 ligand (PD-L1, encoded by *CD274*), inducing immunosuppression upon their binding to TILs.[27,47,22] The ability of tumor-expressed PD-L1 to inhibit tumor-infiltrating lymphocytes expressing PD-1 is considered a major

mechanism by which numerous types of neoplasms achieve immune tolerance.[30,12] Additionally, expression of the PD-1 alternative ligand PD-L2 (encoded by *PDCD1LG2*) has been detected in numerous tumors, although reports vary as to its correlation with expression of PD-L1.[47,3 44] Interesting, few reports have associated the expression of PD-L2 with cancer infiltration and metastasis.[46,14]

Therapeutic use of monoclonal antibodies against PD-1 and PD-L1 for humans has been remarkably effective at reversing immune escape by tumors and increasing patients' median overall survival time.[1,8] Interestingly, patients that developed resistance to anti-PD-L1 therapy had overexpression of PD-L2 [40,38], and at the same time the presence of TILs has been associated with a better outcome and prolonged survival time.[8]

As yet, no effective treatments have been developed for canine melanoma. A DNA vaccine, Oncept, approved by the FDA for canine oral melanoma has shown mixed results in survival times[4,31,41] and is unable to maintain anti-tumor efficacy, perhaps owing to the expression of immunosuppressing checkpoint molecules on vaccine-activated lymphocytes.[4,31,16] As in humans, detection of overexpression of *PD-1* by TILs and *PD-L1* from malignant tumors representing different types of canine cancers has been reported.[19,20] However, only one small pilot clinical study has demonstrated objective anti-tumor response for oral melanoma targeting PD-L1.[21]

A rationale for extension of advances in human melanoma immunotherapy to dogs requires a deeper comparative understanding of disease mechanisms across species, including characterization of the role of tumor-expressed checkpoint molecules in suppressing host immune response. Accordingly, the objectives of the research reported here are to determine the

expression of *PD-1, PD-L1* and *PD-L2* mRNAs in canine melanoma and to evaluate the association of TILs with the expression of these immune modulators.

Material and Methods.

Sample identification

Thirty-one formalin-fixed and paraffin-embedded specimens from biopsies with the diagnosis of non-digital cutaneous (6 cases) or oral melanoma (16 cases) were retrieved from the Virginia Tech Animal Laboratory Services (ViTALS) archives. Melanocytomas (9 cutaneous cases) were used as a control group. These tumor biopsies were from different privately owned dogs, submitted for routine histopathological evaluation. Cases were dated from 2017 to 2019. Diagnostic criteria for melanomas and melanocytomas included mitotic rate and tissue infiltration, as previously described.[36] Briefly, those tumors with a mitotic index $\geq$3 for cutaneous neoplasms and $\geq$4 for oral neoplasms, cytological features such as large nucleoli and atypical mitoses, and the presence of vascular or lymphatic invasion in hematoxylin and eosin-stained slides were considered to be melanomas.[35] Two amelanotic cases were confirmed subsequently by the presence of melanin in biopsies of recurrent tumors. Lymphovascular invasion was assessed morphologically in tissue sections and by clinical staging where provided. No oral melanocytomas were identified.

Real time quantitative PCR (RT-qPCR)

Four to 6 scrolls of 10 μm thickness each were cut serially from paraffin blocks of all cases. RNA was extracted using the QIAamp RNA FFPE tissue kit (Qiagen), and the RNA concentration and purity were estimated by UV spectrophotometry. Only isolates with RNA

concentrations greater than 100 ng/ul were used for this study. Random-primed cDNA was synthesized from 1 ug total RNA using the High Capacity cDNA kit (Life Technologies) and stored at -20°C.

RT-qPCR primers and TaqMan^{TM} exon junction-spanning probe sets targeting canine *PD-L1* (NM_001291972; sense primer: GGTGCTGACTACAAGCGGATTA; antisense primer: GTGACAGGATCCACAGAAATTCTTT; probe: FAM-TGAAAGTTCATGCCCCGTAC-MGBNFQ) and *PD-L2* (XM_847012; sense primer: CGCCTGGGACTACAAATATCTGA; antisense primer: GATCCTGAGGAAATGAGTGTTTATTTT; probe: FAM-TGAAAGTCAAAGCTTCCTACAA-MGBNFQ) were designed using PrimerExpress software (Applied Biosystems) and validated for amplification efficiencies of >95%. Pre-validated primers and probe sets for canine *PD-1* and the internal reference marker 18S rRNA were obtained from Thermo Fisher (cat. nos. 4351372 and 4333760F, respectively).

All reactions were run in triplicate on an Applied Biosystems 7500 thermocycler using the Fast Real-Time PCR program, consisting of an initial incubation of 20 seconds at 95°C, followed by 40 cycles of denaturation (3 secs at 95°C) and annealing/extension (30 secs at 60°C). Triplicate Ct values were averaged and used to determine the relative gene expression of *PD-1*, *PD-L1* and *PD-L2* by the comparative Ct method.[18] For each sample, mean Ct values for each target were first normalized internally to 18S rRNA expression (yielding ΔCt), then compared to the same target's mean normalized expression in melanocytoma controls (yielding $\Delta\Delta$Ct). For display, results were calculated as a fold change relative to mean expression in melanocytoma ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry

Immunohistochemistry was performed on 10 melanoma cases using antibodies against CD79a, CD3, and IBA1. Cases were selected in two groups according to the relative expression of *PD-1* detected by RT-qPCR analysis: the 5 cases with the lowest and the 5 cases with the highest relative expression of *PD-1*. Each of these IHC groups contained 3 cases of oral melanoma and 2 cases of cutaneous melanoma. Sections of 5-µm thickness from each formalin-fixed paraffin-embedded tissue block were deparaffinized and stained for each cell marker. Monoclonal antibodies recognizing CD79a (Biocare Medical clone HM47/A9; 1:250) and CD3 (Dako clone A0452; 1:100) and polyclonal antibodies recognizing IBA1 (FUJIFILM Wako #019-19741; 1:1000) were used at the dilutions indicated. Universal Alkaline Phosphatase Red Detection kit (Ventana UltraView) was used as secondary antibody, and visualization of antibody was obtained with Fast Red chromogen incubation. Slides were processed on an automated Ventana Benchmark XT using standard and previously-validated protocols at Virginia Tech Animal Laboratory Services, an AAVLD-accredited veterinary diagnostic laboratory. Positive controls for each cell marker were tissues from colon and lymph nodes. Sections of melanoma tissue stained without addition of primary antibody served as negative controls. Hematoxylin was used as a counterstain.

Blinded assessment and characterization of the ten cases were performed by a single board-certified veterinary pathologist estimating the percentage of positive cells infiltrating the tumor averaged over ten HPF (2.37 mm^2).

Statistical Analysis

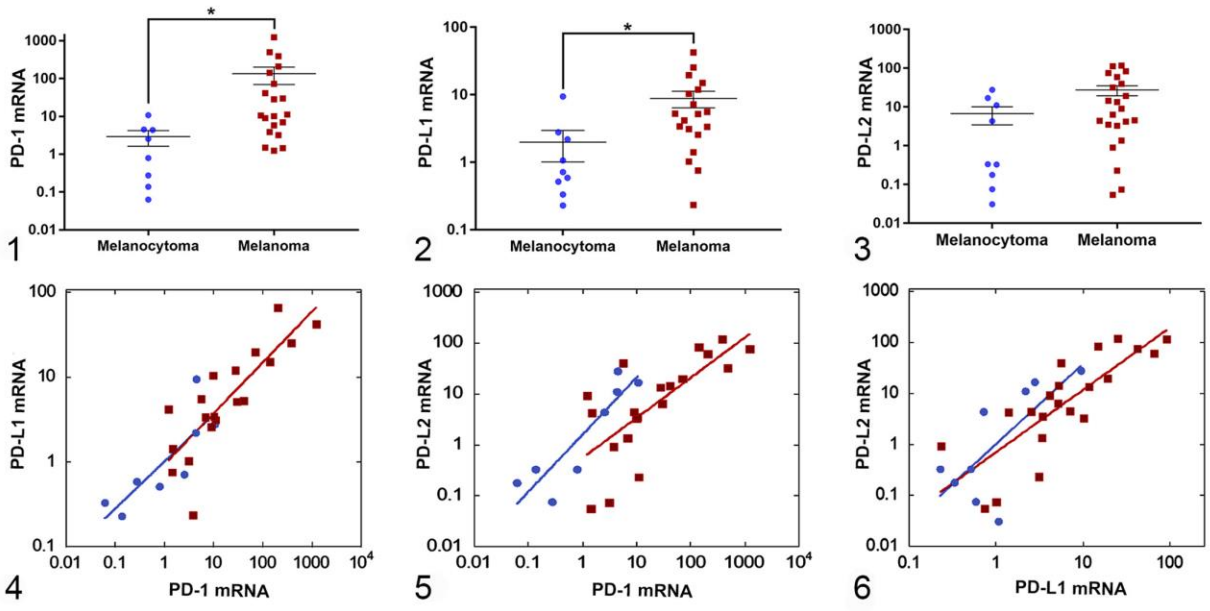
Normalized expression of individual checkpoint molecules (as ΔCt) in melanocytoma and melanoma samples were compared by two-tailed Student's T test using SAS 9.4, after

confirming normality of distribution. The relationships between expressions levels of paired checkpoint molecules (e.g, *PD-L1 vs. PD-1*) within each tumor type were examined by least-squares linear regression analysis of normalized (ΔCt) values and analysis of covariance of curve slopes. Mitotic indices and the densities of $CD3^+$, $IBA1^+$, and $CD79a^+$ cells in stained sections of low *vs.* high *PD-1*-expressing cases were compared by two-tailed Student's T test. In all cases, *p*-values <0.05 were considered statistically significant. The data analyzed in this study are available upon request to the corresponding author.

Results

Checkpoint molecule mRNA expression in melanoma relative to melanocytoma.

RT-qPCR analysis was performed targeting *PD-1*, *PD-L1*, and *PD-L2* using total RNA extracted from 31 formalin-fixed, paraffin embedded melanoma (n=22) and melanocytoma (n=9) tissue blocks. Results for each specimen are expressed as checkpoint molecule mRNA normalized internally to 18S rRNA and relative to mean normalized expression in melanocytoma ($2^{-\Delta\Delta Ct}$; Figures 1-3). Measured expression of all three checkpoint molecules was higher in melanomas relative to melanocytomas; based on 18S rRNA-normalized (ΔCt) values for each marker, the expression differences reached statistical significance for *PD-1* (*p*=0.0012) and *PD-L1* (*p*=0.0043) but not for *PD-L2* (*p*=0.0633, two-tailed Student's t-Test).

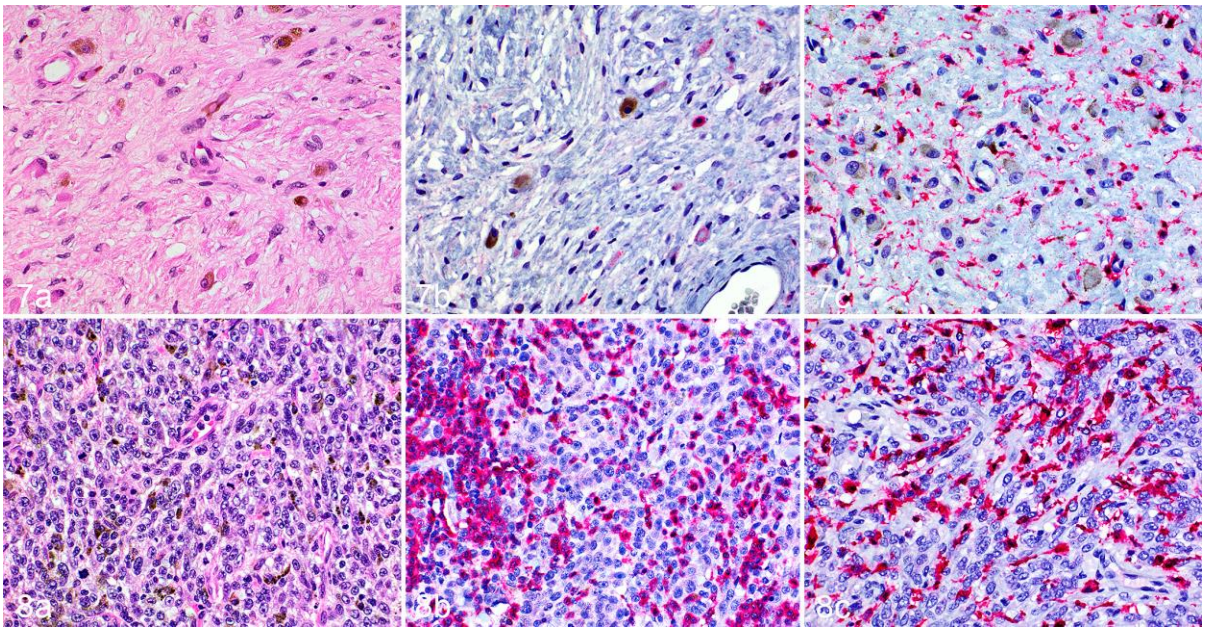


Figures 1–6. Expression of *PD-1*, *PD-L1*, and *PD-L2* mRNAs in canine melanoma and melanocytoma. Each symbol represents checkpoint molecule mRNA expression in an individual melanoma or melanocytoma case, shown as a ratio ($2^{-\Delta\Delta Ct}$) to the mean normalized expression in melanocytoma. **Figures 1–3**. Higher normalized expression of *PD-1* ($P = .0012$, 2-sided *t* test), *PD-L1* ($P = .0043$), and *PD-L2* ($P = .063$) mRNAs were observed in melanomas (red) when compared to melanocytomas (blue). Mean ± SD for $2^{-\Delta\Delta Ct}$ values. **Figures 4–6**. Analysis of covariance of mRNA expression revealed direct positive relationships between expression levels of all paired checkpoint molecules ($P < .05$) in both melanocytomas (blue) and melanomas (red).

To ascertain whether elevation of expression of multiple checkpoint molecules occurred in the same samples, plots of *PD-L1* vs *PD-1*, *PD-L2* vs *PD-1*, and *PD-L2* vs *PD-L1* normalized expression were prepared (Figures 4-6). The slopes of all curves were non-zero for both melanocytoma and melanoma (analysis of covariance $p<0.05$), indicative of positive relationships between expression of the paired checkpoint molecules. For example, relatively higher expression of *PD-1* was associated with relatively higher expression of *PD-L1* in the same sample. While this relationship was observed in all of the comparisons for both the melanocytoma and melanoma groups, slopes of the regression lines did not differ between the two tumor types.

Immunohistochemistry

In order to examine the potential for contribution by infiltrating lymphocytes to the observed *PD-1* expression in melanoma, immunohistochemistry was performed to identify T cells ($CD3^+$), macrophages and monocytes ($IBA1^+$) and B cells ($CD79a^+$) (Figures 7-8). This analysis focused on two subgroups of the melanoma samples: the 5 with the highest observed relative *PD-1* expression and the 5 with the lowest expression (Figure 1). Although these groups differed markedly in *PD-1* expression, they were

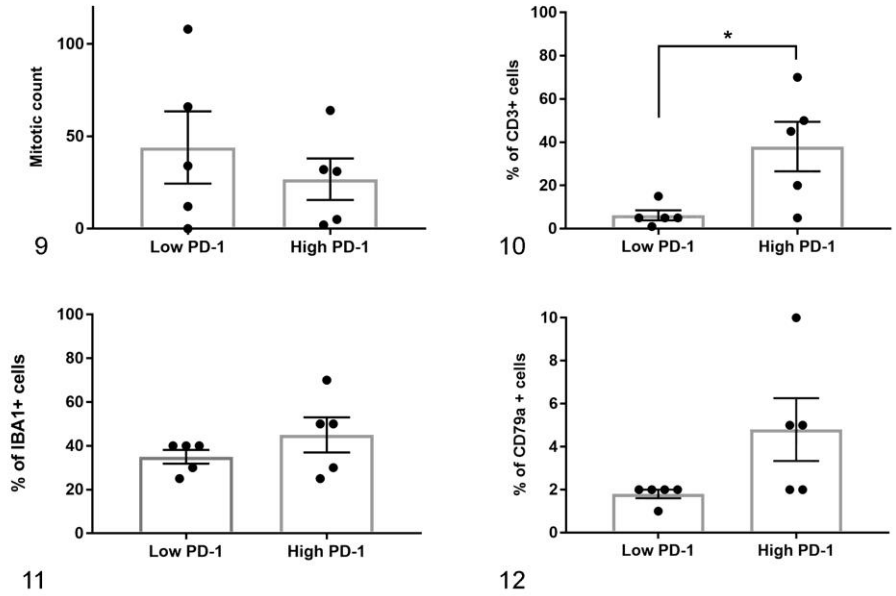


Figures 7–8. Oral melanoma, dog. Compared to a tumor with low *PD-1* expression (**Figure 7**), a tumor with high *PD-1* expression (**Figure 8**) has greater cellularity (a, hematoxylin and eosin), greater abundance of immunoreactivity for CD3 (b), and comparable immunoreactivity for IBA1 (c).

similar in diagnostic features, with the low *PD-1* expressors showing a numerically higher mean mitotic index that was not statistically significant (Figure 9). Each group consisted of 3 oral and 2 cutaneous melanomas.

Infiltration of the respective immune cell types in high and low *PD-1* expressors was estimated by calculating the number of cells positive for each marker as a percentage of total cell numbers, averaged over 10 HPFs (Figures 10-12). Markedly higher relative abundance of $CD3^+$

cells was observed in the group with higher *PD-1* expression when compared with the group with lower expression (38.0±11.5% *vs* 6.2±2.3%, mean ± SEM; p = 0.026; Figure 10), whereas infiltration of IBA1$^+$ cells showed no evident relationship with *PD-1* expression (45.0±8.1% *vs* 35.0±3.2%; p = 0.28; Figure 11). Although there was a small numerical difference observed between in mean abundance of CD79a$^+$ cells in high- *vs* low *PD-1* expressors (4.8+1.5% *vs* 1.8±0.2%), these cells were sparse in both groups and comparison of their means did not indicate a statistically significant difference (p=0.077; Figure 12).



Figures 9–12. Quantification of mitotic figures and immunolabeling for CD3 (T cell marker), IBA1 (macrophage marker), and CD79a (B cell marker) in melanomas with high and low expression of *PD-1* mRNA ("Low PD-1" and "High PD-1") (means ± SEM). A significantly higher percentage of CD3$^+$ cells infiltrating melanomas was observed in the high *PD-1* group (*P* = .026; **Figure 10**), whereas percentages of infiltrating IBA1$^+$ cells were similar in the low and high *PD-1* melanoma groups (*P* = .28; **Figure 11**). Percentages of CD79a$^+$ cells were low in both *PD-1* high and low groups (*P* = .077; **Figure 12**).

Discussion

In this study, we evaluated the expression of mRNAs encoding molecules of the PD axis, specifically PD-1, PD-L1 and PD-L2, in canine melanoma. Expression of these markers in melanoma was compared to that in melanocytoma, a benign neoplasm derived from

melanocytes. Our study of checkpoint molecule expression was limited to analysis of mRNAs extracted from FFPE tissues for several reasons, the principal one being our ability to devise suitably sensitive and quantitative assays based on knowledge of the relevant mRNA sequences. While it would be desirable to confirm expression changes at the protein or cell-type level, approaches such as anti-checkpoint molecule IHC or *in situ* hybridization were outside the scope of this study. Moreover, validated anti-canine checkpoint molecule antibodies are not available that would allow us to adequately interpret an IHC-based analysis of FFPE tissues. However, other investigators have reported a correspondence between transcript and protein for these targets in tumor tissue.[17,9] We chose melanocytoma as a control tissue for comparison of checkpoint molecule expression owing to its shared melanocytic origin, but markedly different disease outcome, relative to melanoma. Since we wish to examine the relationship between checkpoint molecule expression and disease progression, we maintain that melanocytoma is a more appropriate control tissue than normal skin for our study.

Higher mean levels of *PD-1, PD-L1, and PD-L2* mRNAs were detected in the melanoma group compared to melanocytomas, reaching statistical significance for *PD-1 and PD-L1*. Since PD-L1 and PD-L2 can be expressed by antigen presenting cells as well as neoplastic cells, immunohistochemistry was performed to evaluate the potential contribution of lymphocytes and macrophages to checkpoint molecule expression in the tumor microenvironment. Our results revealed a significantly higher proportion of cells with positive cytoplasmic immunoreactivity using antibodies against the T cell marker CD3 in the group of melanomas with higher expression of *PD-1*, whereas the number of cells with positive cytoplasmic immunoreactivity using antibodies against IBA1 (macrophages) was similar in *PD-1* High and *PD-1* Low melanoma groups. Additionally, large numbers of $IBA1^{+}$ cells were seen in both high and low

PD-1 expressing melanomas, including those with a relatively low number of $CD3^+$ cells. The elevation of *PD-L1* and *PD-L2* in these same high *PD-1*-expressing tumors suggests a modulated co-expression of the PD axis molecules in melanoma involving the neoplastic cells and T lymphocytes, rather than macrophages.

Our results are consistent with the expectation that the higher *PD-1* expression in the study samples is contributed by the more abundant T cell population; confirmation of this conclusion awaits cellular localization when appropriate antibodies are available for IHC. Our findings of TILs in all 10 melanoma cases examined are consistent with those of other investigators[28] who reported the presence of TILs (as $CD3^+$ and $CD20^+$ cells) in a high percentage of melanomas (78.1% of oral cases and 64.7% of cutaneous cases). It should be noted that our melanoma group included both oral (16 cases) and cutaneous (6 cases) melanomas. While these forms of melanoma may differ in disease progression and likely oncogenic origin, we did not observe pronounced differences with regard to checkpoint molecule expression or T cell infiltration (data not shown). However, our samples sizes did not allow their rigorous comparison.

The lack of effective therapies for canine melanoma emphasizes the importance of comparative oncology to understand common mechanisms between the species and the potential value of immunotherapy development. Increased PD-1 and PD-L1 expression has been previously associated with shorter survival times and lymph node metastasis in human lung adenocarcinoma[45] and urothelial carcinoma.[42] Increased PD-L2 expression has been linked to increased cell invasion in human patients with colorectal cancer,[14] and increased PD-1, PD-L1 and PD-L2 expression was found to be associated with metastasis when compared with primary tumors in renal carcinoma.[3] These results also correlate with studies in canine cancers, where

PD-L1 was overexpressed in melanoma, osteosarcoma, histiocytic sarcoma, mast cell tumor, mammary carcinoma and prostatic cancer.[20] Additionally, a single study demonstrated overexpression of *PD-L2* in dogs with B cell lymphoma.[39] Since the expression of checkpoint molecules has been related to an increase in tumor infiltrating lymphocytes[8] and a better response to immunotherapy in humans,[44,6] our findings suggest that immunotherapies targeting the PD-1 axis have potential for efficacy in canine melanoma and other cancers, as has been demonstrated.[21]

The majority of studies evaluating checkpoint molecules have compared their expression between malignant neoplasms and healthy control tissue. However, only a few studies performed in human cancers have compared expression between malignant and benign neoplasms of the same cellular origin.[7,32] In the current study, we showed that checkpoint molecule mRNA expression is higher in malignant tumors when compared to the benign counterpart. To our knowledge this is the only study in dogs evaluating the expression of checkpoint molecules using such a control, and brings interesting new insight into the mechanisms of tumor development and anti-tumor immunity.

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CHAPTER IV
INHIBITORY CHECKPOINT MOLECULE EXPRESSION IN CANINE SOFT TISSUE SARCOMA

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Abstract

Canine Soft Tissue Sarcomas (STS) are common neoplasms in dogs and are often considered immune deserts. Tumor infiltrating lymphocytes are sparse in STS and when present tend to organize around blood vessels or at the periphery of the neoplasm. This pattern has been associated with an immunosuppressive tumor microenvironment linked to overexpression of molecules of the PD-axis. PD-1, PD-L1 and PD-L2 expression correlates with malignancy and poor prognosis in other neoplasms in humans and dogs, but little is known about their role in canine STS, their relationship to tumor grade, and how different therapies affect their expression.

The objective of this study was to evaluate the expression of checkpoint molecules across STS tumor grades and subsequent to tumor ablation treatment. Gene expression analysis of checkpoint molecules was performed by qRT-PCR in soft tissue sarcomas that underwent tumor ablation therapy (histotripsy) and from FFPE specimens of STS of different grades from the Virginia Tech Animal Laboratory Services archives. The expression of PD-1, PD-L1 and PD-L2 was detected in untreated STS tissue representing grades 1, 2, and 3. Trends of decreased expression of all markers were observed in tissue sampled from the treatment interface relative to untreated areas of the tumor. The relatively lower expression of these checkpoint molecules at the periphery of the treated area may be related to liquefactive necrosis induced by the histotripsy treatment, and would potentially allow TILs to infiltrate the tumor. The relative trend of increases of these checkpoint molecules in tumors of a higher grade is consistent with previous reports that associate their expression with malignancy.

Introduction

After several years relying only on conventional therapies, such as chemotherapy and radiation, to treat cancer, the introduction of immunotherapies has revolutionized the field, promising numerous advantages for people(1) and dogs.(2) Among the most successful immunotherapies in humans are the ones that aim to block the interaction between Programmed Death -1 (PD-1), a suppressive immunoreceptor expressed in exhausted T cells, and its ligands (PD-L1 and PD-L2).(3-5) PD-L1 is expressed at high levels among malignant tumor cells and, upon the binding of this ligand with the PD-1 receptor on T cells, leads to immunotolerance, allowing tumor progression and increased rate of metastasis.(4, 6-9) Over the last several years, attempts made to develop similar therapies for canine melanoma produced reports of decreases in tumor size, prolonged survival times, and complete remission.(10, 11) Immunotherapies have a great effect on numerous types of tumors in humans, and their efficacy has a strong correlation with the immune profile of the Tumor Microenvironment (TME).(8, 12) Better outcomes are usually associated with "hot" tumors, defined by increased levels of T cell infiltration and immune stimulatory cytokines, while these therapies have less efficacy in "cold" tumors, which maintain an immunoinhibitory TME, with low levels of T cell infiltrates.(2, 12, 13)

Soft Tissue Sarcomas (STS) are fairly common in dogs, representing up to 15% of all cutaneous or subcutaneous tumors.(14) They are a diverse group of neoplasms of mesenchymal origin with similar biological behavior: resection is difficult, the rate of recurrence is high, and metastasis can develop in up to 30% of the cases. Factors that contribute to poorer prognosis are a higher mitotic count, increased pleomorphism, areas of necrosis, and a tumor size larger than 5 cm.(14, 15) STS are usually graded according to guidelines proposed by Trojani et al and modified by Dennis et al 2011. Briefly, a score from 1 to 3 is given based on differentiation of

the neoplasm, another score from 1 to 3 based on mitotic count, and a score from 0 to 2 based on degree of necrosis within the neoplasm.(16, 17)

Furthermore, STSs have long been considered immune deserts and thought to be poor candidates for immune therapies.(18-20). However, further studies reported that infiltration of Tumor Infiltrating Lymphocytes (TILs) depend on tumor type and that highly mutated tumors with elevated Copy Number Alterations have a higher immunogenicity. (19, 21, 22) In fact, expression of PD-L1 has already been described in human STS, where an association with poor prognosis and shorter survival times was reported.(23-26) In contrast, evaluation of the expression of this checkpoint molecule in canine cancers is scare. (11, 27, 28) In STS there is only one report, in which PD-L1 positivity was noted in two of eleven hemangiosarcomas, six of eight malignant fibrous histiocytomas, four of eleven malignant nerve sheath tumors, four of ten hemangiopericytomas, and four of six fibrosarcoma.(29) PD-L1 has also been reported in serum of a group of nine dogs with malignant mesenchymal tumors whose diagnosis included osteosarcoma, liposarcoma, cutaneous hemangiosarcoma, fibrosarcoma, and leiomyosarcoma.(30)

Regardless of the immune status of the tumor, most therapies aim not only to destroy cancer cells, but also to release tumor antigens to the TME to promote its recognition by the immune system, both at the primary site and in circulation, known as abscopal response.(31-34) This approach has been successful in STS, as increasing the immunogenicity of this rather “cold” tumor amplifies T cell recognition and later tumor destruction, giving combined immunotherapies advantage over monotherapies.(8, 35, 36)

Alternative local tumor ablation therapies have been developed over the last 6 years. These therapies aim for a less invasive technique, targeting cancer cells and sparing as many

healthy cells as possible.(37, 38) Tumor ablation therapies include radiofrequency ablation, microwave ablation, laser, high-intensity focused ultrasound, cryoablation, irreversible electroporation, and histotripsy.(38) The first six rely on extreme temperatures to ablate the tumor tissue, whereas histotripsy is one of the first non-ionizing and non-thermal ablation image-guided therapy. Microsecond soundwave pressure pulses cavitate microbubbles within the tissue, generating mechanical stress, and liquifying neighboring cancer cells with precise margins and sparing normal critical structures such as nerves and large blood vessels.(34, 39) The homogenized and liquified tissue contains native tumor antigens with retained antigenicity, which promote the abscopal immune response and can inhibit the process of metastasis by increasing recognition of both antigens by dendritic cells and Damage Associated Molecular Patterns by Pattern Recognition Receptors including Toll-like receptors that feed the cytokine cascade that promote inflammation. (33, 34, 37, 40) After the initial response by the innate immune system, CD8+ cytotoxic and CD4+ helper T cells are activated and can travel to the tumor site via the circulation to interact with the tumor cells.(37, 39, 41)

Although the immune response after histotripsy has been recently described in canine cancers, reports are still limited,(42-44) especially in STS for which a sole report exists.(40) The adaptive response to histotripsy is currently under investigation, and the expression of checkpoint molecules from the PD-axis after therapy is relatively unexplored.

With the evolution of immunotherapies in human medicine, it is imperative that more efforts are made to gain insight in the treatment of veterinary cancer patients and to better understand how immunotherapies could bring answers either as sole therapy or as adjuvants to tumor ablation therapies. To our knowledge, this is the first report that describes the expression of checkpoint molecules in canine STS.

Materials and Methods

Tissue samples

Cases selected for this study consist of 2 different groups. One group includes 29 cases selected from the archives of Virginia Tech Animal Laboratory Services (ViTALS) with the diagnosis of soft tissue sarcoma. These tumor biopsies were from different privately owned dogs, submitted for routine histopathological evaluation. All grade 1 and 2 cases were from 2021. Grade 3 cases were from 2019 (3), 2020 (1), and 2021 (5). The diagnosis of soft tissue sarcoma was further classified as grade 1, 2, and 3 according to guidelines proposed by Trojani et al and modified by Dennis et al 2011. Briefly, a score from 1 to 3 was assigned based on differentiation of the neoplasm, another score from 1 to 3 was assigned based on mitotic count, and a score from 0 to 2 was assigned based on degree of necrosis within the neoplasm. Lymphovascular invasion was morphologically assessed. Following this classification system, 10 cases were assigned as grade 1, 10 as grade 2, and 9 as grade 3.

The second group consisted of cases from a clinical study performed by Ruger et al. 2022,(40) that aimed to determine the safety and feasibility of histotripsy therapy in ten canine patients with soft tissue sarcoma. The histotripsy inclusion criteria were patients with a cytological or histological diagnosis of soft tissue sarcoma, and no prior treatment. Recurrent tumors were not excluded. Excisional biopsies were taken four to six days after histotripsy treatment and submitted for evaluation by a veterinary pathologist with extensive experience evaluating ablated tumor tissue, and tissue sections were stored as formalin-fixed paraffin-embedded (FFPE) blocks. Tissue sections were collected from the area of the treatment interface, and from untreated areas of the tumor. For the current study, one tissue section was selected at the tumor interface area and one from the untreated area for each patient.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

Ten scrolls of 10 um in thickness each were serially cut from FFPE blocks from each of the 49 cases. RNA extraction and cDNA synthesis were performed using commercially available kits (Qiagen RNA extraction kit, and Life Technologies High Capacity cDNA kit respectively) according to manufacturer's protocols. RT-qPCR analysis was performed using previously validated primers and probes as described in Stevenson et al 2021.

For each sample, mean Ct values for each target were first normalized internally to 18S rRNA expression (yielding ΔCt), then compared to the same target's mean normalized expression in Grade 1 tumors or Untreated tumors (yielding ΔΔCt). For display, results were calculated as a fold change relative to mean expression in the group used as control ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry

Immunohistochemical analysis was performed on all 10 cases from the histotripsy clinical study, both at the treatment interface and untreated tissue sections, using antibodies against CD3 (pan T-cell marker) and FoxP3 (a regulatory T-cell marker). Sections of 5 um from each FFPE tissue section were deparaffinized and stained with a monoclonal antibody for CD3 (Dako clone A0452; 1:100), and anti-HuFoxP3 (eBio clone 7979; 1:100). Universal Alkaline Phosphatase Red Detection kit (Ventana UltraView) was used as secondary antibody, and visualization of the antibody was obtained with Fast Red chromogen incubation. Slides were processed on an automated Ventana Benchmark XT using standard and previously validated protocols at Virginia Tech Animal Laboratory Services, an AAVLD-accredited veterinary diagnostic laboratory. Positive controls for each cell marker were tissues from colon and lymph

nodes. Sections of soft tissue sarcoma tissue stained without addition of primary antibody served as negative controls. Hematoxylin was used as a counterstain.

Assessment and characterization of the 16 tissue sections were performed in a blinded fashion by a single board-certified veterinary pathologist counting individual immunopositive cells infiltrating the tumor averaged over 10 high-power fields (HPF; 2.37 mm^2) per each tissue section.

Statistical analysis

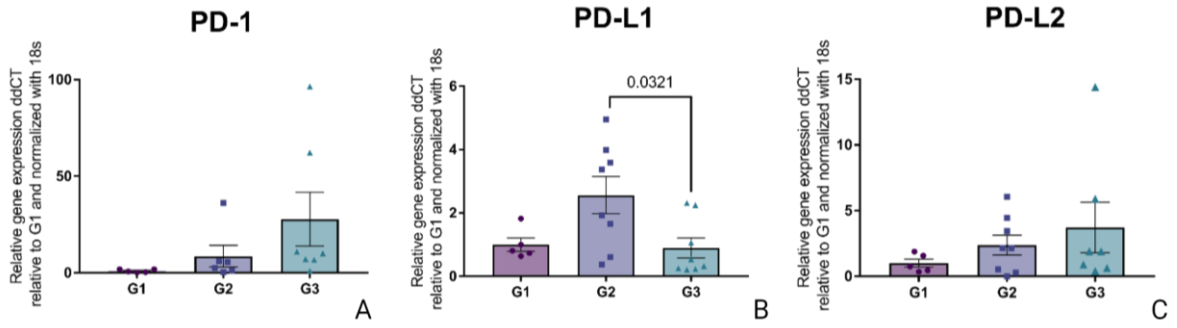
Normalized expression of individual checkpoint molecules (as $2^{-\Delta\Delta Ct)}$) in soft tissue sarcomas of grades 1, 2 and 3, and from treated and untreated tumor sections were compared using one-way ANOVA test to detect differences between groups. Tukey post-hoc test was used for multiple comparison between groups using single pooled variance. Comparisons of degree of differentiation were compared to the gene expression of PD-1, PD-L1 and PD-L2 using an ANOVA test. Results were considered statistically significant when *P* values <0.05. All statistical analysis was performed using Prism 9.4 (GraphPad software).

Results

Expression of inhibitory checkpoint molecules in soft tissue sarcoma of grades 1, 2 and 3.

RT-qPCR analysis was performed targeting *PD-1, PD-L1,* and *PD-L2* using total RNA extracted from 29 formalin-fixed, paraffin embedded soft tissue sarcoma tissue blocks. Results for each specimen are expressed as checkpoint molecule mRNA normalized internally to 18S rRNA and relative to mean normalized expression in Grade 1 tissue sections ($2^{-\Delta\Delta Ct}$).

There was an upward tendency of the measured expression of PD-1 and PD-L2 tissue sections from soft tissue sarcoma of higher grades compared to grade 1 (Figure 1 and 3). Interestingly, PD-L1 relative expression was significantly higher in grade 2 when compared to grade 1, but expression remained similar between grades 1 and 3 (Figure 2).



Figures 1: Expression of PD-1 (A), PD-L1 (B), and PD-L2 (C) mRNA in canine soft tissue sarcoma of grade I, II, and II. Each column represents a different grade, and each point represent an individual case. A trend of increase was observed in the expression of PD-1 and PD-L2. A statistically significantly higher expression was observed for the expression of PD-L1 in grade II soft tissue sarcoma, when compared to those of grade III (P=0.0321).

To evaluate the relationship between anaplasia of the soft tissue sarcomas and the expression of these inhibitory checkpoint molecules, all tissue sections were reclassified based on degree of differentiation of the tumor. All sections were evaluated based only on degree of differentiation using the suggested score system by Dennis et al, 2011. Samples were given the score 1=Mild when the cells resembled normal tissue; score 2=Moderate when there was an identifiable pattern, but only modest resemblance to normal tissue; and score 3= Significant when the tumor was undifferentiated. After this classification, these scores were compared to their expression of PD-1, PD-L1 and PD-L2.

The measured expression of PD-1 and PD-L2 had an upward tendency in tumors with a higher degree of differentiation (Figure 4 and 6). Whereas PD-L1 gene expression remained similar regardless of the degree of differentiation (Figure 5).

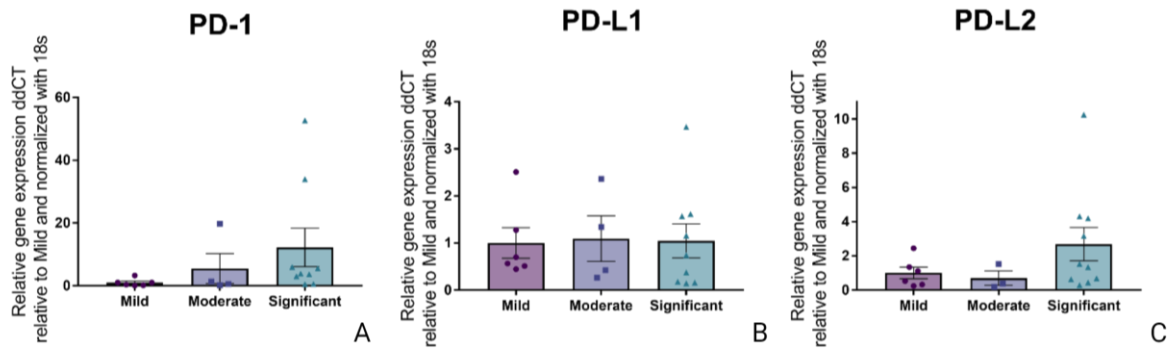
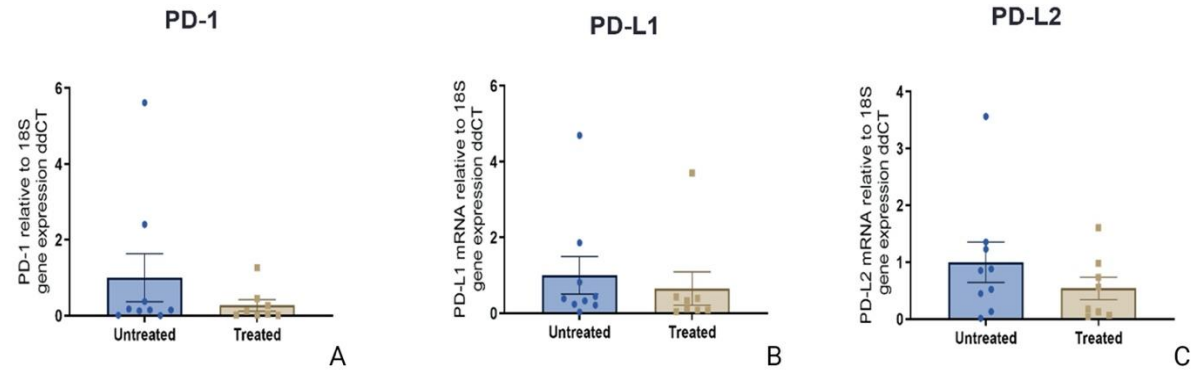


Figure 2: Expression of PD-1 (A), PD-L1 (B), and PD-L2 (C) mRNA in canine soft tissue sarcoma with mild, moderate, and significant degree of undifferentiation. PD-1 and PD-L2 expression is subjectively higher in soft tissue sarcomas with more significant undifferentiation. PD-L1 expression remained similar regardless differentiation grade of the soft tissue sarcoma.

Expression of inhibitory checkpoint molecules in soft tissue sarcomas from a clinical trial using histotripsy.

Measurement of PD-1, PD-L1, and PD-L2 gene expression was performed as described above using RNA extracted from 20 tissue sections, 10 from treatment interface areas, and 10 from untreated areas of soft tissue sarcomas that undergo histotripsy treatment (Figures 7-9). Each specimen's results are calculated as checkpoint molecule mRNA normalized internally to 18S rRNA, and then as relative to the mean normalized expression in the untreated tissue sections ($2^{-\Delta\Delta Ct}$). All 3 checkpoint molecules measured expression were lower in the treatment interface when compared to the untreated areas of the tumor.



Figures 3: Expression of PD-1 (A), PD-L1 (B), and PD-L2 (C) mRNA in soft tissue sarcomas treated with histotripsy. It shows comparison between PD-axis gene expression in tissue sections from the treatment interface, and untreated areas of the neoplasm. PD-1, PD-L1, and PD-L2 expression is subjectively lower in tissue sections from the treatment interface when compared to untreated areas of the neoplasm.

Immunohistochemistry

In order to evaluate the local effects of the histotripsy therapy over the TILs, immunohistochemistry was performed to identify T lymphocytes using CD3 as a marker and T regulatory cells using Foxp3 (Figure 10).

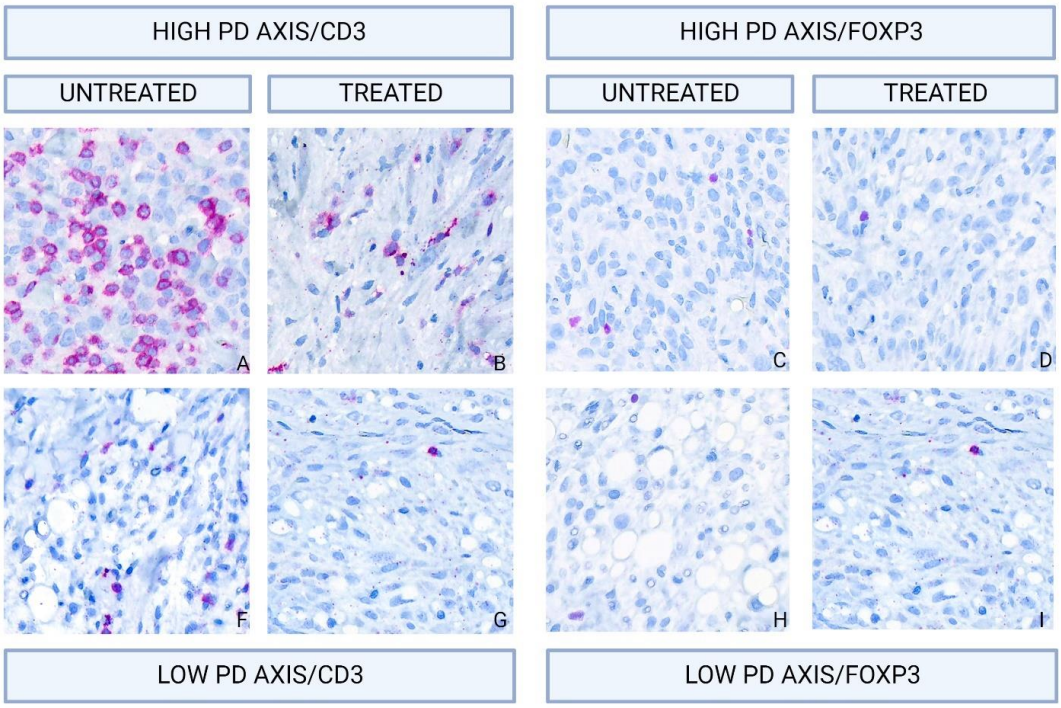
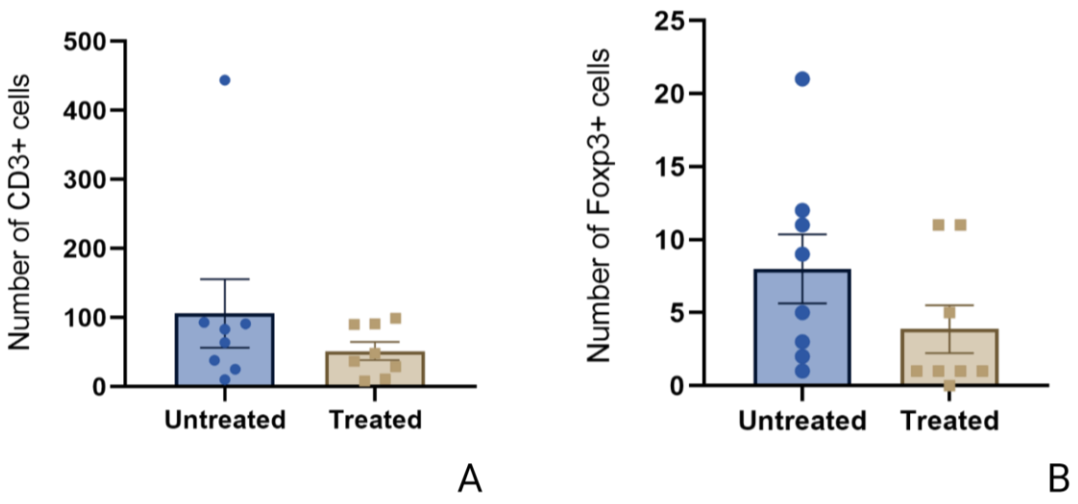


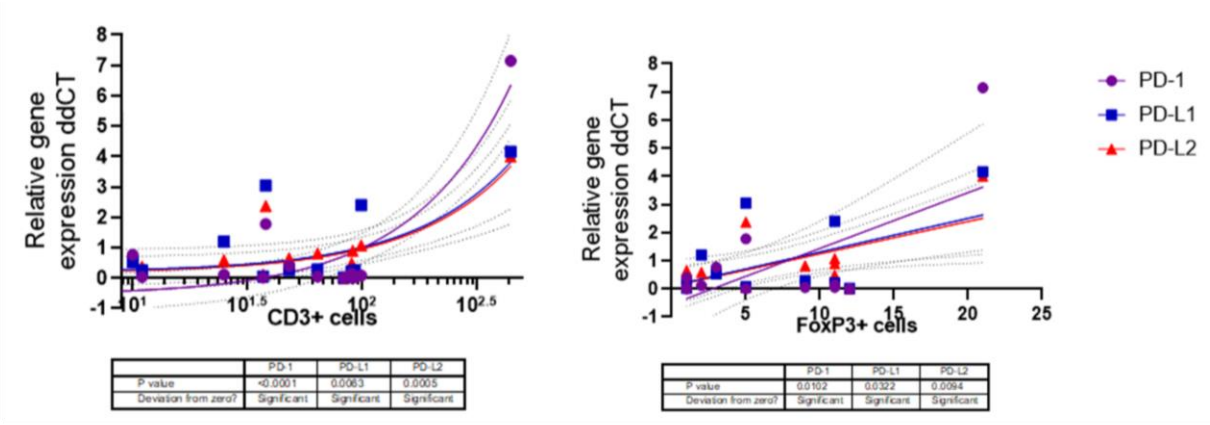
Figure 4: Micrographs from IHC analysis. Images on the top row (A to D) shows a patient with high expression of the PD-axis, and high infiltration of T lymphocytes. Images on the bottom row (F to I) show a patient with low expression of the PD-axis and low infiltration of T lymphocytes. CD3+ cells are highlighted in images A, B F, G, where a higher infiltration of CD3+ positive cells are seen in image A, untreated tumor section. Similarly, Foxp3+ cells are highlighted in images C, D, H, I, where higher numbers of cells were seen in image C.

Although there were no statistically significant changes, evidence of CD3+ and Foxp3+ cell infiltration was lower in sections from the treatment interface when compared to the untreated sections (P= 0.97, and 0.23, respectively) (Figures 11-12).



Figures 5: Number of CD3+ (A) and Foxp3+ (B) cells in sections from untreated areas of the neoplasm compared to counts in sections representing the treatment interface.

Analysis of covariance was done to assess the relationship between the expression of checkpoint molecules and the infiltration of CD3, and Foxp3 positive cells. All coefficients had a significant deviation from zero, indicative of a positive relationship between the expression of these checkpoint molecules and the infiltration of both CD3 and Foxp3 positive cells (Figure 13-14).



Figures 6: Linear regression graphs. Displaying a positive relationship between infiltration of CD3+ (A), Foxp3+ (B), and gene expression of PD-axis. (CD3: PD-1, PD-L1, and PD-L2 R squared = 0.79; 0.44; and 0.61 respectively. Foxp3: 0.40; 0.30; and 0.41 respectively).

Discussion

The present study evaluated the gene expression of checkpoint molecules from the PD-axis (PD-1, PD-L1, and PD-L2) from 2 cohorts of FFPE tissue sections.

The first group comprised STS tissue sections of grades I, II, and III from ViTALs archives, where we expected an increase in the expression of checkpoint molecules in soft tissue sarcomas of higher grades. After analyzing RT-PCR data, we report a trend of an increase in the expression of PD-1 as the STS grade increased. The expression of PD-L1 was higher in grade II STS when compared to STS of grade I, and significantly higher when compared to those of grade III. In addition, those grade III STS had higher expression of PD-L2 despite their low levels of PD-L1. PD-L2 expression has a similar trend of increase as the STS grade increases. Although this trend of increase was anticipated by our hypothesis, STS sarcomas of grade III maintained levels of PD-L2 comparable to grade II, while PD-L1 returned to levels comparable to grade I.

The importance of PD-L2 is evidenced by the analysis of the gene expression of the PD-axis among STS with different degrees of undifferentiation. STS with a higher degree of undifferentiation had higher expression of PD-1 and PD-L2. Interestingly, the expression of PD-L1 remained similar among the 3 undifferentiation classifications. Altogether, these findings suggest potentially different mechanisms that regulate the expression of these two ligands and emphasize the role that PD-L2 could play in malignant neoplasia.

Higher expression of PD-L2 has been reported in human reproductive cancers,(45) oesophageal carcinomas,(46) some soft tissue sarcomas,(47) osteosarcoma, (48) among others, and is associated with tumor cell survival, migration, and resistance to chemotherapy.(22, 47, 49, 50) PD-L2 does not require a conformational change to bind PD-1, as does PD-L1, with a higher

affinity for PD-1 than PD-L1. (51) Yet, PD-L2 has been less thoroughly investigated than has PD-L1, on which the majority of the published data is focused. Although the reports available on PD-L2's role in therapy response and prognosis are contradictory, more recent studies of human neoplasia associate its expression with a poor prognosis.(50, 52-54) More importantly, the expression of this checkpoint molecule has been overlooked in canine neoplasia, with only a sole report by our group in canine melanoma.(27) These results show a trend of increase for both the receptor PD-1 and the PD-L2 ligand, which supports previous reports that suggest that soft tissue sarcomas with higher degree of malignancy often have a higher infiltration of immune cells, but still are considered immunosuppressed. (19, 21, 22) Nevertheless, more data is needed to support these findings and to determine the role that PD-L2 may play in the TME and its association with a poorer prognosis in canine neoplasia.

Major efforts have been made to develop different therapeutic approaches to treat cancer. Still, reports on how these therapies affect the immune system and modulate the TME are scarce in the human medical literature, and in some cases even suggest adaptation of the neoplasms with upregulation of the expression of PD-L1 after treatment.(55-57) The second cohort evaluated in this study were archived pre- and post-treatment tissue sections from a clinical trial of histotripsy in ten canine patients with STS. Tissue sections were collected from the treatment interface and untreated areas of the tumor. We predicted that the expression of checkpoint molecules would be lower on sections obtained from the treatment interface, an expectation that was borne out for the three checkpoint molecules analyzed.

In the IHC analysis, lower numbers of CD3+ and Foxp3+ cells were observed in tissue sections taken from untreated areas of the neoplasm. Additionally, our IHC analysis shows a positive relationship between the infiltration of CD3+ and Foxp3+ cells with the expression of

checkpoint molecules from the PD-axis, as previously reported in canine melanoma.(27) Evaluation of IBA1+/CD206+ (proinflammatory) macrophages was performed by Ruger et al, reporting intensification of these cells towards the treated area in sections taken from the treatment interface.(40) The authors also reported that macrophages at both the treatment interphase and untreated areas were devoid of iNOS, which are usually associated with high expression of PD-L1 through the action of cytokines such as Tumor Necrosis Factor-alpha.(58-60) Considering these findings, it is possible that the timeline of the study favored the evaluation of the innate rather than adaptive response. However, the focal decrease of the expression of the PD-axis would promote future infiltration of lymphocytes.

It is important to note that PD-L1 can be expressed by cells other than cancer cells, such as dendritic cells and macrophages, as part of their normal immune homeostatic activity. On the other hand, PD-L2 can be expressed by macrophages, the tumor cells, and the stroma at the TME and does not require a conformational change to bind PD-1, as does PD-L1, with a higher affinity for PD-1 than PD-L1. (51) The lack of commercially available antibodies for these checkpoint molecules for canine neoplasms limits our ability to localize it within the TME, and thus further studies are needed to determine its expression and confirm its contribution to malignant features of the tumor. However, the higher expression of checkpoint molecules in STS of higher grades suggests an important role in this process that could be further studied by follow-up of these patients that undergo histotripsy, as its higher expression is usually associated with shorter survival times and poor prognosis. The presence of CD3+ lymphocytes in untreated areas, along with the expression of the PD-axis, makes these patients potential candidates for combined therapy with histotripsy and anti-PD-1 immunotherapy.

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CHAPTER V
CONCLUSION AND FUTURE STEPS

Comparative oncology between humans and dogs helps us to understand common factors associated with the development of cancer in both species. As dogs spontaneously develop both melanoma and soft tissue sarcoma, are exposed to the same environmental hazards as humans, and have similar physiology to humans, they are excellent candidates for the future development of immunotherapies.(1) There are currently no effective systemic therapies for the treatment of canine melanoma or soft tissue sarcomas in dogs. However, for humans, the recent development of immunotherapies during the last decade has brought great advantages to the field, either as a sole therapy or in combination with conventional therapies such as chemotherapy and radiation. Immunotherapies against the checkpoint molecules from the PD-axis, whose components are highly expressed in a variety of malignant neoplasia, have been very successful in humans,(2-4) and recently in two pilot studies in dogs. (5-8) The binding of PD-1 receptor with ligands expressed on tumor cells can inhibit T cell anti-tumor effects. Among factors that contribute to the successful inhibition of this immunosuppressive interaction is the presence of TILs within the tumor, (9-21) as blocking this interaction will unleash T cells to attack the cancerous cells.

Challenges to tumor immunotherapy occur when neoplasia lacks high T cell infiltration, but tumor ablation mechanisms such as histotripsy can increase immune cell infiltration. Histotripsy is a focused ultrasound therapy, which is noninvasive and non-thermal, thus preserving the tumor antigens that will allow for later recognition by the immune system.(22-27) After an initial innate response, TILs infiltrate the TME but can still be blocked if there is expression of checkpoint molecules from the PD-axis.(23) Here is where combined therapies play an important role in improving the effectiveness of the treatment. Thus, there is a positive correlation between TILs infiltration and response to checkpoint molecule from the PD-axis

inhibition, but this infiltration will vary greatly on the nature of the neoplasm, and there can be also variation among patients with the same type of neoplasm.

Canine melanoma is a highly malignant and therapy-resistant type of cancer that affects humans and dogs.(5, 8, 28, 29) Despite being considered a highly immunogenic and often high infiltrated with TILs, the melanoma TME is usually immunosuppressed by the expression of checkpoint molecules. In contrast, soft tissue sarcomas are considered an immune desert, that is, generally lacking such robust TILs infiltration; when TILs are present, they are typically restrained at the periphery of the tumor.(30-32) However, this neoplasm is equally considered immunosuppressed, and expression of checkpoint molecules from the PD-axis has been described for humans with association to a poor prognosis as it is in melanoma.(33-36)

The research described in this dissertation aimed to characterize checkpoint molecule gene expression representing the PD-axis and TILs in two types of canine neoplasia with distinct immune profiles. First, we worked with canine melanoma tissue samples comparing them to its benign counterpart, melanocytoma. We found that there is a statistically significant increase of the expression of PD-1, and PD-L1, and a trend of increase of PD-L2, in melanoma tissue samples compared to melanocytoma tissue samples. We found that the expression of these checkpoint molecules had a positive relationship among them, and with the infiltration of TILs.

Next, we worked with canine soft tissue sarcoma samples from two different groups. The first group comprised 29 samples with soft tissue sarcomas of different grades (I, II, and II) to evaluate if there was any association between the expression of checkpoint molecules with the grade of the tumors. For this we found a trend of increase in the expression of mRNAs encoding PD-1 and PD-L2 for tumors from grade III when compared to those from grade I. The expression of PD-L1 was statistically significantly higher in grade II soft tissue sarcoma when compared to

those of grade III. In addition, PD-1 and PD-L2 expression were higher in those samples with significant degree of undifferentiation, whereas PD-L1 remained unchanged, suggesting that different mechanisms regulate these two ligands and emphasizing the role of PD-L2 in high-grade soft tissue sarcomas.

The second group studied focused on expression of these checkpoint molecules in tissue samples from canine patients that had undergone histotripsy for the treatment of soft tissue sarcoma. We noticed a trend of decrease on the expression of the PD-axis in tissue sections that were taken at the treatment interface when compared to untreated areas of the same tumor. This indicates that histotripsy induces a focal decrease in the expression of checkpoint molecules, potentially allowing future infiltration of T lymphocytes. IHC evaluation revealed higher numbers of CD3+ T cells in untreated sections, which supports the potential for combined therapies that use checkpoint inhibitors to unleash T cell's effect in the TME. We reported a positive relationship between the TILs and the expression of the three checkpoint molecules. These findings are consistent with previous work on canine melanoma.

Altogether, our work highlights the dog as a potential candidate for immunotherapies that block the PD-axis, and underscores the use of histotripsy as a mechanism to increase immunogenicity in cold tumors. Further work is needed in order to localize the expression of these checkpoint molecules within the TME, and to determine the mechanisms involved in the regulation of both PD-L1 and PD-L2 ligands. The role of PD-L2 in immune tolerance of tumors is relatively less well understood, but its role in mechanisms of cancer malignancy have come to light in recent years.(37, 38) (39-41) Furthermore, the therapeutic potential of immune checkpoint molecules is not limited to targeting the interaction of the native proteins in the TME,

but may extend to exogenous checkpoint proteins, free or packed into macrovesicles, for systemic administration.

Lastly, more studies in canine tumors are needed in order to identify biomarkers that allows us to not only identify future candidates for developing therapies, but also to anticipate the development of the disease and obstacles that these new treatments may bring, such as resistance to anti PD-L1 therapy. (42, 43)

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