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Canine melanoma in comparative oncology: Translate research advances to develop new diagnostic and therapeutic options

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Canine melanoma in comparative oncology: Translate research advances to develop new diagnostic and therapeutic options

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Editorial: Canine melanoma in comparative oncology: Translate research advances to develop new diagnostic and therapeutic options

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Editorial on the Research Topic
Canine melanoma in comparative oncology: Translate research advances to develop new diagnostic and therapeutic options

Canine melanoma is one of the most studied tumor of the dog, due to its high aggressiveness, especially in its mucosal (oral) form, but also because its similarity with human melanoma. Canine and human mucosal melanoma show several common characteristics, in terms of histological features, biological behavior and genetic modifications (1–3). In both species, malignant melanoma (MM) is a highly aggressive tumor of skin and mucosae, often associated with aggressive malignant behavior with a rapid invasion of surrounding normal tissues, frequent metastasis and resistance to therapy. Despite numerous advances in diagnostic and available treatment options, MM remains fatal in most cases. For this reason, there is currently an urgent need to better understand genetic and molecular mechanisms driving melanoma development and progression, to find valuable new prognostic biomarkers, and novel, effective therapeutic approaches to improve patient survival rates. The aim of this Research Topic was to provide an overview of the current advances in the diagnostic and treatment approaches for canine melanoma in comparative oncology, in light of the translating potential of most of these studies to human melanoma patients (Figure 1). Indeed, most of the research papers published on this Research Topic aimed to improve current diagnostic approaches and treatments for dogs affected by malignant melanoma, but also provided the potential of translating these results to human MM, in particular the rare form of

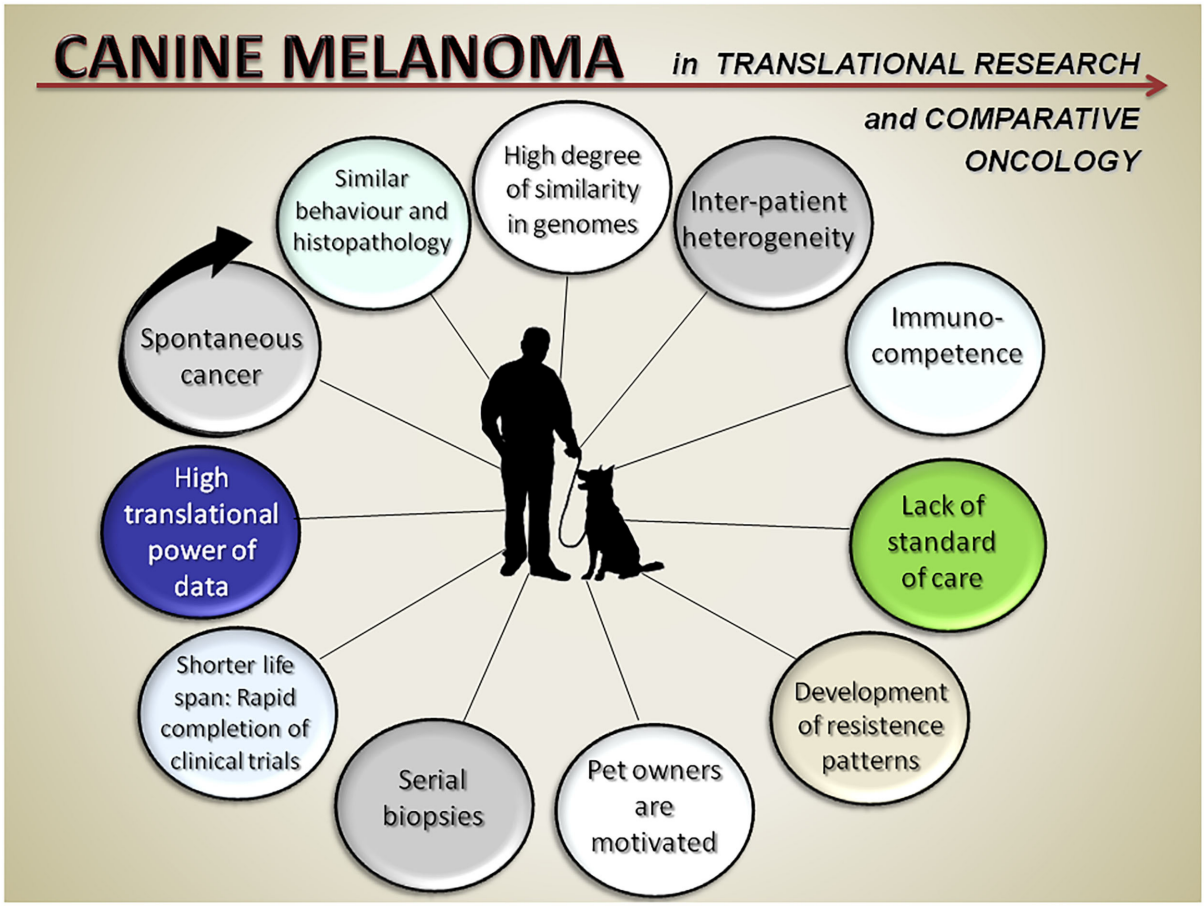

FIGURE 1
Points of strengths of the canine melanoma spontaneous model in translational research and comparative oncology.

human mucosal melanoma. The present Research Topic is made of 12 original articles of various types, with the majority being original research articles, submitted by the most prominent research groups currently working on canine melanoma all over the world. The great participation we found clearly indicates the importance of this Research Topic in the veterinary research community, mirroring the importance of the disease in the daily clinical work of both dogs and men.

We include in the following paragraphs a brief summary of the main findings reported by different authors in their 12 manuscripts that form the bulk of this Research Topic.

A perspective article is included in the present Research Topic, where authors underlined the relevance of translational research performed in last years on canine melanoma. In the paper, the most relevant knowledge on the tumor landscape of canine melanoma is reported. Furthermore, starting on the observation of the great achievements by immunotherapy in the treatment of human melanoma patients, the most promising immunotherapeutic approaches recently investigated, such as anti-cancer vaccination, are reviewed and critically discussed in the paper of Tarone et al.. A brief introduction on the importance of extracellular vesicles as valuable circulating carriers of easily accessible cancer biomarkers is also included, to discuss the potential of these novel approaches to improve the diagnosis and prognosis of canine MM.

The current status of canine melanoma treatment and diagnostics in Brazil, reported from a "Colloquium on Canine Melanoma," a meeting organized by the Brazilian Association of Veterinary Oncology (ABROVET) on December 2020, has been summarized in an opinion paper (Fonseca-Alves et al.). The success of this meeting, with more than 100 attendees, testifies the attention and interest on this Research Topic of the Brazilian oncology community.

A total of six articles that deal with the evaluation of new diagnostic and prognostic markers for canine MM have been published. Some of them have been already reviewed and included in the recently published review paper "*Diagnosis and Histopathologic Prognostication of Canine Melanocytic*

Neoplasms: A Consensus of the Oncology-Pathology Working Group" (4). Global DNA methylation has been investigated and quantified in canine melanotic and amelanotic oral MM samples and in the peripheral blood leukocytes as a novel biomarker, presuming a predominant global DNA hypomethylation in these tumors, potentially related to Ki67 score and tumor pigmentation (Scattone et al.). In their study, Tsoi et al. investigated the IHC and RNA expression patterns of a set of additional markers, besides the well-known melanocyte markers Melan A, PNL2 and TRP-1 and−2, to investigate their potential usefulness for the challenging differentiation of spindloid oral MMs from soft tissue sarcomas (STS), when junctional activity and pigmentation are absent. Authors found that TYR, CD34, and CALD1 were the most discriminatory genes in differentiating between oral MM and STS, and should be evaluated in suspected amelanotic OMMs. The gene expression of H2AFZ and survivin in formalin-fixed, paraffin-embedded canine melanoma samples has been related to malignant histological features and malignant behavior, indicating H2AFZ as a new potentially useful prognostic biomarker and confirming survivin as a useful prognostic biomarker when evaluated also at the RNA levels (Bongiovanni et al.). Leukotriene A4 hydrolase (LTA4H), and Fragile-X-mental retardation-related protein1 (FXR1), both reported as related to metastatic potential in different tumors, appeared to be highly expressed in oral melanoma, at both protein and mRNA level, but did not correlate to known histologic and immunohistochemical prognostic markers. However, their extensive expression in the investigated cases suggests that they may play a role in canine oral melanoma oncogenesis (Nordio et al.). Silveira et al. demonstrated COX-2 overexpression in both oral and cutaneous melanomas and observed that COX-2 expression correlates with established markers of poor prognosis. Porcellato et al. investigated the presence and significance of tumor-associated macrophages in canine melanocytic tumors, through the use of several IHC biomarkers (CD163, CD204, Iba1, and MAC387), revealing an association of these markers with negative prognostic histologic features and a more aggressive biological behavior.

Studies on experimental therapeutic approaches exploiting immune-, gene- or targeted therapy were received and included in this Research Topic, showing interesting and encouraging results.

More than one group of researchers is currently engaged in the study and discovery of new targets for the development of new therapeutic approaches by the use of *in vitro* models of canine melanoma. Silveira et al., by silencing Cox-2 in two melanoma cell lines, demonstrated that cellular proliferation, migration and invasion are COX-2 dependent, establishing that COX-2 is an essential driver of cellular proliferation, migration and invasion, and its expression correlated with malignant behavior in canine melanoma. Alpha-connexin carboxyl-terminal peptide (aCT1) and Bowman-birk protease inhibitor (BBI) treatment, alone or in combination, targeting Cx43, were investigated by Sato et al. on canine oral melanoma cell viability. Authors found that this dual treatment can be combined to achieve anticancer activity. The possibility of blocking the Rb-E2F pathway by using a CDK4/6 inhibitor (Palbociclib) was investigated by Bongiovanni et al. as a potential anti-cancer therapy on four different canine oral melanoma cell lines. The selected drug was effective on three of the four tested cell lines, including a metastatic melanoma-derived one, indicating that CDK4/6 inhibitors could potentially be used as a new anti-cancer treatment for canine melanoma.

The immunologic components of the immune environment and the mechanisms of evasion of the immune response by melanoma cells have been investigated in several papers on this Research Topic. The different T cell phenotypes and functions in melanoma tumor tissue and PBMCs of melanoma-affected patients were evaluated by Sparger et al., revealing a different and unique T cell phenotype of both unstimulated and stimulated T cell populations in melanoma patients compared to healthy dogs. Takeuchi et al. demonstrated that the immunosuppressive cytokine transforming growth factor beta 1 (TGF-β1) is upregulated *in vitro* and *in vivo* in oral malignant melanoma and that its suppressive effect could be reverted *in vitro* by a decoy receptor, developed by the authors, exploring the potential application of this receptor in biologic therapy.

Included in the present Research Topic is also a study investigating the approach of gene therapy using adenoviruses encoding the immunomodulatory gene CD40L (AdCD40L). This strategy has already shown promise in clinical trials on human melanoma patients. Saellström et al. reported results on local AdCD40L treatment in dogs with different types of melanoma (mainly oral and cutaneous), indicating that this type of therapy is safe and could have beneficial effects in canine patients and potential valuable clinical translation to human patients.

Altogether, these manuscripts present new insights on the current researches on canine melanoma with the specific aim to improve our current diagnostic and therapeutic approaches to this highly aggressive and lethal disease of the dog, with a high translational potential to human mucosal melanoma.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

PB receives minority royalty payments from BI Animal Health for the Oncept melanoma vaccine as he is on the xenogeneic DNA patent with co-investigators at MSKCC.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Effects of Alpha-Connexin Carboxyl-Terminal Peptide (aCT1) and Bowman-Birk Protease Inhibitor (BBI) on Canine Oral Mucosal Melanoma (OMM) Cells

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Oral mucosal melanomas (OMM) are aggressive cancers in dogs, and are good models for human OMM. Gap junctions are composed of connexin units, which may have altered expression patterns and/or subcellular localization in cancer cells. Cell-to-cell communication by gap junctions is often impaired in cancer cells, including in melanomas. Meanwhile, the upregulated expression of the gap junction protein connexin 43 (Cx43) inhibits melanoma progression. The α-connexin carboxyl-terminal (aCT1) peptide reportedly maintains Cx43 expression and cell-cell communication in human mammary cells and increases the communication activity through gap junctions in functional assays, therefore causing decreased cell proliferation. The Bowman-Birk protease inhibitor (BBI), a component of soybeans, induces Cx43 expression in several tumor cells as a trypsin–chymotrypsin inhibition function, with antineoplastic effects. This study investigated the effect of aCT1 peptide and BBI treatment, alone or in combination, on TLM1 canine melanoma cell viability. Cell viability after treatment with aCT1, the reverse sequence peptide (R-pep), and/or BBI for 5 days was analyzed by PrestoBlue assay. Immunofluorescence was used to observe Cx43 localization and expression. aCT1 (200 μM) alone did not significantly decrease cell viability in TLM1 cells, whereas BBI (400 μg/ml) alone significantly decreased the TLM1 viability. Combined treatment with both aCT1 (200 μM) and BBI (400 μg/ml) significantly decreased cell viability in TLM1 cells. Cx43 expression, as identified by immunostainings in TLM1 cells, was increased in the cell membrane after the combination treatment with BBI and aCT1. This dual treatment can be combined to achieve the anticancer activity, possibly by increasing Cx 43 expression and affecting Cx43 migration to the cell membrane. In conclusion, a treatment strategy targeting Cx43 with BBI and aCT1 may possibly lead to new effective therapies for canine OMM.

Keywords: melanoma, connexin, peptide, viability, aCT1, Bowman-Birk inhibitor

INTRODUCTION

Melanoma is an aggressive skin and mucosal cancer that develops from melanocytes. This tumor arises due to random genetic mutations, and after the melanoma has spread, it rapidly becomes life-threatening (1). In humans, the diagnosis of early-stage melanomas can facilitate their cure by surgical resection, and approximately 80% of cases are treated in this manner. However, metastatic melanoma is largely refractory to the existing therapies and has a very poor prognosis, being the survival rate for 5-years lower than 15% of the cases (2). Therefore, new treatment strategies are urgently needed. Continued research into more effective therapies for melanoma will improve the treatment and prognosis of these patients.

Canine oral and mucosal melanoma (OMM) are considered good models of human OMM, because they share many similarities including morphology, genetic alterations, and behavior (3–6). OMM is one of the most common oral malignancies in canines. OMM in dogs are considered extremely aggressive tumors, with local invasiveness and high metastatic propensity. The World Health Organization staging scheme for dogs with OMM is based on the size of the tumors (7). MacEwen et al. (8) correlated these stages with survival times. Stage I tumors, with <2 cm diameter has a median survival after surgery of 17 to 18 months. Stage II OMM are 2 to <4 cm in diameter tumors, and the survival time is 5 to 6 months. In stage III tumors of ≥ 4 cm in diameter and/or lymph node metastasis, the median survival is 3 months. Stage IV dogs with OMM have distant metastasis and the prognosis is very poor (9). Therefore, some factors negatively affect the prognosis including the clinical stage, tumor size, evidence of metastasis, and reported histologic criteria for melanoma prognosis. Standardized treatments, such as surgery, radiotherapy, and chemotherapy, have provided minimal to modest stage-dependent clinical benefits, and death in general occurs due to metastasis (9). Notably, most of the medicines used in veterinary medicine are repurposed from drugs indicated for human use and are not being developed specifically for animals. Therefore, it is necessary to intensify the research focus on veterinary oncology, simultaneously testing possible therapeutic alternatives in animal studies and human trials.

Teixeira et al. previously found that canine amelanotic OMM present higher aggressive behavior than their melanotic OMM counterparts (10). This finding could be partly explained by the decreased expression of connexin 43 (Cx43), which probably resulted in an impaired cell-to-cell communication capacity and, consequently, greater cell proliferation. Regarding cell to cell communication, the expression of connexins could be an essential target factor in canine oral melanoma because Cx26 and Cx43 were significantly reduced in amelanotic melanomas (10).

Connexins are integral membrane proteins that form gap junctions or channels between adjacent cells, thereby permitting the bidirectional cytosolic exchange of ions, metabolites, and secondary messengers ($<$1,200 Da). These channels assemble into distinct plasma membrane structures termed gap junctions, and the intercellular communication at the gap junctions play important roles in tissue homeostasis and the regulation of cell growth and differentiation. Additionally, connexins form functional channels (i.e., hemichannels) in the non-junctional areas of the plasma membrane. These hemichannels provide a communication pathway between the intracellular and extracellular milieu, critical for autocrine and paracrine signaling. In addition, connexins present significant channel-independent roles, including their function as signaling hubs; these may occur at the plasma membrane, in the cytoplasm, or even in the nucleus (11). The connexin protein family in humans has 21 members, in which Cx43, named because of its molecular weight of 43 kDa, is the most extensively studied. Very few studies are available on connexins in canine tissues. Cruciani and Mikalsen (12), found the 18 "multi-specie" connexin genes (connexins 26,29/31.3, 30, 30.2/31.9, 30.3, 31, 31.1, 32, 36, 37, 39/40.1, 40, 43, 45, 44/46, 47, 50, and 57/62) in dogs. The expression of connexins in canine cancers has been evaluated in mammary tumors (13–15), bone tumors (16, 17) testes (18), and OMM (10).

Decreased or diminished expression and/or function of connexins have been observed in most tumor cell lines and solid tissue tumors, including melanomas (11). The role of gap junctions in tumor progression has been studied mainly through the ectopic reintroduction of connexin genes into tumor cell lines. The expression patterns of Cx43 have been studied in several cancer types in humans, and it varies depending on the cancer type and stage (19). The ectopic expression of Cx43 has been shown to reduce cell proliferation in many distinct cancer cells, including in mouse melanoma cell lines (20).

The overexpression of Cx43 reduces the proliferative and metastatic capacities of melanoma in mice (20), while the suppression of Cx43 expression by miR-106a promotes melanoma cell proliferation (21) in human-derived cells in *in vitro* studies. Furthermore, Cx43 upregulation is potentially able to inhibit melanoma progression in mice, as shown in an *in vivo* study (22). Therefore, the regulation of Cx43 expression may lead to the developing of an effective treatment strategy for melanomas; scientific evidence suggests that connexins could be an important therapeutic target (11, 19, 23–30).

The alpha-connexin carboxyl-terminal (aCT1) peptide is a 25-amino acid peptide that mimics the carboxyl-terminal of Cx43. At the molecular level, the aCT1 peptide inhibits the activity of Cx43 hemichannels by inducing their sequestration from the perinexus region surrounding the gap junctions, thereby reducing hemichannel density and availability for activation within the cell membrane (31). The aCT1 peptide is expected to clinically improve postsurgical scarring (32). Grek et al. suggested that using aCT1 peptide for targeting the gap junctional distribution and activity of Cx43 is an effective therapeutic strategy in human breast cancer. Furthermore, they demonstrated that aCT1 peptide enhances the activity of therapies like tamoxifen and lapatinib, thereby supporting the clinical potential of combinational strategies, including the modulation of Cx43 by the aCT1 peptide (33). In addition, Murphy et al. (34) indicated that combining aCT1 with temozolomide, an antineoplastic agent, could enhance the therapeutic responses in human glioblastoma cell lines. These findings suggest the possibility of a Cx43 targeting therapy, using aCT1.

Protease inhibitors are known cancer chemopreventive agents, because of their well-established *in vivo* and *in vitro* anti-carcinogenic activity in cancer models (35). The Bowman-Birk inhibitor (BBI) is the most predominant protease inhibitor in soybeans. It consists of a 71-amino acid protein (8 kDa), and a serine protease inhibitor, which has both trypsin and chymotrypsin inhibitory activities (36). BBI is a small water-soluble protein that is present in soybean and almost all monocotyledonous and dicotyledonous seeds, and BBI decreases the proteolytic activities of trypsin, chymotrypsin, elastase, cathepsin G, chymase, serine protease-dependent matrix metalloproteinases, urokinase protein activator, mitogen-activated protein kinase, and phosphoinositide 3 kinase (PI3K), and upregulates Cx43 expression. BBI was found to be an efficient suppressor of carcinogenesis (37). BBI has cancer-protective activities, although its exact mechanism(s) of action is incompletely understood. In previous studies, it was shown that Cx43 induction by BBI contributes to the decreased growth of tumor cells, both *in vivo* and *in vitro* (38, 39).

Therefore, we aimed to investigate the effect of aCT1 peptide and BBI on canine OMM cell viability and evaluate the usefulness of a Cx43-targeting strategy for the treatment of this cancer in dogs.

MATERIALS AND METHODS

Ethical Statement

The study was submitted and approved by the Committee on Ethics on the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science of the University of São Paulo, under protocol number 6968020817.

Reagents

The aCT1 peptide and its reverse sequence peptide (R-pep) were synthesized by the American Peptide Company (Sunnyvale, CA). The aCT1 peptide is a short sequence at the Cx43 C-terminus, and this is linked to an antennapedia internalization sequence (RQPKIWFPNRRKPWKKRPRPDDLEI). The antennapedia internalization peptide sequence is RQPKIWFPNRRKPWKK. The R-pep sequence consists of the reverse sequence of aCT1 attached to an antennapedia sequence for internalization (33).

BBI was obtained from Sigma-Aldrich (#T9777, St. Louis, MO). All culture reagents were purchased from Thermo Fisher Scientific (Waltham, MA), unless otherwise indicated. The primary antibody, Purified Mouse Anti-Connexin 43, was purchased from BD Transduction Laboratories (#610061, San Jose, CA). The secondary antibody, Goat anti-mouse IgG (H+L) linked to Alexa fluor 488, was purchased from Invitrogen (#A28175, Waltham, MA).

Cell Lines

The TLM1 canine oral melanoma cell line was kindly supplied by Dr. Jaime F. Modiano, VMD, PhD (University of Minnesota, Minneapolis, USA). The TLM1 cells originated from a canine (Gordon setter) oral melanoma. The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, #12800-058) supplemented with 10% fetal bovine serum (FBS) and 1%

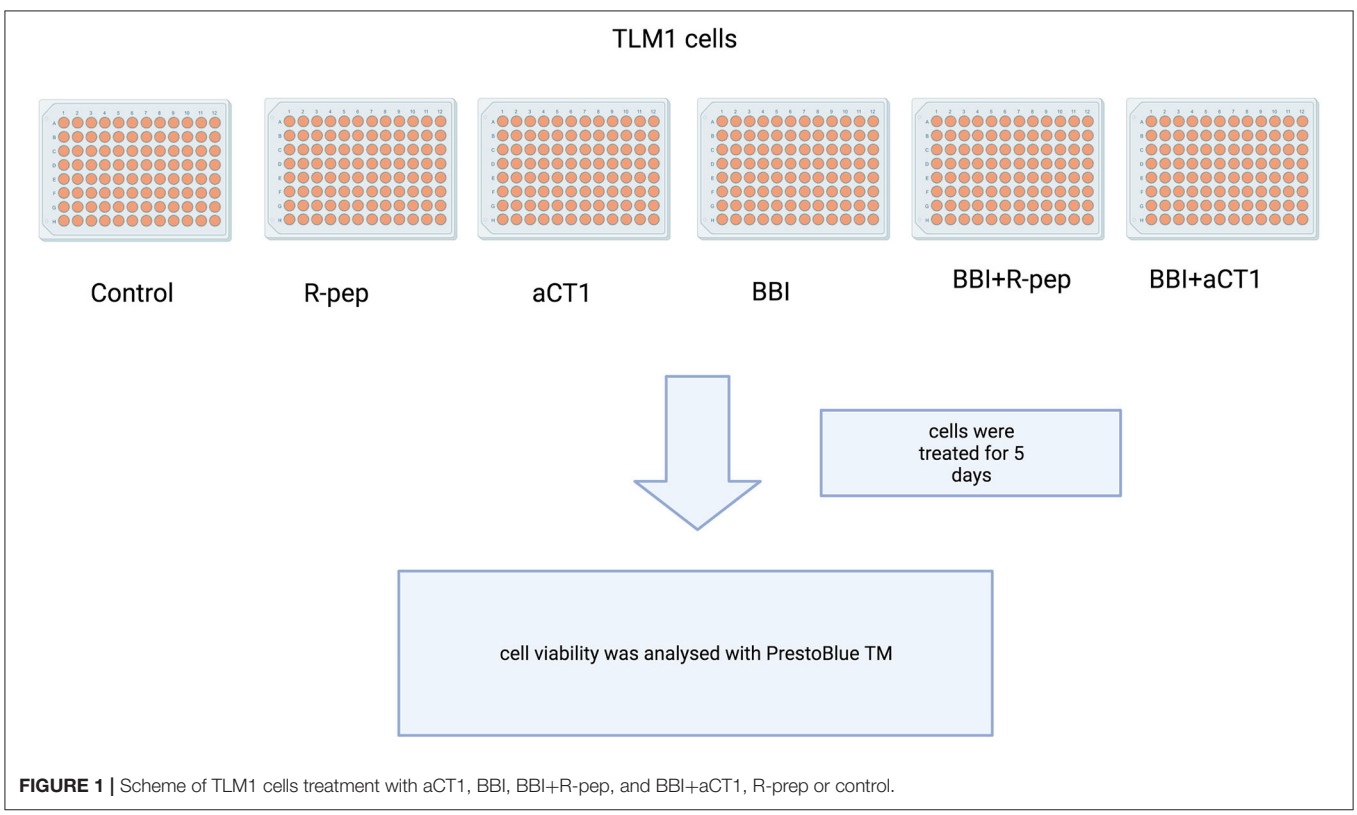

FIGURE 1 | Scheme of TLM1 cells treatment with aCT1, BBI, BBI+R-pep, and BBI+aCT1, R-prep or control.

Antibiotic–Antimycotic at 37°C in a humidified atmosphere with 5% CO_2.

Cell Viability Assay

Cells (2×10^3 cells/well) were seeded in culture plates with 96 wells with DMEM, containing 1% FBS, and cultured for 1 day. After incubation with each of the treatment reagents (i.e., Control, R-pep, aCT1, BBI, BBI+R-pep, and BBI+aCT1) for 5 days, cell viability was determined using PrestoBlue™ Cell Viability reagent (#A13261, Thermo Fisher Scientific). Non-toxic resazurin in the PrestoBlue was converted to red-fluorescent dye within viable cells. The fluorescence at 570 nm was measured using a microplate reader (**Figure 1**).

Immunofluorescence

In a 24-well plate, round coverslips of 12-mm diameter were placed in each well and prepared by irradiation using ultraviolet light for 15 min. Each cell line (1×10^5 cells/well) was seeded in the plate with DMEM, containing 1% FBS, and cultured at 37°C in a humidified atmosphere with 5% CO_2 for a day. Following the removal of the culture medium, 0.5 ml DMEM, containing aCT1 peptide or R-pep and/or BBI was added, and the plate was incubated at 37°C for 3 days. After washing with phosphate-buffered saline (PBS) two times, the cells were fixed by 0.5 ml 4% paraformaldehyde solution for 40 min at 4°C. The fixed cell samples were permeabilized with PBS with Tween 20 (PBS-T), 0.1% Triton X-100 and 5% Skim milk dissolved in PBS, for 30 min at room temperature (RT). Then, 100 μl/well of primary antibody solution (1:100 dissolved in PBS-T) was added to the cell sample. After leaving at 4°C overnight, PBS wash was undertaken three times. 100 μl/well of the secondary antibody solution (1:100 dissolved in PBS-T) was added to the cell sample. After leaving it at RT for 1.5 h in the dark, PBS wash was done three times. VECTASHIELD Antifade Mounting Medium with DAPI (#H1200, Vector Laboratories, Burlingame, CA) was dropped on each glass slide, and coverslips were placed on it. The slides were left overnight at 4°C in the dark to dye the cells with DAPI. Fluorescence microscopy, using ECLIPSE E800 (Nikon, Japan) with a setting of 40× lens, was undertaken, and stained cytoplasmic membranes and nuclei were imaged (.jpeg), and the resulting images were merged using Image J software (Bethesda, MD).

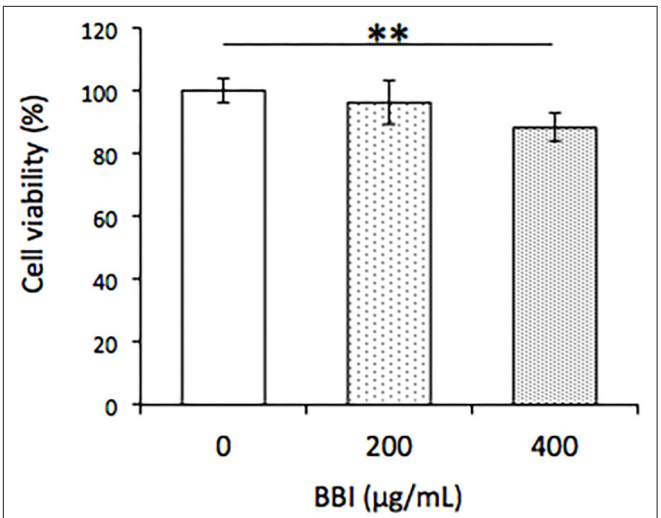

FIGURE 3 | Effect of BBI on cell viability in TLM1 cells. The cells were treated with indicated concentrations of BBI for 5 days. Cell viability was evaluated by a PrestoBlue assay. Columns represent means ± standard deviations ($n = 6$). $^{**}p < 0.01$ vs. 0 μg/ml, using Dunnett's test.

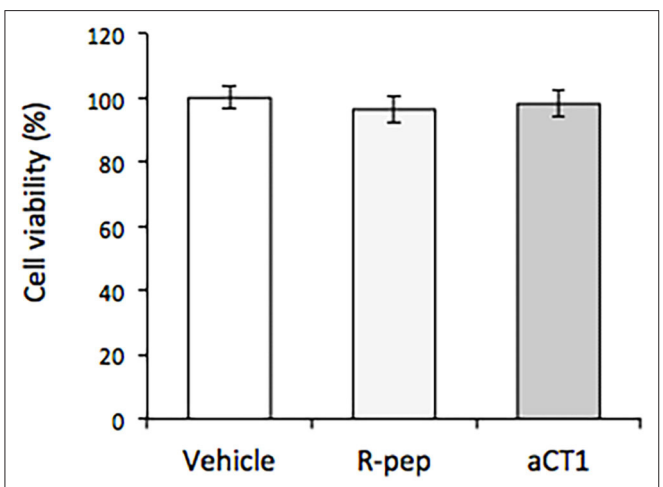

FIGURE 2 | Effect of aCT1 peptide on cell viability in TLM1 cells. The cells were treated with 200 μM aCT1 or the reverse sequence peptide (R-pep) for 5 days. Cell viability was evaluated by a PrestoBlue assay. Columns represent means ± standard deviations ($n = 5$).

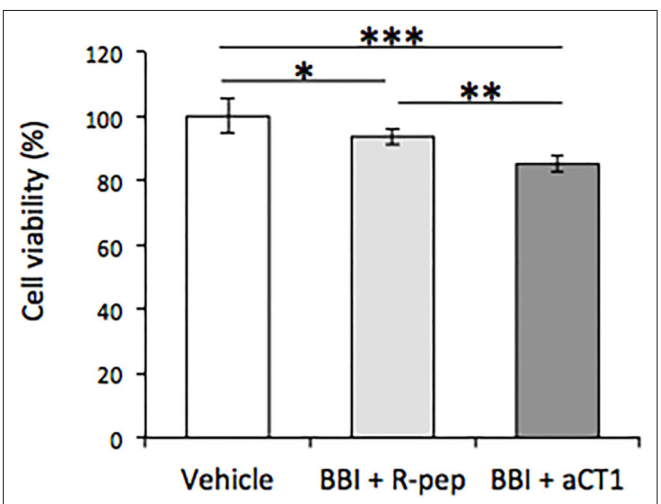

FIGURE 4 | Effect of combination treatment of BBI and aCT1 peptide on cell viability in TLM1 cells. The cells were treated with 400 μg/ml BBI and 200 μM aCT1 (or R-pep) for 5 days. Cell viability was evaluated by a PrestoBlue assay. Columns represent means ± standard deviations ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, using Tukey's test.

Statistical Analysis

Differences in group means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test or Dunnett's. The GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) was used for these calculations. *P*-values < 0.05 were considered significant.

RESULTS

The effects of aCT1 and R-pep on cell viability in TLM1 cells were evaluated. Vehicle (water) was used to treat the control group. As shown in **Figure 2**, aCT1 peptide alone, or the R-prep, showed no effect on cell viability in TLM1 cells.

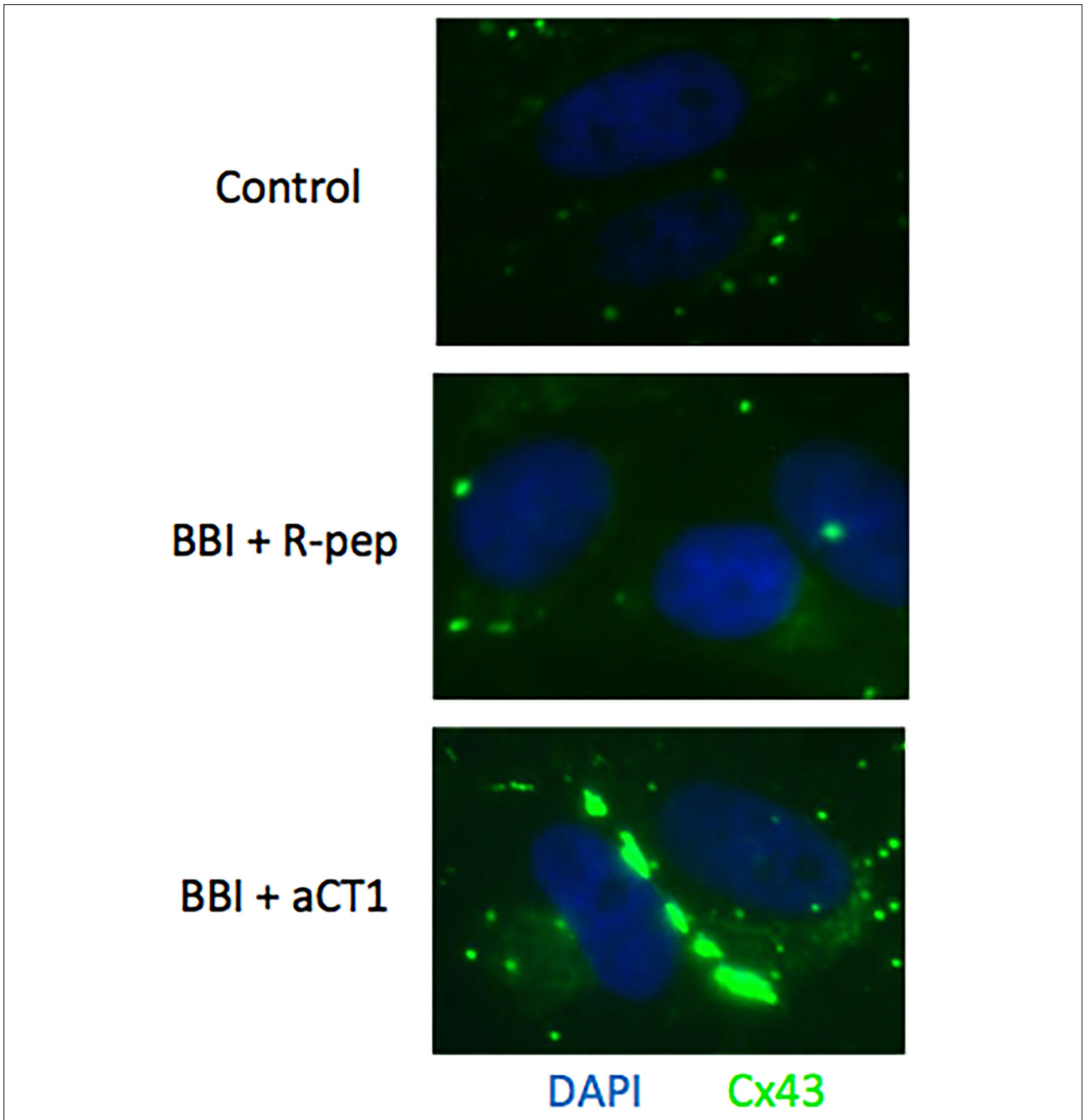

FIGURE 5 | Cx43 expression after BBI and aCT1 peptide treatment in TLM1 cells. Cx43 plaques seen as green dots in the membrane of TLM1 cells submitted to treatment with 400 μg/ml BBI and 200 μM aCT1 peptide (or R-pep) for 3 days.

The effects of different concentrations of BBI (0, 200, and 400 μg/ml) on cell viability in TLM1 cells were evaluated. As shown in **Figure 3**, 400 μg/ml BBI treatment significantly decreased cell viability in TLM1 cells.

Subsequently, the effects of combination treatment with BBI and aCT1 or R-pep were evaluated. As shown in **Figure 4**, combination treatment with BBI and aCT1 significantly decreased cell viability compared with combination BBI and R-pep treatment in the TLM1 cells.

Immunofluorescence was carried out to compare the Cx43 localization in BBI and aCT1 treated cells with BBI and R-pep treated cells. As shown in **Figure 5**, combination treatment with BBI and aCT1 increased Cx43 expression compared with the combination treatment of BBI and R-pep in the cell membrane in the TLM1 cells, and a strong positivity to connexins was seen in the cell membranes.

DISCUSSION

Cell viability assays were carried out to evaluate the effects of aCT1 peptide in canine OMM TLM1 cells by treating them with 200 μM aCT1 peptide or R-pep. aCT1 peptide alone showed no effect on cell viability in the TLM1 cells. Additionally, we pre-tested the different treatment periods (2–4 days) of the peptide, and there were no significant differences on the cell viability (data not shown). An assay tested different concentrations of aCT1 peptide (0–400 μM), but the outcomes were similar to **Figure 1** (data not shown). It is reported that aCT1 peptide (200 μM) inhibited human breast cancer cell proliferation in an *in vitro* study (33). The effect of aCT1 peptide may depend on the cell types. Murphy et al. (34) have also reported the possibility that different effects of aCT1 are shown in several cell lines. It was thought that in the absence of or with minimal Cx43 expression in the cell membrane, the aCT1 peptide could not exert its effect. In this context, Alaga et al. (40) revealed that Cx43 was detected in intracellular compartments, but not assembled in the gap junctions, and they suggested that the melanocytes do not form the Cx43 homocellular gap junctions. Although Cx43 levels increase during melanoma progression, connexin rarely assembles in gap junction structures (40). Therefore, we focused on a component, BBI, which may induce Cx43 expression.

BBI induced the expression of Cx43 genes in mice with M5076 ovarian tumor and decreased the tumor growth in this *in vivo* model (38). A similar effect of BBI was demonstrated in an osteosarcoma cell line (39). Tang et al. reported that treatment of prostate cancer cells (LNCaP) with 500 μg/ml BBI resulted in the inhibition of viability as measured in WST-1 assays, with the induction of Cx43 and expression of cleaved caspase-3 protein (41).

As shown in **Figure 2**, BBI treatment decreased cell viability in TLM1 cells at a pharmacological concentration (400 μg/ml). The mechanism has not been elucidated in detail in this study; however, it is known that BBI might improve the cell-to-cell communication because of its trypsin and chymotrypsin inhibitory activities. Furthermore, BBI has implications for Cx43 expression and induces apoptosis via factors such as a VEGF secretion inhibitory effect (42), besides the mitochondrial impairment and oxidative damage following proteasome 20S inhibition (43). These are reasons why BBI has been shown to have strong anti-carcinogenic activity in animal carcinogenesis model systems compared to other potential cancer chemopreventive agents in soybeans (44). Interestingly, a phase II clinical trial in patients with oral leukoplakia demonstrated a dose-dependent reduction in the oral lesion size after a 1-month treatment with BBI concentrates at doses of up to 1,066 CI units (45), and BBI is expected to prevent oral cancer (46). These findings have a potential for application in the development of new oral melanoma therapies.

Notably, as shown in **Figure 3**, the suppression effect of cell viability through the combination treatment of BBI and aCT1 peptide in TLM1 cells was remarkable. Moreover, immunofluorescence staining demonstrated that the combination treatment of BBI and aCT1 peptide induced a high expression of Cx43 in the cell membrane in TLM1 cells (**Figure 4**). These results indicate that Cx43 is important for canine OMM cell growth. Overall, the possibility of an enhancement effect of anticancer drugs by aCT1 peptide was emphasized similarly as in previous reports (33, 34); although further studies are needed to validate this finding. A finding that resveratrol enhances chemosensitivity in mouse melanoma model through Cx43 upregulation (47) supports this suggestion. Thus, Cx43 may influence the response of tumor cells to cancer therapies by facilitating the spread of antitumor drugs or death signals between neighboring tumor cells.

In conclusion, the findings of this study suggest that Cx43 upregulation may be useful for OMM treatment and warrants further research.

CONCLUSIONS

In this study, it has been shown, for the first time, that the combined treatment with aCT1 peptide and BBI decreases cell viability in TLM1 canine melanoma cell line, which can possibly be used as a new therapy for canine oral melanomas.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AS executed the study and wrote the manuscript. IF and MN technical support and mentoring. GT technical support on cell cultures. RO technical support on immunocytochemistry. FH-B mentoring and technical support on immunocytochemistry. TY and EY mentoring and supply of materials. MD mentoring, financial support, manuscript writing, and editing. All authors contributed to the article and approved the submitted version.

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Canine Transforming Growth Factor-β Receptor 2-Ig: A Potential Candidate Biologic for Melanoma Treatment That Reverses Transforming Growth Factor-β1 Immunosuppression

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Cancer cells can evade host immune systems *via* multiple mechanisms. Transforming growth factor beta 1 (TGF-β1) is an immunosuppressive cytokine that induces regulatory T cell (Tregs) differentiation and is involved in immune evasion mechanisms in cancer. The inhibition of the TGF-β1 signaling pathway can suppress cancer progression and metastasis through the modulation of anticancer immune responses. However, to best of our knowledge, no implementation of treatments targeting TGF-β1 has been reported in dog cancers. This study aimed to examine whether TGF-β1 is upregulated in canine cancers. We measured TGF-β1 concentrations in culture supernatants of canine melanoma cell lines and in serum samples from dogs with oral malignant melanoma. TGF-β1 production was observed in several cell lines, and serum TGF-β1 levels were elevated in dogs with oral malignant melanoma. Interestingly, the addition of recombinant TGF-β1 to canine peripheral blood mononuclear cell cultures decreased Th1 cytokine production and increased differentiation of $CD4^+CD25^+Foxp3^+$ lymphocytes, suggesting that TGF-β1 is immunosuppressive in canine immune systems. We developed a decoy receptor for TGF-β, namely TGF-βRII-Ig, by identifying an open reading frame of the canine *TGFBR2* gene. TGF-βRII-Ig was prepared as a recombinant fusion protein of the extracellular region of canine TGF-βRII and the Fc region of canine IgG-B. As expected, TGF-βRII-Ig bound to TGF-β1. In the presence of TGF-β1, the treatment with TGF-βRII-Ig increased Th1 cytokine production and decreased the differentiation of $CD4^+CD25^+Foxp3^+$ lymphocytes. Our results suggest that TGF-βRII-Ig competitively inhibits the immunosuppressive effects of TGF-β1 and thereby activates immune responses. This study demonstrated the potential of TGF-βRII-Ig as a novel biologic for canine melanoma.

Keywords: canine, melanoma, immunosuppression, TGF-β1, biologic, cancer immunotherapy

INTRODUCTION

Transforming growth factor beta 1 (TGF-β1) is a multifunctional cytokine that is associated with cancer progression and suppression of immune response (1, 2). TGF-β1 directly inhibits cytotoxic T cell function by suppressing cell proliferation and the expression of cytotoxic genes (3, 4). TGF-β1 also inhibits the effector function of natural killer cells (5), contributing to immune evasion of cancer cells (6). In addition, TGF-β1 indirectly mediates immunosuppression by inducing regulatory T cells (Tregs) from peripheral naive T cells (7). Tregs inhibit interleukin (IL)-2 competitively through the increased expression of the IL-2 receptor, CD25, and suppress T cell function by producing immunosuppressive cytokines such as IL-10 and TGF-β1 (8). Therefore, TGF-β1 is considered a critical suppressor of Th1 responses in normal and diseased conditions, including cancers.

In a mouse model of metastatic breast cancer, TGF-β antagonism by an anti-TGF-β antibody resulted in significant enhancement of antitumor immune responses mainly mediated by $CD8^+$ T cells (9). In a human phase I clinical trial, an anti-TGF-β antibody (GC1008) induced a partial response in one patient and stable disease in 6 of 28 patients with malignant melanoma and renal cell carcinoma (10). Similarly, galunisertib, a small-molecule inhibitor of transforming growth factor-β receptor 1, improved overall survival in patients with unresectable pancreatic cancer when combined with gemcitabine (11). These reports suggest that TGF-β1 inhibition is a possible strategy in developing immunotherapy against cancers.

In canine oncology, little information is available on the association of anticancer immunity with the TGF-β pathway. A previous study found the mean plasma TGF-β1 levels in tumor-bearing dogs were significantly higher than those in healthy controls (12). Canine transmissible venereal tumor, a unique, contagious cancer in canids, has been reported to produce TGF-β1 and suppress lymphokine-activated killer activity against tumor cells (13, 14). MiR-145, which is reported to regulate TGF-β, was downregulated in oral canine malignant melanoma tissue when compared with healthy oral mucosa tissue (15). In addition, previous studies have shown that canine mastocytoma and osteosarcoma cell lines produce TGF-β1 (16, 17) and TGF-β1 serum concentrations were higher in dogs with malignant perianal tumors (18). Furthermore, immunohistochemistry tests revealed that canine perivascular wall tumors and squamous cell cancers expressed TGF-β1 (19, 20). These studies suggest that the TGF-β1 pathway might be a common mechanism for evading immune responses exploited by canine cancers. However, there has been no report on TGF-β1 expression in other canine cancer types, and its immunosuppressive functions in dogs are still largely unknown.

In this study, we examined the potential of TGF-β1 as a therapeutic target in canine melanoma by confirming TGF-β1 production in melanoma cell lines and evaluating TGF-β1 serum concentrations in dogs with oral malignant melanoma (OMM). Next, we explored the immunosuppressive effects of TGF-β1 in canine peripheral blood mononuclear cell (PBMC) cultures. After that, we prepared, canine transforming growth factor-β receptor 2-Ig (TGF-βRII-Ig), which consists of an extracellular region of TGF-βRII and an Fc region of canine IgG-B, to investigate its efficacy as a candidate anticancer biologic, expecting it to inhibit the TGF-β1 pathway as a decoy receptor. Finally, the ability of TGF-βRII-Ig to bind TGF-β1 and its effect on immune response were investigated *in vitro*.

MATERIALS AND METHODS

Canine Samples

The use of animal samples throughout this study was approved by the Institutional Animal Care and Use Committee (#15-0149), Faculty of Veterinary Medicine, Hokkaido University, which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Peripheral blood samples were obtained from healthy beagles (3–6 years old) kept at the Experimental Animal Facility, Faculty of Veterinary Medicine, Hokkaido University. Peripheral blood samples of dogs with OMM were obtained at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University. Written informed consent was obtained from the owners for the participation of their animals in this study.

Cell Cultures

Canine melanoma cell lines LMeC (21), CMeC (21), CMM-1 (22), CMM-2 (22) were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-glutamine (Thermo Fisher Scientific), 200 μg/mL streptomycin (Thermo Fisher Scientific), and 200 U/mL penicillin (Thermo Fisher Scientific) at 37°C, 5% CO_2. Canine PBMCs were isolated from 10 mL of peripheral blood samples by density gradient centrifugation on Percoll medium (GE Healthcare, Little Chalfont, UK) and were cultured in RPMI 1640 medium supplemented with 10% inactivated FBS, 2 mM L-glutamine, 200 μg/mL streptomycin, and 200 U/mL penicillin at 37°C, 5% CO_2. To activate PBMCs, 1 μg/mL Staphylococcal enterotoxin B from *Staphylococcus aureus* (SEB; Sigma-Aldrich) was added to the culture medium. ExpiCHO-S cells (Thermo Fisher Scientific) were cultured in ExpiCHO Expression Medium (Thermo Fisher Scientific) at 37°C, 8% CO_2 on an orbital shaker.

Measurement of TGF-β1 Concentration

The amino acid sequence of canine TGF-β1 is 100% identical to human TGF-β1. Thus, canine TGF-β1 concentration in cell culture supernatants and sera were measured by human TGF-β1 DuoSet ELISA (DY156, R&D systems, Minneapolis, MN). The absorbance was measured at 450 nm with an MTP-900Lab (CORONA ELECTRIC, Ibaraki, Japan).

RNA Extraction and cDNA Synthesis

Canine white blood cells were prepared from lysed whole blood of a toy poodle and a papillon. Total RNA was extracted from PBMCs and white blood cells using TRI reagent (Molecular Research Center, Cincinnati, OH, USA). Residual genomic DNA was digested using DNaseI (Thermo Fisher Scientific), and cDNA

was synthesized from 1 μg total RNA using PrimeScript Reverse Transcriptase (TaKaRa, Otsu, Japan) and oligo dT primer.

Identification of Canine *TGFBR2* Gene

A primer pair for canine *TGFBR2* gene was designed based on the predicted dingo *TGFBR2* mRNA sequence registered in the GenBank database (XM_025461082.1). The open reading frame (ORF) of canine *TGFBR2* was amplified from beagle, toy poodle, and papillon cDNA by PCR using the primers 5′-TCG GTC TAT GAC GAG CAG C-3′ and 5′-GCT GCC TCT GTT CTT TGG TG-3′. The amplicon was purified using the FastGene gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan) and cloned into T-Vector pMD20 (TaKaRa) using TA-cloning. The nucleotide sequence was analyzed using the GenomeLab GeXP Genetic Analysis System (SCIEX, Framingham, MA, USA), and the identified ORF was translated and aligned using BioEdit software (23). Unrooted neighbor-joining phylogenetic trees were constructed using Mega version 7 software (24, 25).

Expression and Purification of Canine TGF-βRII-Ig and Control IgG-B

Recombinant canine TGF-βRII was expressed as an Fc-fusion protein (TGF-βRII-Ig) in a mammalian-cell based transient expression system. To predict the signal peptide sequence and transmembrane region, the deduced amino acid sequence of canine TGF-βRII was analyzed using the prediction tools: SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) for signal peptide prediction and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) for transmembrane region prediction. The gene sequence encoding the predicted signal peptide and the extracellular region of canine TGF-βRII that was fused to the Fc region of canine IgG-B (AF354265.1) were designed, codon-optimized, and synthesized with an *Asc*I/*ASi*SI restriction site (GenScript, Piscataway, NJ, USA). The gene sequence was cloned into the expression vector pDC62c5-U533 (kindly provided by Prof. Yasuhiko Suzuki, Hokkaido University) using *Asc*I (New England Biolabs, Ipswich, MA, USA) and *Asi*SI (New England Biolabs), and the plasmid was purified using NucleoBond Xtra Midi (TaKaRa). To prepare a negative control for TGF-βRII-Ig, the expression vector that only encodes the canine IgG-B Fc region was similarly constructed using the signal peptide of canine antibody light chain (26). The expression plasmids were transfected into ExpiCHO cells using the ExpiFectamine CHO Transfection Kit (Thermo Fisher Scientific) and the expressed proteins were purified using Ab-Capcher ExTra (ProteNova, Kagawa, Japan). The buffer was replaced with phosphate-buffered saline (PBS; FUJIFILM Wako Pure Chemical, Osaka, Japan) using PD MidiTrap G25 (Cytiva, Tokyo, Japan). The concentration of the expressed proteins was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 2 × Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA) containing 2-mercaptoethanol in reducing and non-reducing conditions (without 2-mercaptoethanol). The samples were incubated at 96°C for 5 min and separated by electrophoresis using SuperSep Ace (FUJIFILM Wako Pure Chemical). Precision Plus Protein All Blue Standards (Bio-Rad) was used as a protein standard. The gel was stained using the Quick-CBB kit (FUJIFILM Wako Pure Chemical).

Detection of TGF-βRII-Ig Binding to TGF-β1

The binding of TGF-βRII-Ig to TGF-β1 was evaluated by ELISA. A 96-well ELISA microplate (SUMITOMO BAKELITE, Akita, Japan) was coated with 10 μg/mL of TGF-βRII-Ig or Control IgG-B diluted in PBS at 37°C overnight. After washing with PBS containing 0.05% Tween20 (KANTO KAGAKU, Tokyo, Japan), the plate was blocked with Super Block (Thermo Fisher Scientific) at 37°C for 1 h. Recombinant human TGF-β1 (240-B/CF, R&D systems) was added to the plate at various concentrations (4-fold dilution from 8 to 0.125 ng/mL), followed by incubation at 37°C for 1 h. Then, 1 μg/mL biotinylated chicken anti-human TGF-β1 antibody (R&D systems) was added and incubated at 37°C for 1 h. The reaction was developed using peroxidase-conjugated NeutrAvidin protein (Thermo Fisher Scientific) and TMB One Component Substrate (Bethyl Laboratories, Montgomery, TX, USA) and then stopped with 0.18 M H_2SO_4. As a dilution buffer for recombinant TGF-β1, anti-TGF-β1, and NeutrAvidin, PBS containing 1% bovine serum albumin was used. The absorbance was measured at 450 nm with the MTP-900Lab (CORONA ELECTRIC).

Measurement of Cytokine Production From Canine PBMCs

Canine PBMCs from healthy beagles were cultured with SEB at 1 μg/mL for 72 h. Recombinant human TGF-β1 (0.78 nM) alone or in combination with TGF-βRII-Ig (78 nM) were added to the medium. Control IgG-B was used as negative control for TGF-βRII-Ig. IL-2, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α concentrations in the culture supernatants were measured by Canine IL-2 DuoSet ELISA (DY1815, R&D systems), Canine IFN-γ DuoSet ELISA (DY781B, R&D systems), and Canine TNF-α DuoSet ELISA (DY1507, R&D systems), respectively. The absorbance was measured at 450 nm with the MTP-900Lab (CORONA ELECTRIC).

Flow Cytometric Analysis of $CD4^+CD25^+Foxp3^+$ Lymphocytes

Cells were collected from PBMC cultures as described above and blocked with PBS containing 10% goat serum (Thermo Fisher Scientific) at 25°C for 20 min. Subsequently, 1 μg/mL of anti-canine CD4 mouse monoclonal antibody (R&D systems) and 1 μg/mL phycoerythrin (PE)-conjugated anti-canine CD25 mouse monoclonal antibody (Thermo Fisher Scientific) were added and incubated at 25°C for 30 min. The dead cells were stained using Fixable Viability Dye eFluor780 (Thermo Fisher Scientific). Anti-canine CD4 was labeled with Alexa Fluor 488 using Zenon Mouse IgG2b Labeling Kits (Thermo Fisher Scientific). PE-labeled mouse IgG1 (Thermo Fisher Scientific) was used as an isotype control antibody for CD25 staining. The cells were washed twice with PBS, and then fixed using FOXP3 Fix/Perm Buffer (BioLegend, San Diego, CA, USA) and permeabilized using FOXP3 Perm Buffer (BioLegend). In the permeabilization buffer, 2% normal rat serum (Sigma-Aldrich)

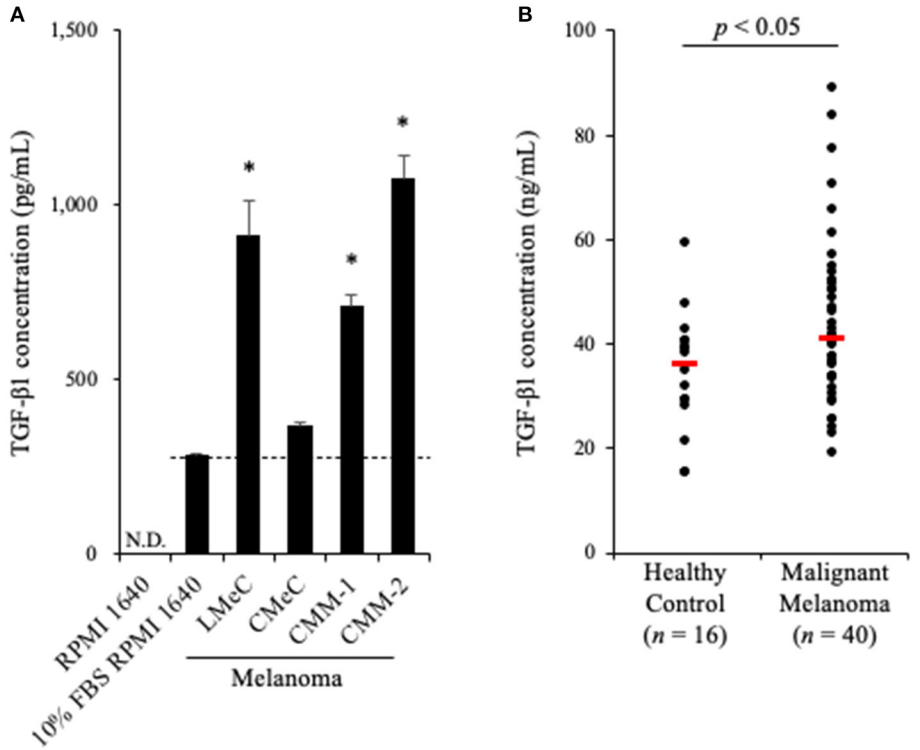

FIGURE 1 | TGF-β1 expression in canine cancers. **(A)** TGF-β1 production by canine cancer cell lines. TGF-β1 concentration in 10% FBS RPMI 1640 is shown as the background (a dashed line). One-way ANOVA with *post-hoc* Dunnett's-test was used for statistical analysis under the assumption of equal variance between samples (*$p < 0.05$ vs. 10% FBS RPMI 1640). **(B)** TGF-β1 serum levels in dogs with oral malignant melanoma. The red bar shows the median value. The Mann-Whitney *U*-test was used for statistical analysis.

was added to block non-specific reactions. The cells were stained with 10 μg/mL of allophycocyanin-conjugated anti-canine Foxp3 rat monoclonal antibody (Thermo Fisher Scientific) at 4°C for 30 min. Allophycocyanin-labeled rat IgG2a kappa chain isotype control (Thermo Fisher Scientific) was used as a negative control. The cells were washed twice with FOXP3 Perm Buffer and analyzed using a FACS Verse flow cytometer (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

One-way ANOVA with Tukey's *post-hoc* test or Dunnett's *post-hoc* test were used for multiple comparison. The Wilcoxon singed-rank sum test was used to compare the paired samples. The Mann-Whitney *U*-test was used for comparison of two groups. A *p*-value <0.05 was considered statistically significant. JMP 14 (SAS Institute, Cary, NC, USA) was used for all statistical analysis.

Nucleotide Sequence Accession Numbers

The nucleotide sequence of the canine *TGFBR2* gene has been submitted to the DDBJ/EMBL-Bank/GenBank database under accession number LC600803.

RESULTS

Production of TGF-β1 by Canine Melanoma Cell Lines

To examine whether TGF-β1 is produced in a tumor microenvironment, we measured TGF-β1 concentrations in culture supernatants of canine melanoma cell lines. Melanoma cell lines were seeded into 6-well cell culture plate at a density of 2.0×10^5 cells in 2 mL medium. After 48 h, when the cells were 60–80% confluent, the cell culture supernatant was collected. The cell culture was prepared in triplicate. Since FBS was expected to contain bovine TGF-β1, the mature amino acid sequence, which is 100% identical to canine and human TGF-β1, TGF-β1 concentration in RPMI 1640 medium supplemented with 10% FBS was measured as a background control. Of the four cell lines, LMeC, CMM-1, CMM-2 had significantly higher TGF-β1 concentrations in the culture supernatant than in the background (**Figure 1A**). These results confirmed that canine melanoma cells can produce TGF-β1.

TGF-β1 Serum Levels in Dogs With OMM

Since we proved that canine melanoma cells produce TGF-β1, we hypothesized that the TGF-β1 serum levels in dogs with melanoma are upregulated. To examine this, serum samples were obtained from dogs with metastatic OMM ($n = 40$, **Supplementary Table 1**) and healthy beagles ($n = 16$),

and the TGF-β1 serum levels were measured by ELISA. As expected, elevated TGF-β1 serum levels in dogs with metastatic OMM was observed (median, 36.4 ng/mL in healthy control vs. 41.1 ng/mL in metastatic OMM; **Figure 1B**). The range of TGF-β1 serum levels was 15.2–59.2 ng/mL in healthy control and 18.9–88.8 ng/mL in metastatic OMM. These results support the potential of targeting TGF-β1 as a melanoma treatment.

Effects of TGF-β1 on Cytokine Production and Tregs Induction in Canine PBMC Cultures

To analyze the immunosuppressive effects of TGF-β1, we performed cytokine production assay in PBMCs cultures. PBMCs obtained from healthy beagles ($n = 6$) were seeded into 48-well cell culture plate at a density of 2.0×10^6 cells in 1 mL medium and cultured for 3 days in the presence of TGF-β1. Th1 cytokine concentrations in the culture supernatant were measured by ELISA. IL-2, IFN-γ, and TNF-α concentrations were significantly decreased in the presence of TGF-β1 (**Figures 2A–C**). In addition, we tested the effects of TGF-β1 on the differentiation of $CD4^+CD25^+Foxp3^+$ lymphocytes. These lymphocytes are considered a regulatory T cell subset in dogs and involved with immune response suppression (27). Flow cytometric analysis using 1×10^5 cells per each condition was performed at the day of sample collection and revealed that the addition of TGF-β1 significantly increased the percentage of $CD25^+Foxp3^+$ cells among $CD4^+$ lymphocytes (**Figure 2D**). The gating strategy is shown in **Supplementary Figure 1**.

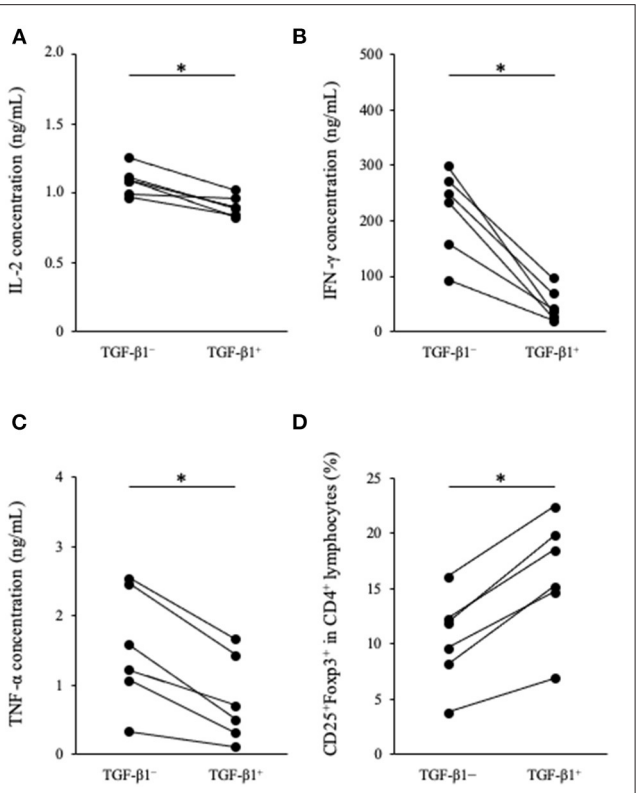

FIGURE 2 | Effect of TGF-β1 on Th1 cytokine production and Tregs differentiation in PBMC cultures. **(A)** IL-2, **(B)** IFN-γ, and **(C)** TNF-α concentrations in cell culture supernatant were measured by ELISA. **(D)** The percentage of $CD25^+Foxp3^+$ cells among $CD4^+$ lymphocytes was measured by flow cytometry. The Wilcoxon signed-rank sum test was used for statistical analysis ($n = 6$, $^*p < 0.05$).

Identification of Canine *TGFBR2* Gene

To reverse the immunosuppression by TGF-β1 in the tumor microenvironment, we attempted to develop a decoy receptor that competitively binds to TGF-β1. Among TGF-β1 receptors, TGF-βRII acts as a direct binding partner to TGF-β1 (28, 29). However, the *TGFBR2* gene, which encodes TGF-βRII, had not been identified in dogs. Therefore, we firstly identified the canine *TGFBR2* gene using cDNA from a beagle, a toy poodle, and a papillon. The ORF of canine *TGFBR2* was 1,704 bp in length and was identical among these three dog breeds. The deduced amino acid sequences of canine TGF-βRII showed high homology with those of other animals that were registered in the GenBank database (**Figure 3A**). The phylogenetic tree was inferred in relation to the deduced TGF-βRII amino acid sequences of other animals. Canine TGF-βRII formed a cluster with cattle and human TGF-βRII and was relatively distant to the rodent and chicken homologs (**Figure 3B**). Taken together, it is suggested that canine TGF-βRII has a similar function to TGF-βRII in other animals.

Preparation of TGF-βRII-Ig and Control IgG-B

Based on the obtained sequence for the canine *TGFBR2* gene, we designed TGF-βRII-Ig, a Fc-fusion protein of the TGF-βRII extracellular region, and its control Ig protein, Control IgG-B (**Figures 4A,B**). Using a transient expression system, the Ig proteins were expressed and purified from the culture supernatant by affinity chromatography using a protein A derivative. Then, the TGF-βRII-Ig and Control IgG-B purities were analyzed by SDS-PAGE. The molecular weights of TGF-βRII-Ig and Control IgG-B were estimated at ∼45 and 30 kDa, respectively, in reducing condition (**Figure 4C**). The hinge region of canine IgG-B Fc contains two cystine residues that would lead to the formation of a parallel dimer. Expectedly, the Ig proteins migrated at a higher molecular weight in non-reducing conditions, suggesting a homodimer formation with interchain disulfide bonds (**Figure 4C**).

Binding of TGF-βRII-Ig to TGF-β1

To examine the binding of TGF-βRII-Ig to TGF-β1, an ELISA was developed using recombinant TGF-β1 and anti-TGF-β1 antibody. Various TGF-β1 concentrations were captured on a TGF-βRII-Ig-coated microwell plate, and the TGF-β1 binding was detected using anti-TGF-β1 antibody. As expected, the TGF-β1 binding was observed in a concentration-dependent manner, whereas no increase in the absorbance was observed in a microwell plate coated with Control IgG-B. These results indicated that TGF-βRII-Ig specifically binds to TGF-β1 (**Figure 4D**).

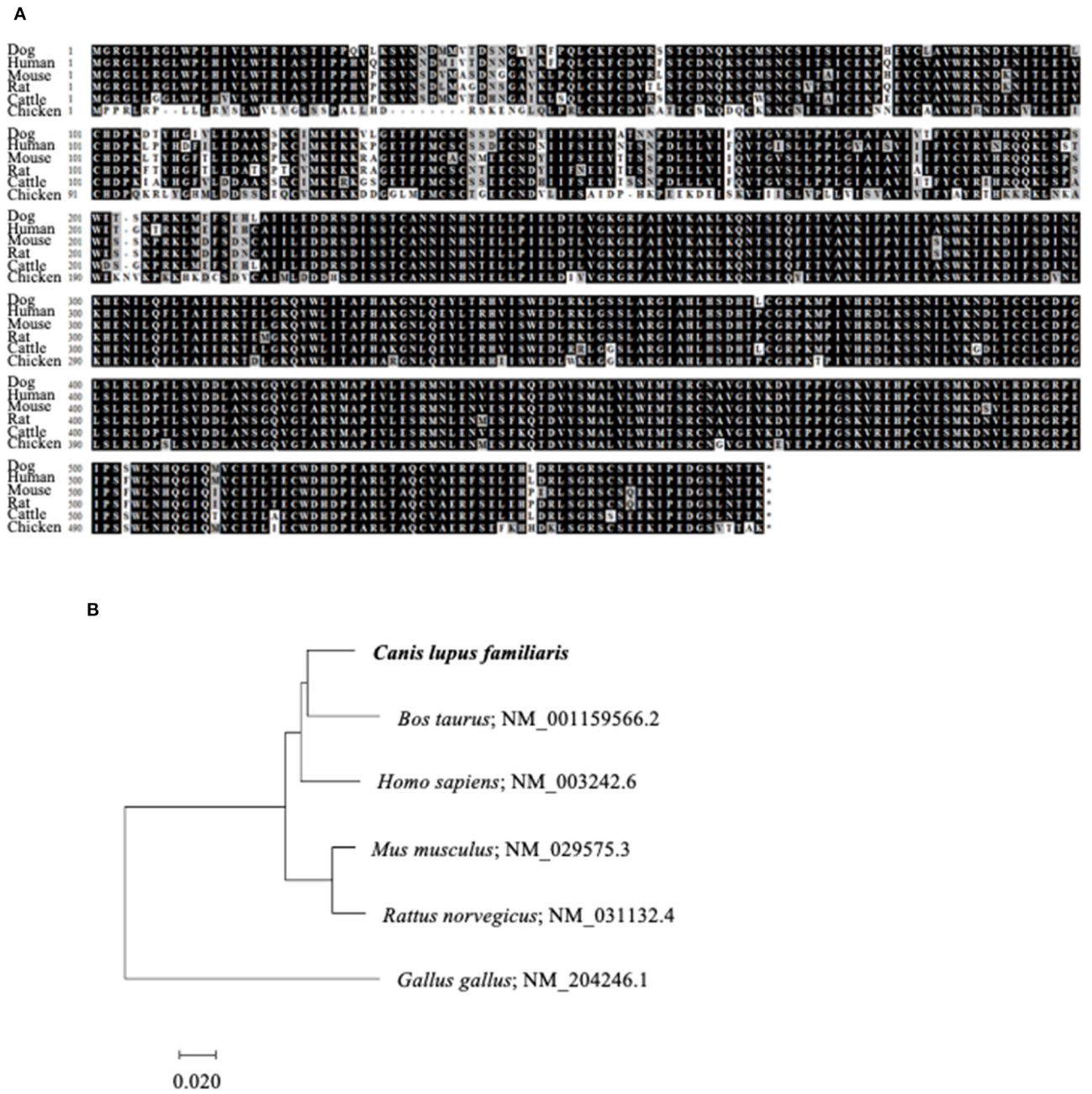

FIGURE 3 | Identification of canine TGFBR2 gene. **(A)** Multiple alignment of TGF-βRII. The deduced amino acid sequence of canine *TGFBR2* gene was aligned with those of other species. **(B)** The phylogenetic tree of canine TGF-βRII in relation to those of other species. GenBank accession numbers were listed beside the scientific names. The scale bar indicates the branch length.

Effects of TGF-βRII-Ig on Cytokine Production and Tregs Induction in Canine PBMC Cultures in the Presence of TGF-β1

To analyze the function of TGF-βRII-Ig, TGF-βRII-Ig was added to the PBMC cultures in the presence of TGF-β1. TGF-βRII-Ig was added at a 100:1 molar ratio to exogenous TGF-β1 to block the effect of TGF-β1 completely. Cytokine production and Tregs induction were analyzed as described above. IL-2, IFN-γ, and TNF-α production in culture supernatants were significantly increased by treatment with TGF-βRII-Ig compared with Control IgG-B (**Figures 5A–C**). In addition, the TGF-βRII-Ig treatment significantly decreased the percentage of $CD25^{+}Foxp3^{+}$ cells among $CD4^{+}$ lymphocytes (**Figure 5D**).

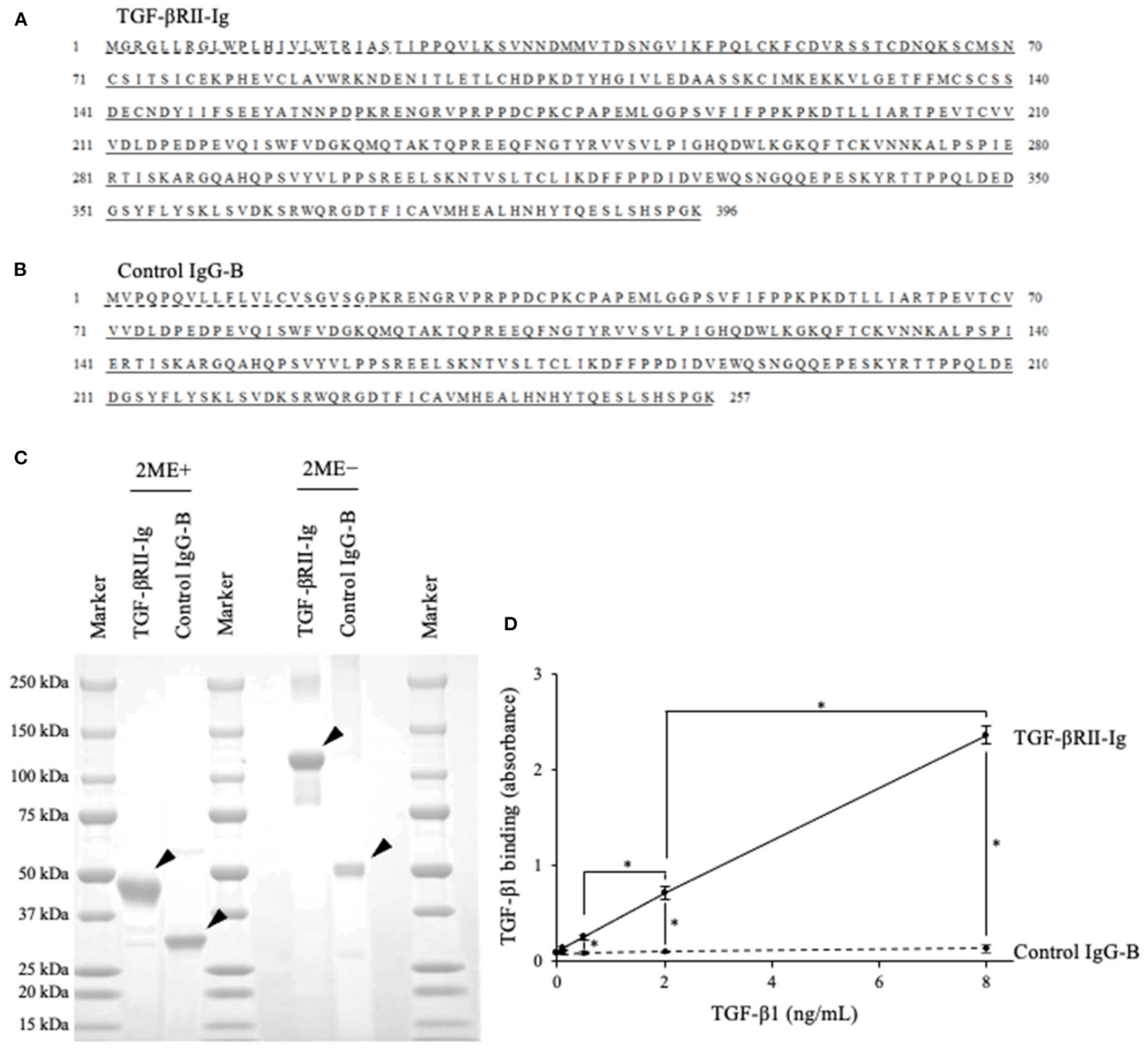

FIGURE 4 | Preparation of recombinant TGF-βRII-Ig and Control IgG-B. **(A)** The amino acid sequences of TGF-βRII-Ig. The predicted signal peptide of TGF-βRII, extracellular region, and canine IgG-B Fc region are marked with a dashed line, underline, and double underline, respectively. **(B)** The amino acid sequences of Control IgG-B. The signal peptide of the canine antibody light chain and canine IgG-B Fc region are marked with a dashed line and double underline, respectively. **(C)** SDS-PAGE of the purified Ig proteins. Analysis in reducing (left) and non-reducing (right) conditions. Arrowheads indicate the protein bands with expected molecular weights. **(D)** Binding of TGF-βRII-Ig to TGF-β1. Each dot represents the average of absorbance obtained from three independent experiment. Error bars represent the standard deviation. One-way ANOVA with *post-hoc* Tukey's-test was used for statistical analysis (*$p < 0.05$).

DISCUSSION

In this study, we confirmed the production of TGF-β1 by the specific melanoma cell lines tested, possibly contributing to the elevation of TGF-β1 serum levels in melanoma dogs. The addition of TGF-β1 decreased Th1 cytokine production from PBMCs and induced Tregs differentiation, indicating its immunosuppressive effect. The decoy receptor TGF-βRII-Ig bound specifically to TGF-β1 and reversed cytokine production and Tregs differentiation induced by TGF-β1. These data strongly suggest that TGF-βRII-Ig competitively inhibits immunosuppressive effects of TGF-β1, resulting in the reversal of Th1 immune response and inhibition of Tregs differentiation. Hence, targeting TGF-β1 using TGF-βRII-Ig is a potential therapeutic strategy for canine melanoma treatment.

To our knowledge, this is the first report that observed TGF-β1 production in canine malignant melanoma cell lines and elevation of serum TGF-β1 in dogs with metastatic OMM. Since TGF-β1 is involved in morphological changes associated with cancer cell migration and metastasis (1), we studied dogs

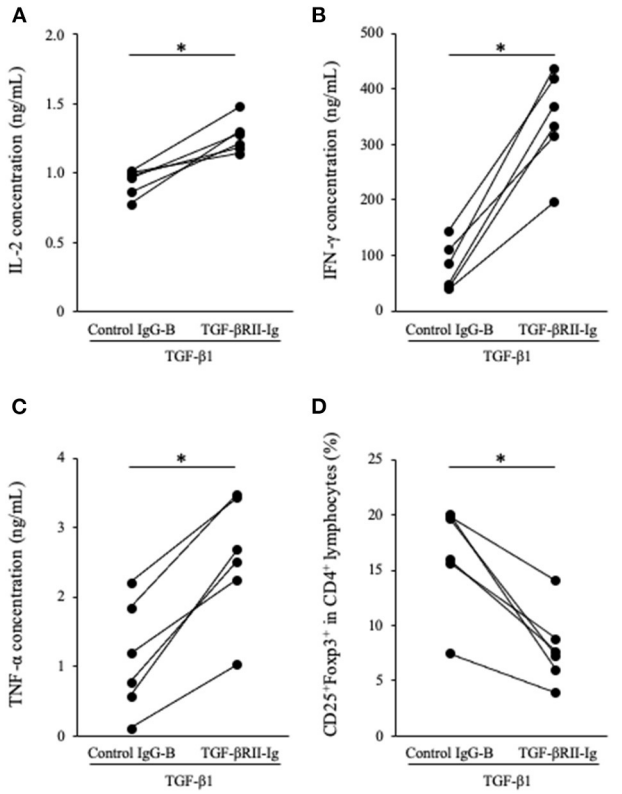

FIGURE 5 | Effect of TGF-βRII-Ig on Th1 cytokine production and Tregs differentiation of in PBMC cultures in the presence of TGF-β1. **(A)** IL-2, **(B)** IFN-γ, and **(C)** TNF-α in cell culture supernatants were measured by ELISA. **(D)** The percentage of $CD25^+Foxp3^+$ cells among $CD4^+$ lymphocytes was calculated by flow cytometry. The Wilcoxon signed-rank sum test was used for statistical analysis ($n = 6$, $^*p < 0.05$).

diagnosed with metastatic OMM presenting with lymph node metastasis or distant metastasis. Due to the limited access to clinical samples of non-metastatic OMM, we could not evaluate TGF-β1 serum levels in dogs with non-metastatic OMM. Some dogs with metastatic OMM had lower TGF-β1 serum levels than healthy control. Considering the different levels of TGF-β1 production from melanoma cell lines, it may reflect the different cancer phenotype. Because various cell types, including tumor-infiltrating lymphocytes, can produce TGF-β1 in a tumor microenvironment (30), the source of serum TGF-β1 in canine metastatic OMM remains unclear. Further studies that include immunohistochemistry analysis or single-cell RNA sequencing using tissue samples of OMM might be beneficial to address this issue. Nonetheless, TGF-β1serum levels and expression in cancer tissues should be analyzed in various types of cancers to explore the clinical indications of TGF-βRII-Ig as a potential therapeutic agent.

In humans, higher TGF-β1 in plasma was associated with reduced overall survival in patients with locally advanced or metastatic pancreatic cancer (31). In addition, TGF-β1 plasma levels in lung carcinoma patients were correlated with the disease status at long-term follow-up after radiotherapy (32). Osteosarcoma patients with metastatic disease also had higher serum TGF-β than those without metastasis (33). Therefore, TGF-β1 serum levels could be used as a prognostic factor in human cancers. In dogs, the association between TGF-β1 serum levels and cancer prognosis remains unclear. Further studies involving samples coupled with clinical data are needed to examine whether TGF-β1 serum levels can be used as a prognostic marker in dogs with cancer.

Tregs are involved in the suppression of immune responses to foreign antigens, such as viruses (34), and immune evasion mechanisms in cancers (35). Interestingly, the percentage of Tregs in the peripheral blood of dogs with OMM and carcinoma was higher than that of healthy control dogs (36, 37). In addition, the increased number of intratumoral Tregs was related to shorter overall survival in dogs with OMM, oral squamous cell carcinoma, and pulmonary adenocarcinoma (38). Given that the present study confirms that serum TGF-β1 levels were higher in metastatic OMM dogs and that the addition of TGF-β1 induced Tregs differentiation, it is possible that TGF-β1 is responsible for Tregs induction in canine cancers, which leads to poor prognosis.

A limitation of this study is the lack of assessment of the immunostimulatory potential of TGF-βRII-Ig in the absence of TGF-β1. Since PBMC culture contains a certain amount of TGF-β1 derived from stimulated PBMC and FBS, the immunostimulatory potential of TGF-βRII-Ig might not be separated from its effects of reversing TGF-β1 immunosuppression.

In this study, we observed that TGF-β1 serum levels were elevated in dogs with metastatic OMM, and TGF-βRII-Ig successfully enhanced Th1 immune responses in PBMC cultures. The immunostimulatory effects of TGF-βRII-Ig should be further investigated in clinical studies including OMM dogs.

In conclusion, we confirmed that canine melanoma cells can produce TGF-β1 and TGF-β1 serum levels in dogs with melanoma are upregulated. In addition, TGF-β1 suppressed immune response in dogs. We successfully obtained TGF-βRII-Ig, which, as expected, reversed the TGF-β1 immunosuppression. Our results highlighted the potential of TGF-β1 as a target for canine cancer immunotherapy. Furthermore, the current findings support the further investigation of TGF-βRII-Ig as a candidate biologic enhancing anticancer immunity. The antitumor efficacy and safety of TGF-βRII-Ig should be evaluated in clinical studies targeting dogs with cancers as a single agent or in combination with existing treatment modalities.

DATA AVAILABILITY STATEMENT

The nucleotide sequence of the canine TGFBR2 gene has been submitted to the DDBJ/EMBL-Bank/GenBank database under accession number LC600803.

ETHICS STATEMENT

The animal study was reviewed and approved by the use of animal samples throughout this study was approved by the Institutional Animal Care and Use Committee (#15-0149),

Faculty of Veterinary Medicine, Hokkaido University, which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Peripheral blood samples were obtained from healthy beagles kept at the Experimental Animal Facility, Faculty of Veterinary Medicine, Hokkaido University. Peripheral blood samples of dogs with OMM were obtained at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SKo, SM, and KO designed and supervised the project. HT and NM performed the experiments. HT, SKo, NM, and TO analyzed the data. ST, HO, NS, SKi, and YS contributed reagents, materials, and analysis tools. HT, SKo, and NM prepared the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.656715/full#supplementary-material

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Adenoviral CD40 Ligand Immunotherapy in 32 Canine Malignant Melanomas–Long-Term Follow Up

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Malignant melanoma is a serious disease in both humans and dogs, and the high metastatic potential results in poor prognosis for many patients. Its similarities with human melanoma make spontaneous canine melanoma an excellent model for comparative studies of novel therapies and tumor biology. Gene therapy using adenoviruses encoding the immunostimulatory gene CD40L (AdCD40L) has shown promise in initial clinical trials enrolling human patients with various malignancies including melanoma. We report a study of local AdCD40L treatment in 32 cases of canine melanoma (23 oral, 5 cutaneous, 3 ungual and 1 conjunctival). Eight patients were World Health Organization (WHO) stage I, 9 were stage II, 12 stage III, and 3 stage IV. One to six intratumoral injections of AdCD40L were given every seven days, combined with cytoreductive surgery in 20 cases and only immunotherapy in 12 cases. Tumor tissue was infiltrated with T and B lymphocytes after treatment, suggesting immune stimulation. The best overall response based on result of immunotherapy included 7 complete responses, 5 partial responses, 5 stable and 2 progressive disease statuses according to the World Health Organization response criteria. Median survival was 285 days (range 20–3435 d). Our results suggest that local AdCD40L therapy is safe and could have beneficial effects in dogs, supporting further treatment development. Clinical translation to human patients is ongoing.

Keywords: immuno oncology, adenoviral vectors, translational medicine, canine malignant melanoma, clinical trials, AdCD40L

INTRODUCTION

Canine melanoma is a cancer presenting in many different forms, each with different prognosis, just like its human counterpart. Prognosis is dependent on anatomical location, stage at clinical presentation and histologic grade. Oral and digital (ungual) malignant melanomas carry the worst prognosis with a metastatic rate ranging from 40 to 100% at initial diagnosis and a median overall survival (OS) after radical surgery of less than five months (1–3). Cell proliferation is reported to

predict survival and a mitotic index >2 is considered of prognostic significance (3). In contrast to humans, where most melanomas in the skin are classified as a skin cancer (4), only about 20% of dermal melanomas in dogs are malignant, but the high-grade types still result in a poor prognosis. Ocular melanomas are in general benign, with a few important exceptions where severe aggressive behavior can be seen, and also here mitotic index seems the most reliable prognostic indicator (5, 6). Histological grade, in particular nuclear atypia and proliferation index using Ki-67, has been shown to correlate to prognosis (7, 8). Median survival times for dogs with oral melanoma treated with surgery are approximately 17–18, 5–6, and 3 months with stage I, II, and III disease, respectively, clearly indicating that there is an unmet need for developing adjuvant treatment modalities in high-stage disease. Aggressive facial and digital surgery in dogs can severely influence normal function and quality of life post-operatively. A multimodal therapy approach can therefore contribute to decreased surgical intensity, while still extending overall survival with fewer adverse events, for improved animal welfare. Spontaneous models of tumors in pet dogs have been receiving increased attention as a comparative asset for many cancers in humans, including malignant melanoma (9–11).

Immune evasion is one hallmark of cancer and is identified in both human and canine malignant melanomas (12–14). Several mechanisms are involved in this process, including T regulatory (Treg) cell expansion, myeloid derived suppressor cells, (MDSC), M2 tumor-promoting macrophage switch and immunosuppressive cancer-derived exosomes (15, 16). Immunoediting is a stepwise process and can be evolved over several years (17). In general, the cancer tries to induce and maintain a chronic inflammatory state and suppress signals and pathways, creating an acute inflammatory environment (18, 19). The role of dendritic cells is crucial for genesis and maintenance of the tumor (20). CD40 is a co-stimulatory molecule belonging to the tumor necrosis factor superfamily and is essential in activation of dendritic cells (21). In many tumors this expression is suppressed and the dendritic cells are inactivated (22). Hence, reactivation of dendritic cells with CD40 upregulation is an attractive strategy in immunotherapy of different tumor models *in vitro*, in animal models and in human clinical trials. Adenoviral cancer therapy has long been recognized as a promising approach and has developed from monotherapy gene transfer into a combinational approach. Here oncolytic genetically modified adenoviral systems generate neoantigen exposure to the tumor-associated immune system. The transgene product simultaneously activates the immune system suppressed by the tumor. Adenoviral cancer therapy is generally seen as an attractive system because of low toxicity with a convenient and reliable system of gene editing and gene expression, and was the first viral vector developed for gene therapy, being approved for clinical trials in 1990 (23). Even if only a small part of the tumor becomes infected, the cascade developed in the tumor can still be sufficient to induce a tumor response as well as creating tumor-specific immune cells that can move away from the treated tumor and seek and destroy cancer cells in other locations, and hence an abscopal effect is generated.

Malignant melanoma in humans (HMM) often occurs in the skin, but it originates from the neural crest; hence malignant melanoma also can occur on mucous membranes and in the gastro-intestinal canal, as well as the CNS, here primarily as metastases. Local low-stage disease has an excellent prognosis, whilst the 10-year survival in stage IV melanoma is only 10%. Canine malignant melanomas are in many aspects suggested as a good comparative model to HMM, particularly the rare non-UV-induced human melanomas, especially mucosal melanomas (10). *In vitro* stimulation in malignant melanomas as well as intratumoral activation of CD40 in melanoma in mouse models have shown promising results (24, 25). Two earlier studies of replication-deficient AdCD40L intratumoral treatment of spontaneous canine malignant melanoma have been reported (26, 27). Hence, the rationale is obvious for perusing human malignant melanoma in a clinical setting (28, 29).

The aim of this current study was to include a larger number of dogs than previously reported, and with a life-long follow-up, to present survival data and finally identify long-term effects of episomal CD40L gene therapy.

MATERIALS AND METHODS

Study Design

This is a long-term follow-up study of a pilot study using AdCD40L immunotherapy. In addition to the 19 previously reported dogs (26), 13 additional dogs were added and all dogs were followed up until death. This data was collected by contacting the dogs' owners, as well as retrieving individual patient information available in the electronical medical record system (Trofast, Sanimalis, Sweden) at the University Animal Hospital UDS, SLU. Owners were asked for the date on which their dog passed away/was euthanized, the reason for ultimate euthanasia, and if recurrence of melanoma was known to have occurred.

Between May 2005 and May 2013, 32 client-owned dogs with spontaneously occurring malignant melanoma participated in a study of local intratumoral AdCD40L immunotherapy. AdCD40L therapy and sample collection were approved by the Swedish Animal Ethical Committee and the Swedish Animal Welfare Agency (C228/6). A written consent form from the dog owners was obtained. The therapy was performed within the University Animal Hospital (UDS), at the Swedish University of Agricultural Sciences (SLU).

Dogs were treated with weekly intratumoral and/or metastatic lymph node injections (n = 1–6) of a human adenovirus serotype 5 carrying a human CD40 ligand administered under subcutaneous sedation with medetomidine/butorphanol (0.01 mg/0.1 mg/kg), with local lidocaine (100 mg/ml) anesthetic spray for oral treatments. All sedated animals were reverted with intramuscular injection of atipamezole (0.05 mg/kg) post AdCD40L injection.

At each treatment, blood samples were collected; CBC, standard clinical chemistry profile, and immunoglobulin gel electrophoresis were performed. The AdCD40L vector

was prepared by diluting 37 μl of stem solution (viral titer 7×10^{10} Pfu/ml) to a volume of 1 ml (2.6×10^9 Pfu) with sterile isotonic sodium chloride. This GMP-produced AdCD40L was constructed at Baylor College of Medicine, Huston, TX, USA and full description of the virus is described in (30). Between 1 ml and 3 ml AdCD40L were injected each time using a syringe and a 25-gauge needle.

Being a toxicity study on AdCD40L administration in dogs with high stage/high grade malignant melanomas, initial dogs started on low doses and few injections, carefully increasing the dose (volume and titer) to monitor any toxicity. As the toxicity did not differ between 0.5 and 3 ml, it was decided to standardize the dosing regimen to 1.0 ml x 3. Exceptions were made where owners insisted to have additional treatments as the vaccine had a positive effect on tumor burden and quality of life. After an initial dose-escalation phase, the suggested dose (1 ml−2.6×10^9 Pfu), was considered optimized, as a larger volume could be difficult to administered in smaller lesions. The protocol also is analog to human trials, where the final selected dose is used, regardless on tumor size or weight of the patient. In some trials, the full dose is given in one selected lesion and in other protocols the dose can be diluted and divided into several lesions. In our study, a split dose regime was used. Hence, the dose volume was adjusted with dilution buffer to render the same injection volume per tumor lesion for split dose treatments as for full dose treatment. Detailed information on oncolytic viral dose calculation in human malignant melanoma is described in (31). Although, the study is using a conditionally replicative oncolytic herpes virus, it is still instructive and pivotal in explaining principles in dose calculation regardless of tumor or patient size and is the first FDA approved oncolytic virus therapy for human refractory melanoma (T-VEC, or Imlygic®).

Tumor tissues sampled pre- and post-treatment were investigated for immune cell infiltration in 20 of the dogs. Histological staining was performed according to the method described previously (27). Briefly, tumor specimens were fixed in a buffered 10% paraformaldehyde solution and paraffin embedded. Tissue sections were prepared from the paraffin-embedded tissues for routine hematoxylin-eosin staining and immunohistochemistry and subjected to staining procedures with either monoclonal mouse anti-human CD3 (clone F7.2.38, DakoCytomation, Glostrup, Denmark) or monoclonal mouse anti-human CD79 α (clone HM57, DakoCytomation). The complex expressions were visualized by using the DakoCytomation EnVision+R system HRP anti-mouse (K 4001) with diaminobenzidine as substrate. Nuclear counterstaining was performed with Mayer's hematoxylin. Investigations were done according to histologic remission and immune cell infiltration. Briefly, histologic remission means less viable tumor cells present after treatment and histologic complete remission—that is, no tumor cells present post treatment (or post mortem). Immune cell infiltration was graded 0–3, with 3 being the highest grade of infiltration. Macroscopic tumor immune reaction was clinical observation of swelling at the injection site, ulceration and regional lymphadenopathy occurring days post immunotherapy.

Clinical Assessment of Response

Response to therapy was categorized in accordance with WHO criteria, where the products of bidimensional lesion measurements are summed and the change is calculated from baseline while on therapy in order to quantify response (32). More specific CR is specified as complete regression of measurable soft tissue disease, PR (partial regression, >50% but <100% regression in one dimension of measurable soft tissue disease), SD (stable disease, ≤50% regression of soft tissue disease or ≤25% progression), and PD (progressive disease, >25% increase in measurable disease or appearance of new lesions). The minimal time interval required between two measurements for determination of stable disease response was 6 weeks. Best overall response was defined as the best response recorded from the start of the treatment until disease progression or recurrence. Macroscopic tumor lesions were measured by digital caliper and documented with photography (**Figure 1**). In addition, dogs were followed with diagnostic imaging (computer tomography, radiography and/or ultrasound), using the best suitable modality for each case. Treatment adverse events were graded according to the VCOG-CTCAE, version 1.1 (33).

Ultrasound was used very infrequent to follow tumor response (most commonly to follow non-target lesions or for staging purposes) and used only if any other modality was considered inappropriate. If used, the guidelines described in (34) was used, and more specific, the same machine and operator performed all examinations for the individual patient. Dogs were rechecked for as long as clinically necessary to determine survival for each dog. Overall survival was defined as the time from first treatment with AdCD40L until subsequent death. All dogs were followed up until death.

Statistical Analyses

Survival-time distributions were estimated using the Kaplan–Meier method. Statistical analyses were carried out using JMP Pro 15.2.1 (SAS Institute Inc., Cary, NC) statistical discovery software. Univariate survival analysis was carried out for each parameter. Parameters determined to be of statistical significance in the univariate analysis according to log-rank testing were used to stratify Cox proportional hazard models. Parameters included in this study were weight, sex, breed, WHO stage, melanotic vs. amelanotic primary tumor, primary tumor location, presence of local or/and distant metastases, number of AdCD40L treatments, adverse effects after treatment, response, surgery after treatment initiation, histologic tumor type and grade. A parameter with a P-value < 0.05 was considered significant. Adjusting for multiple testing was performed by Bonferroni correction (35). The Bonferroni correction compensates for that increase by testing each individual hypothesis at a significance level of α/m, where α is the desired overall α level (0.05) and m is the number of hypotheses.

Kaplan–Meier survival graphs were calculated for stage, tumor grade, histological type, weight and response, as well as for patients who underwent surgery in combination with immunotherapy vs. patients who did not.

For the purpose of this study, tumors were histologically described as being melanotic if any part of the histological sample

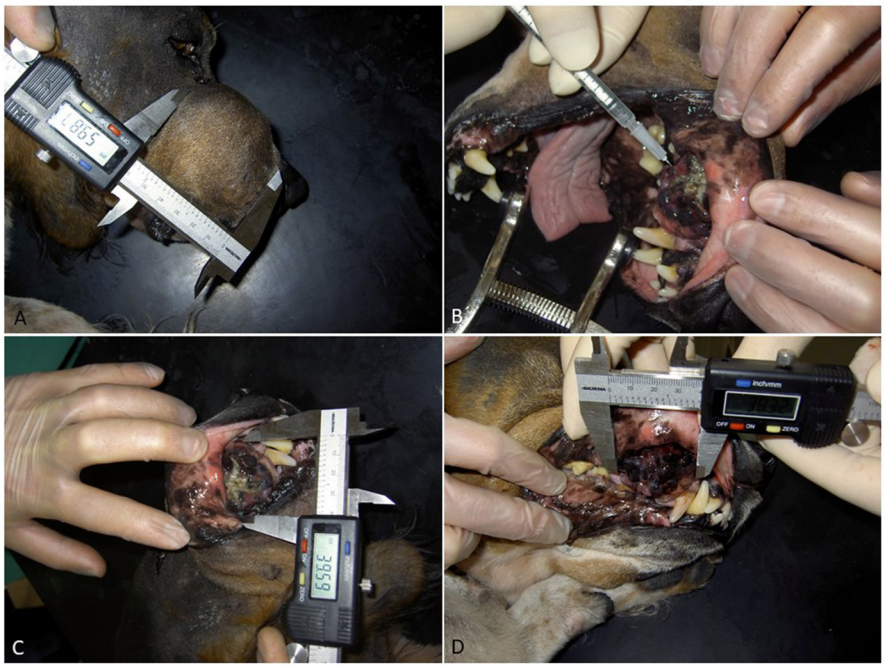

FIGURE 1 | A dog with oral malignant melanoma was presented with bone involvement and lymph node metastases and was considered refractory to surgery. **(A,B)** Tumor size measurements were made with a digital caliper and documented with photography with dogs under sedation. Picture **(C)** shows the first injection of AdCD40L. Picture **(D)** shows the second injection of AdCD40L, 7 days after first injection. Please note the rapid organization of the tumor and significantly less necrotic tissue. The dog started eating as soon as 2 days post first injection, clearly showing an improved quality of life.

was deemed to contain pigment. Multiple tumors which were histologically described as "mainly amelanotic" have therefore been classified as melanotic for the purposes of statistical analysis. For the purpose of WHO staging, ungual and conjunctival tumors were classified according to the protocol for dermal or epidermal tumors, because of the lack of a universal staging system for tumors of these locations. Patients were also grouped according to weight and stage for statistical analyses. Weight was divided into two groups: <20 kg ($n = 17$) and >20 kg ($n = 15$). Similarly, patients were subdivided into three age groups: <9 years ($n = 11$), 9–11 years (10), and >11 years ($n = 11$).

When analyzing histologic cell type, groups were dived into epithelioid, spindle shape, mixed and balloon cell (clear cell) melanoma. Grade was divided into low and high, with mitotic index above 2 as the determinant.

RESULTS

Patient Population

The patient population consisted of 32 dogs with spontaneously occurring malignant melanoma: 22 oral, 5 cutaneous, 3 sub ungual, 1 nasal and 1 conjunctival (**Table 1**). Patients were staged according to the WHO staging system (36). Of the patients included, 8 were stage I, 9 stage II, 12 stage III and 3 were stage IV. Median age at the start of therapy was 120 months (48–168 months). Median weight was 32.2 kg (5–49 kg). Thirteen of the dogs were intact female, 3 were spayed females, 13 were intact males and 3 were neutered males. Twenty of the 32 dogs underwent surgery, 7 before immunotherapy was initiated and 13 after. Three dogs underwent surgery both prior to and after therapy initiation. The vast majority of surgeries performed were cytoreductive and not radical with curative intent. Two dogs underwent a second surgery post AdCD40L immunotherapy after initial unclean margins. At second surgery no remaining tumor cells could be detected and the margins were clean, thus we classified only these two surgeries as curative-intent. One dog had undergone chemotherapy as part of previous treatment, but tumor control had not been achieved. The number of AdCD40L treatments ranged from 1 to 6 with a mean of 3; 15 dogs got 3 treatments and an injection volume between 0.5 and 3 ml depending on tumor volume.

Clinical Response to AdCD40L Treatment

Best overall response according to WHO response criteria, based only on macroscopic change post immunotherapy was 7 complete response (CR), 5 partial response (PR), 5 stable disease (SD), and 2 progressive disease (PD) (**Table 1**). In all cases, a clinically evident inflammation occurred in the tumor after the course of AdCD40L injections, with initial edema often followed by increased firmness and signs of organization. Dogs achieving a complete or partial response had a significantly longer median survival ($p < 0.0001$) (**Figure 2**). Details related to survival compared with different responses to therapy are presented in **Table 2**.

TABLE 1 | Study demographics.

Breed	Age (Y)	Weight (Kg)	Gender	Stage	Grade	Histologic type	Treatment*	BOR	Time Sx	Surgery	Survival (Days)
Golden retriever	8	32	F	III	H	Spindle, amelanotic	1.5 ml X2	NA	9	Y	401
American cocker spaniel	11	12.5	M	I	L	Epithelioid	1,5–3 mlX5	NA	−30, 40	Y	1,141
Siberian husky	11	26.9	F	I	H	Mixed	1 mlX6	CR	85	Y	645
Dachshund	4	5.2	F	II	L	Epithelioid	1 mlX5	NA	−35, 37	Y	3,435
Irish setter	9	37.8	M	II	L	Epithelioid	1 mlX5	CR	81	Y	500
Golden retriever	12	32.8	F	III	H	Balloon cell	2 mlX4	NA	16	Y	130
Mixed	13	32.4	FS	III	H	Mixed, amelanotic	2 mlX3	NA	28	Y	180
Golden retriever	7	31	F	III	H	Epithelioid, anaplastic	2 mlX3	NA	23	Y	279
Irish setter	12	24	F	III	H	Epithelioid	2 mlX3	NA	25	Y	78
Mixed	11	36.5	F	I	L	Spindle	1 mlX3	CR	−30	Y	1,083
Flat coated retriever	4	37.5	M	I	L	Epithelioid	1 mlX3	NA	15	Y	2,057
Rottweiler	5	49	M	I	L	Mixed	1 mlX3	NA	−15	Y	495
Mixed	5	35	FS	I	L	Epithelioid (cytology)	1 mlX1	NA	15	Y	1,340
Golden retriever	12	33	M	II	H	Mixed	1 mlX3	CR	55	Y	225
Rottweiler	8	34.5	F	I	L	Mixed	1 mlX3	NA	35	Y	1,818
Golden retriever	14	31	M	II	L	Epithelioid	1 mlx3	CR	−30	Y	356
Labrador retriever	12	40	MC	II	L	Spindle	1 mlx6	CR	−46	Y	336
Giant poodle	14	13	FS	II	H	Epithelioid	1 mlx3	CR	248	Y	260
Risenschnauzer	7	47	F	III	H	Epithelioid	1 mlx2	NA	−7, 400	Y	500
Mixed	12	5	MC	II	H	Epithelioid	1 mlx3	NA	35	Y	308
Jack russel	9	7.8	M	I	H	Spindle	1.5 ml X6	PR		N	290
Basset artesién	14	15.4	M	IV	H	Mixed	0.5–2 ml X3	PR		N	60
Golden retriever	9	33.8	M	III	H	Epithelioid	2–3 ml X3	PD		N	100
Border terrier	12	9.9	M	III	L	Epithelioid	1 ml X1	SD		N	160
Flat coated retriever	8	39.6	M	III	H	Epithelioid	2 ml X5	SD		N	131
Swedish lapphound	11	23	M	III	H	Mixed	2 mlX3	PR		N	48
Danish-Swedish farmdog	9	9.2	M	III	H	Mixed	1 mlX3	SD		N	128
Risenschnauzer	8	38	F	IV	H	Mixed	2 mlX2	PD		N	20
German shepherd	14	40	MC	IV	H	Mixed	2 ml X2	PR		N	55
Pumi	13	11.2	FS	III	H	Mixed, amelanotic	2 mlX2	SD		N	41
Rottweiler	9	34	F	II	H	Mixed	1 mlX4	SD		N	42
Risenschnauzer	6	22	F	II	L	Epithelioid	1 mlx6	PR		N	1,211

*BOR, Best overall response; CR, Complete response; PR, Partial response; SD, Stable disease; PD, Progressive disease; NA, Not Applicable. Time of surgery (Sx) in days related to AdCD40L treatment. Some Sx were performed prior to immunotherapy. Two dogs achieved clean margins post 2nd Sx post AdCD40L treatment. *Virus concentration was 2.6 – 10^9 Pfu/ml.*

Overall median and mean survival times were 285 and 558 days, respectively. Median and mean survival for dogs in stages III–IV were 128 and 154 days (**Figure 3**). Median and mean values for all stages are shown in **Table 3**. Stage was significant, both as separate stages ($p < 0.0001$) with Bonferroni correction for multiple comparisons ($p < 0.008$), where stage III was prognostic, as well as where stage was grouped into low (I+II) and high (III+IV) respectively.

Median and mean survival times for patients who underwent surgery were 448 days (78–3,435) and 778 days respectively. For patients who did not undergo surgery combined with immunotherapy, median and mean survival times were 80 days (41–1,211) and 191 days respectively. Survival was significantly increased in dogs that also underwent surgery ($p < 0.0001$) (**Figure 4**).

Parameters significant in univariate survival analysis were breed, age, presence of local/distant metastases, histologic grade, tumor stage, tumor type, and surgery after therapy initiation. These parameters were then used to stratify a Cox proportional hazard model and significance remained for breed (Golden Retriever) ($p < 0.00001$), stage (0.00006), age (dogs below 9y lived longer) ($p = 0.0119$) and surgery together with immunotherapy (0.0001). All details regarding survival with or without combined surgery and AdCD40L treatment are shown in **Table 4**.

Clinical Immunology

Immunological responses, based on serum cytokine analysis (TNF-α, IL-8, and the T-cell response suppressor IL-10) have been reported before for 19 treated dogs, including neutralizing antibodies in

100% post the third immunotherapy in six tested dogs (26, 37).

In one case where a metastatic prescapular lymph node was injected, distant immunoreaction was identified in a previously undetected metastatic lesion in the brain. This dog developed seizures three days after each injection with AdCD40L vector and was euthanized one week post second injection. The CNS metastatic lesion was examined post mortem and compared to adjacent normal brain tissue; the metastatic lesion was infiltrated with T-lymphocytes. The histologic findings, together with the clinical appearance of seizures occurring three days post injection, suggest abscopal effect of the AdCD40L injection.

Safety and Toxicity

All adverse events were recorded. Adverse reactions (considered related to treatment) were seen in twenty out of thirty-two dogs. Mild transient fever developed in seven, and five had mild inappetence/anorexia. Twenty dogs had swelling at the injection site, and this was most prominent in dogs that received AdCD40L therapy in a metastatic lymph node. Five dogs had a mild and transient increase in liver enzymes after treatment; one had a grade 2 increase in both alanine amino transferase (ALT) and alkaline phosphatase, and another had a grade 2 rise in ALT (graded according to the Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events [VCOG-CTCAE] following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1) (33). No other adverse effects were noticed. The adverse reactions after AdCD40L injection seemed to decline after one or two treatments, which corresponded to an increase in adenovirus-specific neutralizing antibodies in serum post therapy.

Due to the low toxicity and that no increase in toxicity could be registered even when the virus titer increased up to 3 ml (2.6×10^9 Pfu/ml), and that 100% of the dogs developed neutralizing antibodies after three injections, it was decided to use a regimen of 3 times 1 ml injection, irrespective of tumor size or number of lesions in the latter half of the trial. Exceptions were made, where owners requested additional injections where a clear tumor response was achieved.

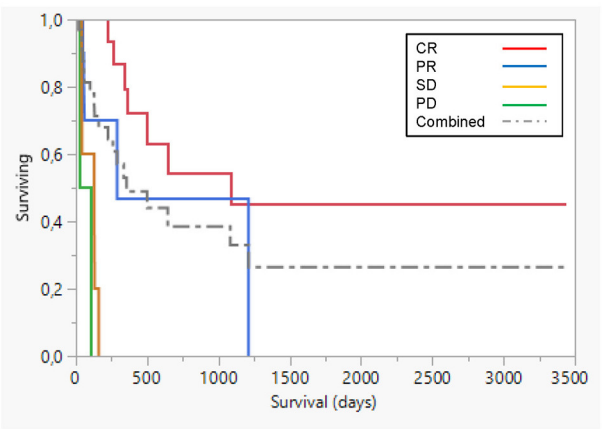

FIGURE 2 | Survival plot comparing prognosis for different responses. Best overall response according to WHO response criteria, based on macroscopic change due to AdCD40L therapy was 7 complete response (CR; red line), 5 partial response (PR; blue line), 5 stable disease (SD; yellow line), and 2 progressive disease (PD; green line). Dogs achieving a complete or partial response had a significantly longer median survival ($p < 0.0001$). Dotted line combined group.

Histopathology

Histological evaluation was performed in 31 dogs (one case only diagnosed with cytology), showing 15 epithelioid melanomas, 4 spindle cell melanomas, 12 mixed type melanomas and 1 balloon cell melanoma (**Table 1**). The histological diagnosis of melanoma was based on the histological classification of tumors in domestic animals according to cytological appearance, number of mitoses, pleomorphism, and presence of anaplastic and poorly differentiated cells (38). However, histological evaluation alone is not always a reliable prognostic indicator; rather the combination of oral location and morphology indicating malignancy support a worse outcome of melanoma in dogs. In the case where diagnosis was based on fine needle aspiration biopsy, a board-certified veterinary clinical pathologist analyzed the cytology and the tissue specimen was reported to contain a high frequency of mitoses and a round cell population of markedly pigmented cells. Thus, a tentative definition of epithelioid, high-grade malignant melanoma was made.

Several publications report proliferation as a consistent prognostic marker of malignant melanoma in dogs. Hence, a grading into low and high grade was made with mitotic index (MI) of >2 per 10 high power field (HPF) as the determinant. With this subdivision, 12 tumors were low grade and 20 were high grade and this was significant ($p < 0.0001$) (**Figure 5**).

TABLE 2 | Details related to survival compared with different responses to therapy in 19 dogs with macroscopic response to AdCD40L therapy.

Response	Number	Mean (D)	Std error	Median (D)	Lower 95%	Upper 95%	p
CR	7	772*	104.6	1,083	336	.	<0.0001
PD	2	60	40	60	20	100	
PR	5	649*	239.7	290*	48	1,211	<0.0001
SD	5	100.2	24.7	128	41	160	
Combined	19	601	97	356	160	1,211	

*Dogs achieving CR and PR had a significant longer survival compared to the rest of the groups. CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; *significant $p < 0.008$, Bonferroni correction for multiple comparisons.*

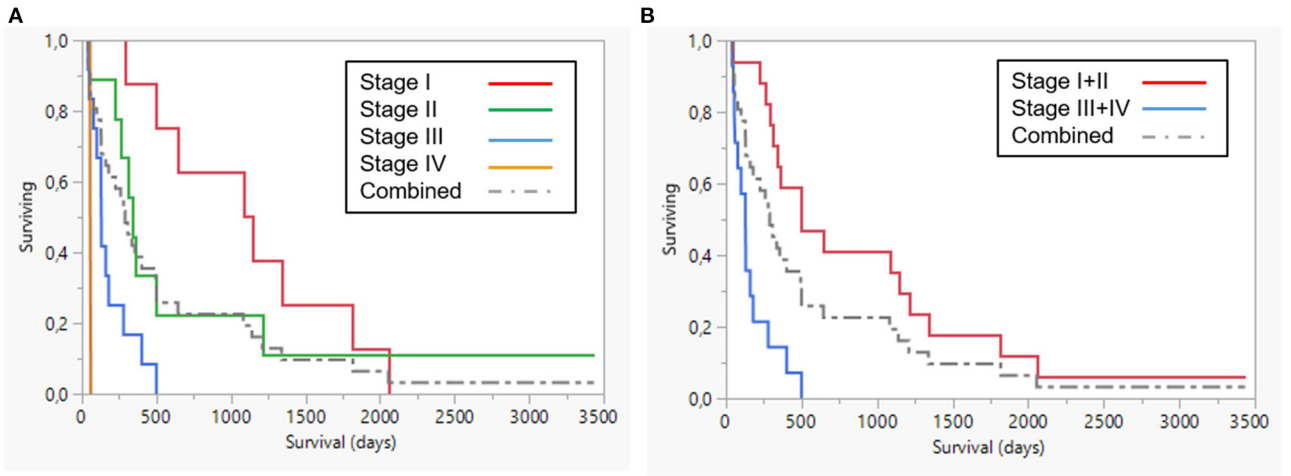

FIGURE 3 | Survival plot **(A)** all stages **(B)** Stages I+II (red) and III+IV (blue). Dotted line combined group.

TABLE 3 | Comparison of survival between different stages.

Stage	N	Mean	Std error	Median (D)	Lower 95%	Upper 95%	*p*
I	8	1108.6	220.4	1,112	290	1,818	
II	9	741.4	354.0	336	42	1,211	
III	12	181.3	41.1	131¤	48	279	0.0001
IV	3	45	12.6	55	20	60	
I+II	17	914.2	212.8	500	290	1,211	
III+IV	15	154.1	35.7	128*	48	160	0.0001
Combined	32	558	131.6	285	130	495	

*Stages also merged in low (I+II) and high (III+IV) to equal group size. Survival was significantly lower in stage III compared to all other groups. When only comparing low (I+II) and high (III+IV) stage, it was a significantly worsened prognosis in high stage. ¤Significant, $p < 0.008$, Bonferroni correction due to multiple comparisons. *Significant, $p < 0.05$.*

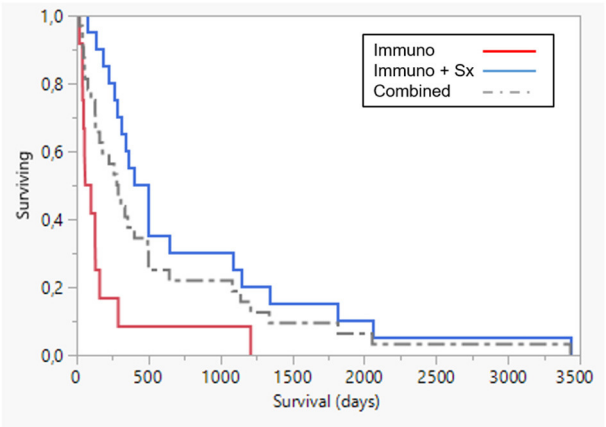

FIGURE 4 | Survival plot comparing AdCD40L immunotherapy only ($n = 12$, red line), median 80 days and combined with surgery (Sx) ($n = 20$, blue), median 448 days. Survival was significantly longer in dogs that also underwent surgery combined with immunotherapy $p < 0.0001$. Dotted line combined group.

For full details of survival data in different histology grades, see **Table 5**. Immune activation by AdCD40L therapy was seen in several of the patients. An increase in the infiltration of immune cells was seen in 8 of 11 dogs. Histological grading described in (26) was based on lymphocyte counts of random representative areas in the tumors, and defined as: none = 0, 1–10 lymphocytes per high power field = 1, 11–30 lymphocytes per high power field = 2, and >30 lymphocytes per high power field = 3. Tumor regression was seen histologically in 25 of 32 cases. There was an initial significant relationship between histological tumor type and overall survival, with the mixed type showing a worse outcome ($p < 0.035$), although with Bonferroni correction for multiple comparison ($p < 0.008$) this significance was lost (**Figure 6**). Details of the outcome depending on histologic type are presented in **Table 6**. No statistical significance was seen in the relation between the increase of immune cell infiltration after treatment and overall survival. A comparison was made on survival depending on melanotic ($n = 25$) or amelanotic ($n = 7$). No statistical difference on survival between the groups were seen ($p < 0.11$).

TABLE 4 | Comparison of survival between dogs only treated with immunotherapy and dogs also undergoing surgery.

Group	N	Mean (D)	Std error	Median (D)	Lower 95%	Upper 95%	*p*
AdCD40L only	12	191	95.2	80	41	160	
Surgery plus AdCD40L	20	778	187.8	448*	260	1,083	<0.0001
Combined	32	558	131.6	285	130	495	

*Survival was significantly longer in dogs that also underwent surgery combined with immunotherapy ($p < 0.0001$). *Significant, $p < 0.05$.*

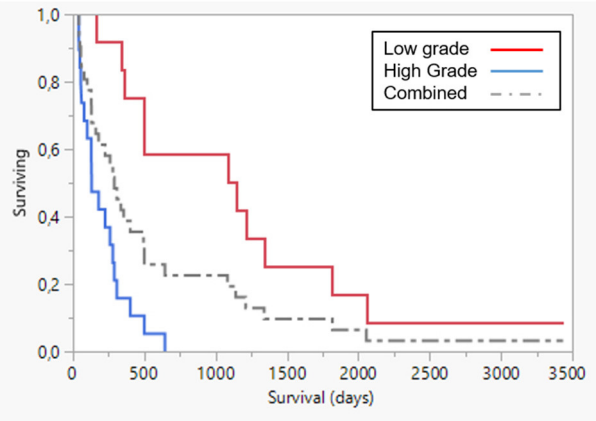

FIGURE 5 | Survival plot: low grade (red line, $n = 12$) vs. high grade (blue line, $n = 20$) ($p < 0.0001$). Dotted line combined group. Survival was significantly lower in high grade disease.

DISCUSSION

We describe responses, overall survival and toxicity in a large group of dogs with a single tumor type, where there still is an unmet need regarding treatment modalities. Reported median survival time for dogs with stage II melanoma treated with curative intent surgery alone is less than five months, and in dogs with grade III or IV it is less than two to three months (1, 3, 39). In the current study, median survival for dogs in all stages was 285 days, whilst for dogs in stage II it was 336 days, and for stages III–IV was 128 days. Although this was not a randomized study aiming at proving efficacy, these survival times compare favorably to the previously reported figures. This suggests that AdCD40L immunotherapy has potential value in therapy of canine malignant melanoma CMM, either as adjuvant in a multimodal treatment setting, or for use as a primary therapy form when alternatives are not feasible. Finally, surgeries performed in this study were cytoreductive. In contrast to radical surgery, partial removal of the tumor leads to rapid recurrence locally, with short subsequent survival (39). The addition of AdCD40L immunotherapy as a consolidating therapy allowed survival comparable to situations when radical surgery is possible. Importantly, in contrast to radical surgery, immunotherapy plus cytoreductive surgery allowed high quality of life and functioning, with only mild and transient adverse reactions. Hence, the results encourage further investigation of the potential of adenoviral gene therapy in dogs with malignant melanoma.

Many different immunotherapy studies in canine malignant melanoma have been published (40). As many different techniques and case selection are involved, comparisons are hard to conduct. Different methodologies for gene transfer has been tested, such as CSPG4 (41), xenogenic Tyrosinase (42) and hgp100 (43). Collectively, macroscopic responses to immunotherapy are modest, but toxicity are tolerable and usually overall survival is reported to be favorable when combining with other therapies, preferentially surgery. To our knowledge, our studies are still the only one reporting responses in naturally occurring malignant melanomas in dogs using oncolytic viral therapy, although the technique is considering a promising modality also in dogs and many *in vitro* studies have been conducted (44, 45). The long-follow up time in this study is a strength and also rather unique, but comparable with at least one gene therapy clinical trial with a similar long-term follow-up published in the field (46). Oncolytic viral treatment in other canine malignancies are also reported (47).

A single pathologist (T.S.) reviewed the majority, but not all of the tumors. Since some of the tumors could not be re-evaluated, interpretation was done using the original histology reports. Currently, there is no consensus grading of canine malignant melanoma. Ramos-Vara and colleagues made the most promising attempt in 2000, using 338 oral malignant melanoma cases (38). Here several prognostic features were reported, and a score was described. Both in this study and as previously reported, proliferation stands out as a fairly robust prognostic indicator, where a mitotic index (MI) >2 is reported to shorten overall survival in many different types of malignant melanomas regardless of anatomic location (3, 38, 48). We therefore grouped the tumors into low and high grade if proliferation was reported to be MI >2. With this rather simple categorization, grade was prognostic with a median overall survival (OS) of 1,112 days with low-grade and only 131 days with high-grade tumors. One tumor was only diagnosed by cytology, but as the tumor was examined by a board-certified veterinary clinical pathologist and had so many cytological features of a high-grade malignant melanoma (and many visible mitotic figures), it was classified as an epithelioid high-grade malignant melanoma.

At least one clear case of abscopal effects of distant reaction in a CNS metastatic lesion was registered in the study. In future prospective studies with stronger funding allowing e.g. more frequent use of advanced digital imaging, the true occurrence of abscopal effect can be verified in

TABLE 5 | Comparison of survival between different histologic grades.

Histology grade	N	Mean (D)	Std error	Median (D)	Lower 95%	Upper 95%	*p*
Low	12	1,161	270.5	1,112	336	1,818	
High (MI) >2	20	196.1	37.9	131*	55	279	<0.0001
Combined	32	558	131.6	285	130	495	

*Survival was significantly lower in the cases with a high mitotic index. *Significant, $p < 0.05$.*

dogs with melanoma treated with AdCD40L therapy. Comparing the OS data in this study with historic controls, especially as many dogs received no other treatment than immunotherapy for macroscopic malignant melanoma, the likelihood of abscopal effects in more dogs is, however, very high.

In human oncology several clinical trials have been conducted with AdCD40L therapy, prior to radical surgery (22, 49). (22, 49). Here, reference to the current dog study is of comparative value, as many of the dogs did not receive any surgery at all and the vast majority of those did not undergo radical surgery. These two factors have been reported to significantly worsen prognosis in dogs (39). A clinical trial with 15 treatment-refractory patients with malignant melanoma received intratumoral injections of AdCD40L (28). Nine of the patients also received low-dose cyclophosphamide conditioning before the first and fourth AdCD40L injection. As in dogs with malignant melanoma, the side effects were few with mild transient reactions. No macroscopic objective responses were recorded by MRI, but local and distant responses were seen on FDG-PET. Survival at six months appeared improved when cyclophosphamide was added to AdCD40L. The patients with the best survival developed the highest levels of activated T cells and experienced a pronounced decrease of intratumoral IL8. The results are encouraging for proceeding with adenoviral and CD40 directed treatment modalities.

No dogs were treated with immune interfering medication before AdCD40L injections. This enables a clean description of toxicity, but also the true efficacy of injections alone. Based on current understanding within immuno-oncology, the initial suppression of e.g. Tregs will potentiate the following immune therapy given. In one study, Mitchell and colleagues reported that the veterinary-registered TKI toceranib (Palladia, Zoetis, USA) had similar reduction potential of Tregs as classic low-dose cyclophosphamide (50). Toceranib is a multi-kinase inhibitor, closely related to sunitinib (51). The use of toceranib, as a conditioner in association with immune therapy in cancer patients, would also promote an antagonistic action toward VEGF, as well as add to the collective action to tilt the inflammatory environment in the tumor toward a less angiogenic environment with fewer immunosuppressive lymphocytes and more Th1 cells and M1 macrophages (1, 3, 38, 52–56).

The parameters found to be significant in association with OS were: Breed, Age, Stage, Grade, and Surgery together with immunotherapy.

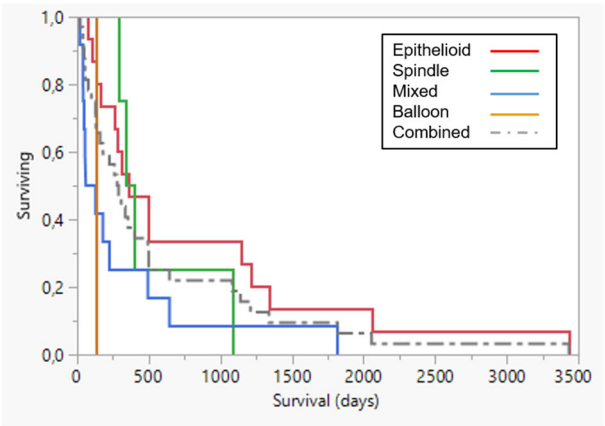

FIGURE 6 | Survival plot comparing prognosis in different histological groups. Epithelioid ($n = 15$, red line), spindle type ($n = 4$, green line), mixed type ($n = 12$, blue line) and one balloon cell (clear cell) melanoma. Dotted line combined group. Mixed cell type had a p-value of $p < 0.035$, which was not considered significant when addressing the Bonferroni correction for multiple comparisons ($p < 0.008$).

Stage being a significant parameter complies with earlier reports (1–3). Considering that stage is a widely used prognostic tool it was expected that a correlation between stage and OS would be observed. This supports the validity of the WHO staging system as a prognostic tool. An expanded, revised staging system has been suggested, with more prognostic factors included (39). Herein the need to compensate for body weight is suggested, as a primary tumor with a diameter of 4 cm in a small-sized dog of 5 kg should be considered worse than the same tumor size in a dog weighing 40 kg. In our study we found a trend toward survival benefit for dogs weighing >20 kg (median OS of 495 vs. 225 days), although this was not statistically significant. If the study had been larger, the possibility of this finding being significant would likely increase. On the other hand, it is reported that increased bodyweight is negatively correlated with longevity in dogs (57).

Breed has also been shown in one previous study to be associated with OS (58). The most common breed in this study was the Golden Retriever, making up 6 of 32 patients, supporting the suggestion that this breed is particularly predisposed to CMM (38), although the patient group cannot be considered representative of the population as a whole because of the small sample size. No conclusions can be drawn in regard

TABLE 6 | Comparison of survival between different histologic types.

Histology type	N	Mean (D)	Std error	Median (D)	Lower 95%	Upper 95%	*p*
Epithelioid	15	790	240.5	356	131	1,141	
Spindle	4	528	186.6	369	290	1,083	
Mixed	12	313	148.3	94	41	495	0.035
Balloon cell	1	130	0	130	0	0	
Combined	32	558	131.6	285	130	495	

The initial deceased survival detected in the mixed group was lost when using Bonferroni correction ($p < 0.008$) for multiple comparisons. MI, Mitotic index.

to specific breeds' positive or negative correlations with OS, although an unpowered significant difference was observed due to the fact that there were in most cases only one or two patients representing each breed.

Age was correlated to OS as dogs below 9y lived longer ($p = 0.0119$; Bonferroni correction ($p < 0.012$), when the dogs were categorized into three equal sized groups; <9, 9–11 and >11 years. Younger dogs have fewer co-morbidities and owner's may be more prone to invest in advanced treatments for younger animals. Still the significance is borderline and not interpreted as a major finding in this study.

Surgery showed a positive correlation with OS. This result may, however, be biased, for a number of reasons. Unfeasibility of radical surgery was one of the entry criteria for the clinical trial, emphasizing the exceptionally long survival time observed in the study. Whether or not a dog underwent surgery as part of treatment at a later stage may also have been influenced by a number of factors including the owners' personal feelings, age of the dog, previous treatment history, financial situation, etc. However, our results suggest that cytoreductive surgery may be useful in combination with immunotherapy for achieving the best results. The underlying mechanism may relate to the immunosuppressive nature of large tumors, partly conducted through tumor exosomes and generation of a pre-metastatic niche (59). Physically removing some of the immunosuppressive mass may facilitate the long-term effects of immunotherapy.

Tumors were staged according to the WHO staging scheme (36). The WHO staging, with a few exceptions, is divided into localization-specific tumor protocols. This study included tumors originating from cutaneous, oral, nasal, conjunctival and ungual tissue. However, due to a lack of specific protocol for conjunctival and ungual tumors according to the WHO staging scheme, these tumors were staged according to the criteria for dermal or epidermal tumors.

Spontaneous tumors in dogs have been suggested as good comparative models to human cancers. They occur spontaneously, in immunocompetent animals, often have similar or identical histologic appearance, share metastatic preferences and pattern, respond to the same type of treatment protocols (surgery, chemotherapy, radiotherapy and immunotherapy) and share the same environment as their owners (60–63). The pet dog has also been suggested as a good model to inform drug development and cancer treatment trials in humans (64). The comparative aspects of malignant melanoma in dogs have been extensively reviewed, showing both molecular biological similarities and thanks to a spontaneous developing tumor in an immune competent animal, ideal for investigating new treatment modalities, especially immunotherapy (10, 11, 14, 65). Finally, the rapidly increasing resolution of the dog genome sequence makes canine comparative oncology studies easier to perform with high accuracy (66).

The findings in this study, with encouraging survival data, low toxicity and the achievement of good quality of life during and post treatment in such a complicated tumor in dogs, supports the usefulness of the canine melanoma model for developing and informing parallel clinical studies in humans with malignant melanoma.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Swedish Animal Ethical Committee and the Swedish Animal Welfare Agency (C228/6). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SS, MD, and HR treated and monitored the patients at the animal hospital, AH, AL, OK, TT, SS, and HR contributed to conception and design of the study. SS, HR, and AH organized the database and performed the statistical analysis. AS, EE, AL, and TT performed the *in vitro* studies and lab work. TS performed the pathology examinations. SS wrote the first draft of the manuscript. HR, AL, and AH wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Quantitative Expression of *TYR*, *CD34*, and *CALD1* Discriminates Between Canine Oral Malignant Melanomas and Soft Tissue Sarcomas

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Canine oral malignant melanomas (OMMs) exhibit a variety of morphologic phenotypes, including a spindloid variant. The microscopic diagnosis of spindloid OMMs is based on junctional activity and/or the presence of melanin pigment. In the absence of these features, spindloid OMMs are difficult to differentiate from soft tissue sarcomas (STS). An antibody cocktail (MDX) that includes Melan-A, PNL2, and tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2) is the current gold standard for identifying amelanotic OMMs by immunohistochemistry (IHC). However, MDX is less sensitive for diagnosing spindloid amelanotic OMMs. This raises concern for biopsy specimens that lack overlying epithelium, making it potentially difficult to differentiate OMM from STS by IHC. The goal of this study was to identify additional markers to help differentiate between STS and OMMs that lack pigment and junctional activity. SOX-10 has recently been proposed as a sensitive marker for melanocytes in humans but has not been validated in dogs. Similarly, RNA expression for various genes has been analyzed in humans, but not in the context of diagnosing canine melanocytic neoplasms. For this retrospective study, formalin-fixed, paraffin-embedded tissues from 20 OMMs, 20 STS, and 20 oral spindle cell tumors (OSCTs) that lacked junctional activity and pigmentation were selected. IHC for MDX, SOX-10, and laminin, in parallel with RT-qPCR of *TYR*, *SOX10*, *CALD1*, *CD34*, *DES*, and *LAMA1*, was performed in all cases. *TYR*, *CD34*, and *CALD1* were the most discriminatory genes in differentiating between OMM and STS, all having 100% specificity and 65, 95, and 60% sensitivity, respectively. While all 20 OMMs were immunohistochemically labeled for SOX-10, two STS were also labeled (100% sensitivity and 90% specificity). MDX IHC labeled all 20 OMMs and no STS. Surprisingly, none of the 20 OSCTs expressed *TYR* RNA above the cutoff, and 14/20 OSCTs expressed *CALD1* or *CD34* RNA above the cutoff, thereby confirming them as STS. Four OSCT were suspect STS, and no OSCTs were confirmed as OMMs based on IHC and RNA expression patterns. In conclusion, the RNA levels of TYR, CD34, and CALD1 should be evaluated

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in suspected amelanotic OMMs that are negative for MDX to accurately differentiate between OMM and STS.

Keywords: melanoma, soft tissue sarcoma, tyrosinase, CD34, caldesmon, *SOX10*, SOX-10, canine

INTRODUCTION

Oral malignant melanomas (OMMs) are the most common malignant oral neoplasm in dogs (1–3). They are locally invasive and have a high rate of metastasis to local lymph nodes and to the lungs (1, 2). An overall median survival time of 6 months has been reported (from surgical removal to time of death) (1, 2, 4). The mean age at diagnosis is 10–11 years of age, and certain breeds appear to be overrepresented, including poodles, golden and Labrador retrievers, Rottweilers, Yorkshire terrier, cocker spaniels, chow-chows, Scottish terriers, and dachshunds (2). Canine OMMs exhibit variable morphologic phenotypes, including epithelioid, spindloid, mixed, whorled, balloon cell, signet ring cell, clear cell, and adenoid cell types (5). In dogs, OMMs most commonly present, similarly to human mucosal melanomas, with an intraepithelial component (junctional activity), lentiginous spread, and extensive subepithelial vertical growth that causes the actual mass effect (5, 6). The spindloid phenotype represents 34% of OMMs according to Ramos-Vara et al. (7) and is diagnosed histologically based on the presence of melanin pigment and junctional activity. In the absence of these morphologic features, spindloid amelanotic OMMs are difficult to differentiate from oral soft tissue sarcomas (STS) microscopically (8). Such distinction is important as STS, in contrast to OMMs, rarely metastasize and are primarily treated with surgery and/or radiation therapy with a significantly longer reported median survival time of 540 days (8). While some antibodies such as Melan-A and PNL2 have been shown to be highly specific in differentiating OMMs from STS, other antibodies with high sensitivity for detecting OMMs, such as MITF-1 and S100, have poor specificity and also label a large percentage of STS (3). Currently, the most sensitive and specific method to diagnose amelanotic OMMs in dogs is immunohistochemical labeling with a cocktail of four antibodies: Melan-A, PNL2, and tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2) (3). We previously demonstrated that this antibody cocktail has 100% specificity and 93.9% sensitivity in detecting canine oral melanocytic neoplasms compared to STS (3). In that study, the spindloid phenotype was the least likely variant to label with the individual specific melanocytic markers and the antibody cocktail. In three out of nine cases of spindloid OMMs, the antibody cocktail did not label the spindle cell component in the subepithelial portion of the mass (vertical growth portion). Furthermore, intraepithelial neoplastic cells are the most likely neoplastic cells in OMMs to label with melanocytic markers as they are the most differentiated cells (3). As the epithelium overlying canine oral malignancies is commonly ulcerated, surgical biopsies of spindloid amelanotic OMMs often present a challenge in routine diagnostics. The true incidence of OMMs within this specific group of oral spindle cell tumors (OSCTs) that lack pigmentation and junctional activity is unknown, and identification of novel markers capable of differentiating between OMMs and STS would advance our diagnostic capabilities.

Antigens such as CD34, SOX-10, caldesmon, laminin, and desmin have been shown to be expressed in various types of STS in dogs (9); however, their utility to differentiate spindloid amelanotic OMMs from oral STS in dogs has not been studied in detail. In human melanomas, not only has the expression of melanoma-associated proteins been investigated with immunohistochemistry (IHC) but also numerous studies have investigated gene expression patterns for their diagnostic and prognostic utility (10, 11). The expression of tyrosinase has been especially investigated as it is both immunogenic and essential in key processes of melanogenesis (12). The expression of *TYR* mRNA has been detected in 100% of investigated human melanoma cell lines in some studies (10), and others found that high mRNA levels of *TYR* in human metastatic melanomas were predictive of overall improved survival (11). Similar studies have not been conducted in dogs. The goal of this study was to investigate the RNA and protein expression of melanoma-associated markers, as well as markers associated with STS, to establish expression patterns for these two entities and, ultimately, to accurately differentiate spindloid amelanotic OMMs from non-melanocytic OSCTs.

MATERIALS AND METHODS

Case Selection

Three groups, including OMMs, subcutaneous STS, and OSCTs, of 20 cases each were selected from the Michigan State University Veterinary Diagnostic Laboratory archives of formalin-fixed, paraffin-embedded tissues that had been submitted for routine surgical biopsy between 2008 and 2020. All samples had been fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin wax, sectioned at 5 μm, and routinely stained with hematoxylin and eosin. Diagnoses were independently established for all cases through review by two board-certified pathologists (MK and TT). The first group (OMM group) included 20 dogs with OMMs (**Figures 1A,B**) that were predominantly spindloid and exhibited junctional activity but were poorly pigmented (<5% of neoplastic cells). All cases in this group were positive for the melanoma diagnostic antibody cocktail (MDX) that contains antibodies against Melan-A, PNL2, TRP-1, and TRP-2. The second group (STS group) included 20 dogs with subcutaneous STS that were immunohistochemically negative for MDX and exhibited features of malignant fibrous tumors, malignant nerve sheath tumors, and malignant perivascular wall origin tumors (**Figures 2A,B**). The last group (OSCT group) included 20 dogs with OSCTs (**Figures 3A,B**), defined as being predominantly composed of spindloid neoplastic cells that had no pigmentation,

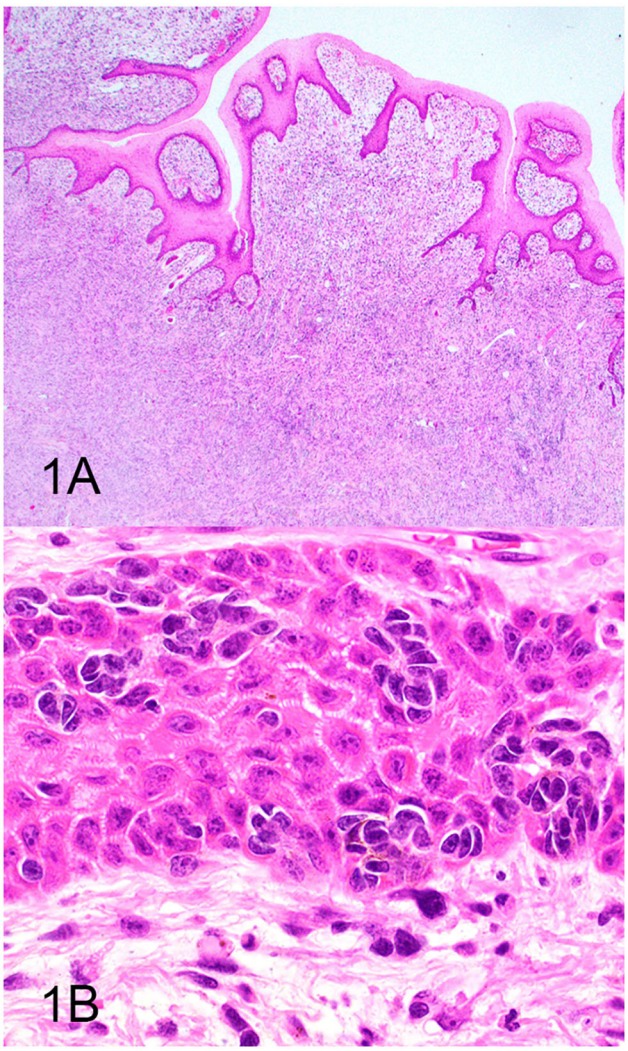

FIGURE 1 | Oral malignant melanoma (OMM), hematoxylin and eosin staining. **(A)** OMM with junctional activity and composed predominantly of subepithelial spindloid cells with <5% containing pigment. Magnification, ×4. **(B)** Higher magnification of **(A)** highlighting junctional activity (intraepithelial nests within the basal layer of the epithelium). Magnification, ×40.

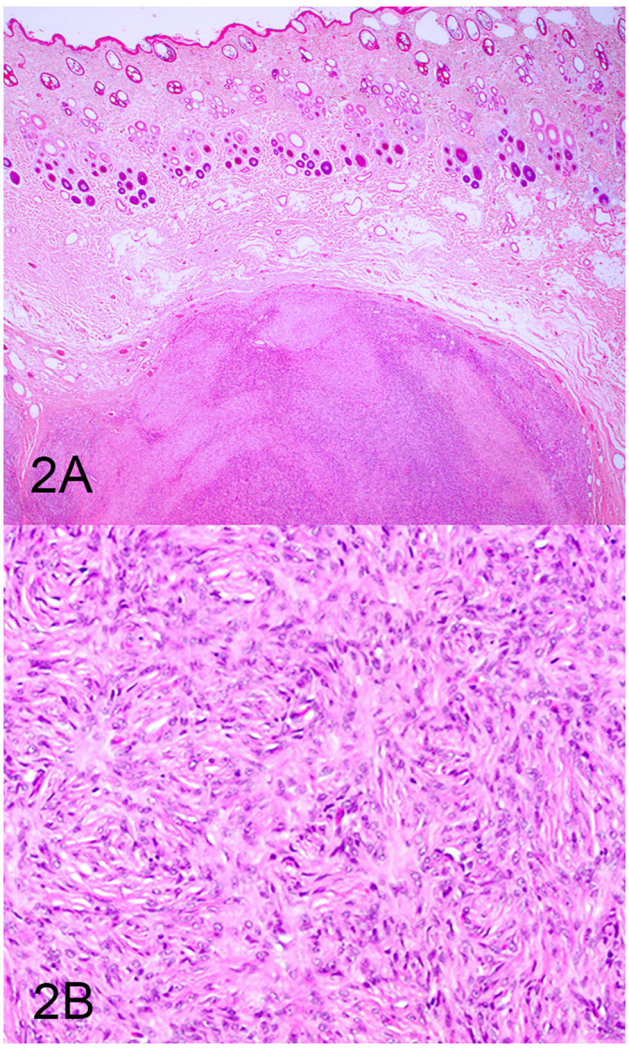

FIGURE 2 | Soft tissue sarcoma (STS), hematoxylin and eosin staining. **(A)** STS composed of sheets of spindloid cells. Magnification, ×4. **(B)** Higher magnification of **(A)** highlighting spindle cells arranged in a fingerprint pattern. Magnification, ×20.

that were negative for MDX and that were present at the epithelial–subepithelial junction but lacked junctional activity (neoplastic cells were not identified within the basal layer of the epithelium). These neoplasms were all classified as malignant based on their high cellularity, poor degree of differentiation, and invasion into the adjacent stroma.

RNA Extraction and RT-qPCR

Regions of high tumor cellularity (at least 80%), away from the overlying epithelium, from inflammation, and from necrosis, were selected (manually shaved) for RNA extraction from each case. Total RNA was isolated from formalin-fixed, paraffin-embedded tissue using RecoverAll™ Total Nucleic Acid Isolation Kit (ThermoFisher Scientific, catalog#AM1975) according to the protocol of the manufacturer. Following deparaffinization with CitroSolv, tumor tissue was incubated in proteinase K containing lysis buffer. RNA was quantified using Qubit (ThermoFisher Scientific, catalog#Q32852). Six hundred nanograms of total RNA was treated with TURBO DNA-free™ (ThermoFisher Scientific, catalog#AM1907) to remove contaminating DNA. First-strand cDNA synthesis was performed using SuperScript III Reverse Transcriptase (ThermoFisher Scientific, catalog#18080044) with random primers (Promega, catalog#C1181). The cDNA was then column-purified by using QIAquick PCR Purification Kit (QIAGEN, catalog# 28104) and eluted with distilled nuclease-free water at 5 ng/μl. For the determination of specific gene expression, each primer was designed with Primer3 software (13, 14). The primers for *TYR*, *SOX10*, *CALD1*, *CD34*, *DES*, and *LAMA1* are

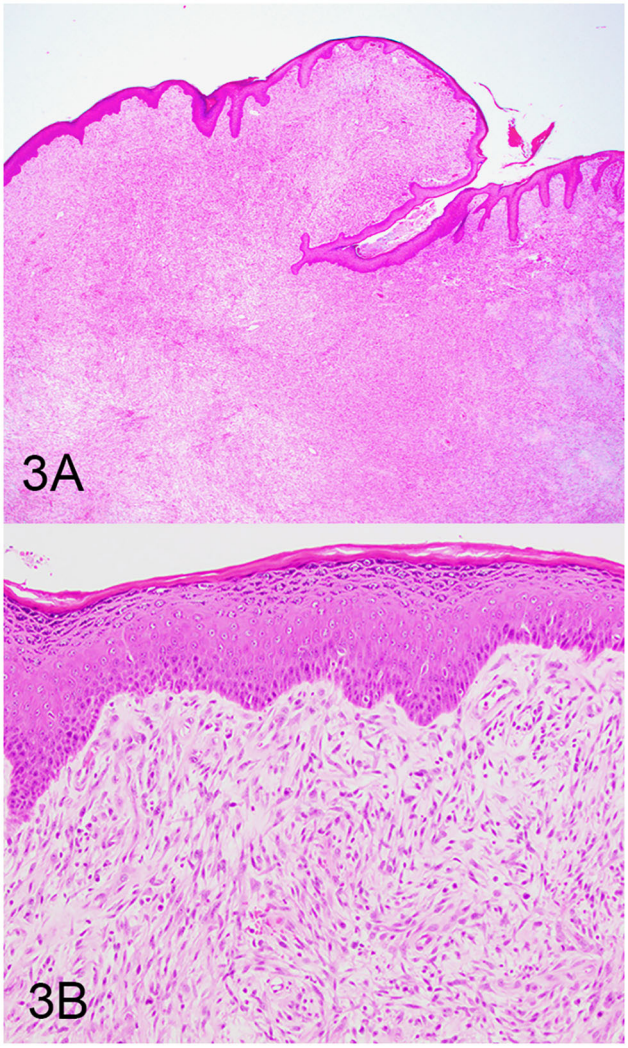

FIGURE 3 | Oral spindle cell tumor (OSCT), hematoxylin and eosin staining. **(A)** OSCT with no junctional activity, but the neoplastic cells are present at the epithelial–subepithelial junction, are predominantly spindloid, and have no pigment. Magnification, ×4. **(B)** Higher magnification of **(A)** highlighting neoplastic cells at the epithelial–subepithelial junction. Magnification, ×20.

listed in **Supplementary Table 1**. Ten nanograms of cDNA was used as the template in the reaction mixture for quantitative real-time PCR, using SYBR Green (ThermoFisher Scientific, catalog# 4309155) according to the recommendations of the manufacturer. The protocol was as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at a temperature suitable for each gene marker for 10 or 20 s, and extension at 72°C for 10 s. The baseline was set automatically, and the threshold Ct was defined as the number of cycles in which the fluorescence exceeded the automatically set threshold. A normal gingival tissue sample was chosen as a calibrator, and the ratio of each target gene to the beta-2 microglobulin expression for each tumor sample was normalized by the same ratio for the normal tissue sample using the delta-delta Ct ($\Delta\Delta$Ct) method. Each sample was assayed in triplicate. A control and a reference were included in every run.

Immunohistochemistry

Routine immunohistochemical labeling was performed as previously described (3) using MDX (which contains antibodies against Melan-A, PNL2, TRP-1, and TRP-2) and antibodies against SOX-10, laminin, desmin, and S100 (**Supplementary Table 2**). IHC for MDX, SOX-10, and laminin was performed on all three groups. In addition, IHC for desmin was performed on STS and OSCTs only, and IHC for S100 was performed on OSCTs only. For desmin, laminin, and S100, IHC was performed on a Bond autostainer automated system using BOND Polymer Refine Detection kit (Leica Microsystems). For MDX and SOX-10, IHC was performed on Dako Omnis using EnV FLEX HRP Magenta (Agilent). Positive and negative controls for each antibody were used appropriately in every run (**Supplementary Table 2**). IHC for tyrosinase was attempted using two different antibodies but was unsuccessful (data not shown).

Histological Examination

Immunohistochemically labeled sections were scored as previously described (3) by two board-certified pathologists (RS and MK). Briefly, percentages of positively labeled neoplastic cells were semi-quantitatively scored using the following cutoffs: ≤10% (considered negative and identified as *N*), 11–50% (assigned a score of 1), 51–80% (assigned a score of 2), or 81–100% (assigned a score of 3). For MDX, neoplasms were also considered positive if intraepithelial nests, consisting of at least five clustered neoplastic cells, labeled positively or if there were aggregates of at least 20 neoplastic cells that labeled positively anywhere within the neoplasm as previously described (3). The sensitivity of SOX-10 IHC as a melanocytic marker was determined based on the ability of the antibody to positively label the 20 OMMs, and specificity was determined based on the absence of labeling of the 20 STS, which served as negative controls.

Statistical Analyses

Data are presented as means ± SEM. The differential expression of OMM relative to STS genes was analyzed by Student's *t*-test. Cutoff values for relative RNA levels were determined using receiver operating characteristic (ROC) curve. Sensitivity was determined based on the number of cases (OMM or STS) above the established cutoff value; specificity was determined based on the number of (OMM or STS) cases below the established cutoff value. A value of $P \leq 0.05$ was considered statistically significant. Analyses were performed using Prism software, v.6.01 (GraphPad Software).

RESULTS

Based on the selection criteria, MDX IHC correctly labeled all 20 OMMs and did not label any of the 20 STS and none of the 20 OSCT (**Figure 4**; **Table 1**). In two OMM cases, MDX IHC labeling was limited to the intraepithelial nests of neoplastic

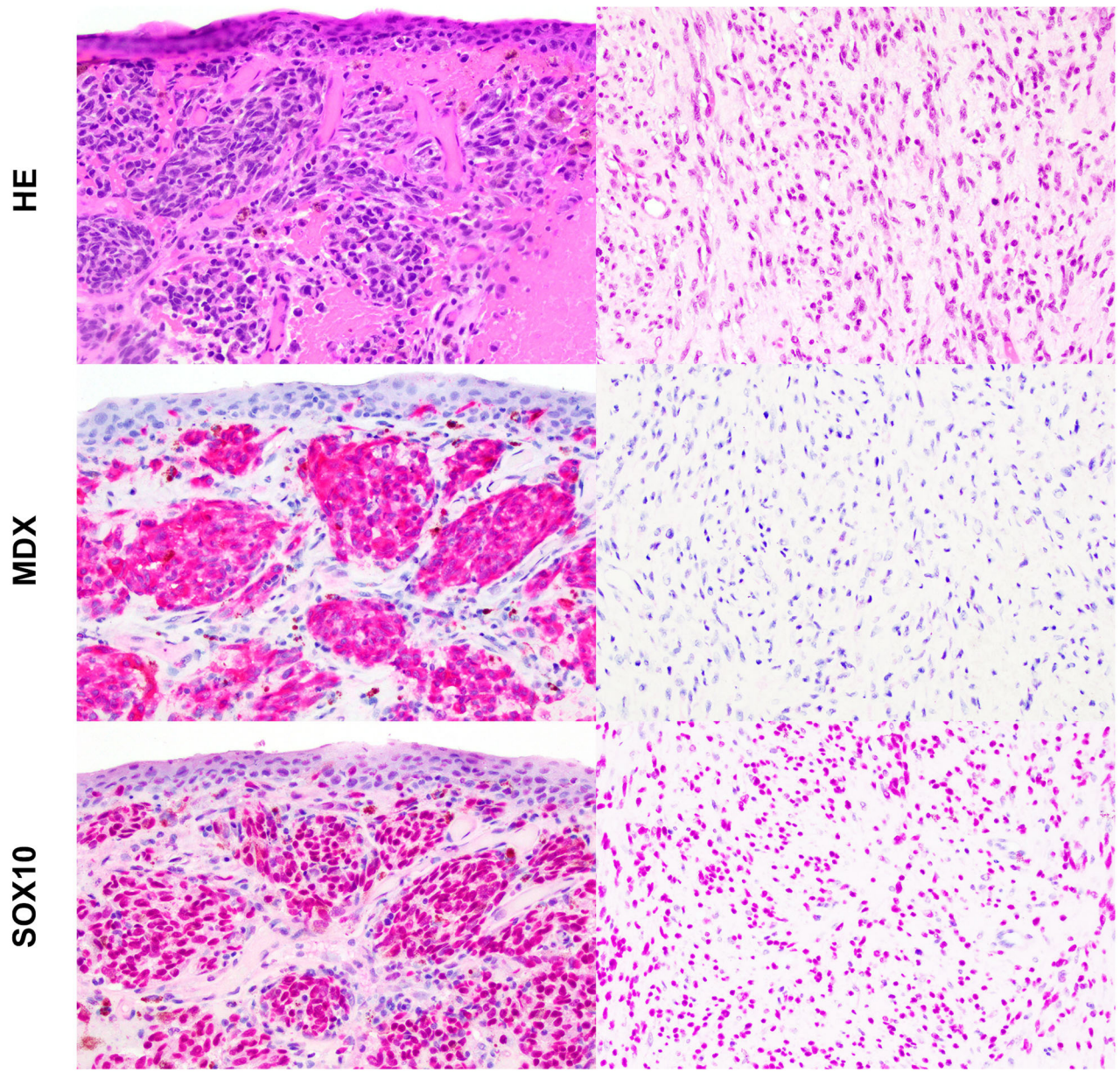

FIGURE 4 | Oral malignant melanoma (OMM) and soft tissue sarcoma (STS) stained with hematoxylin and eosin or immunohistochemically labeled with the melanoma immunodiagnostic cocktail (MDX; red cytoplasmic labeling) or SOX-10 (red nuclear labeling) with hematoxylin counterstaining. OMM with red cytoplasmic labeling for MDX with a score of 3 and red nuclear labeling for SOX-10 with a score of 3. STS negative for MDX and red nuclear labeling for SOX-10 with a score of 3. Magnification, ×20.

melanocytes and was absent in the subepithelial neoplastic spindloid melanocytes. SOX-10 IHC labeling was also seen in all 20 OMMs, but two STS labeled as well (100% sensitivity and 90% specificity in diagnosing OMM). Six of the OSCTs labeled for SOX-10 (**Figure 5**; **Table 2**). IHC labeling for laminin was detected in the basement membranes surrounding neoplastic cells in six OMMs and 18 STS (90% sensitivity and 70% specificity in diagnosing STS). Only one STS had cytoplasmic labeling for desmin. Sixteen OSCTs labeled for laminin and two for desmin (**Figure 5**). In addition, five OSCTs labeled for S100.

Following RT-qPCR for *TYR*, *SOX10*, *LAMA1*, *DES*, *CD34*, and *CALD1*, the cutoff values for relative RNA levels were determined using ROC curves (**Table 3**). Thirteen OMMs expressed *TYR* RNA levels above the cutoff value, all 20 OMMs

TABLE 1 | Number of cases with immunohistochemical expression of tested antibodies in oral malignant melanomas (OMM) and soft tissue sarcomas (STS).

	OMM			STS			
	MDX	**SOX-10**	**Laminin**	**MDX**	**SOX-10**	**Laminin**	**Desmin**
Epi only[a]	2	0	0	NA	NA	NA	NA
≤10%[b]	0	0	14	20	18	2	19
11–50%	5	0	4	0	0	10	1
51–80%	7	3	2	0	1	6	0
81–100%	6	17	0	0	1	2	0
Total # positive	20	20	6	0	2	18	1

NA, not applicable.
[a]*Expression limited to intraepithelial nests.*
[b]*Neoplasms with ≤10% of cell labeling were considered negative.*

expressed *SOX10* RNA above the cutoff value, and six and one OMM expressed *LAMA1* RNA and *DES* RNA above the cutoffs, respectively (**Table 4**). None of the OMMs expressed *CD34* RNA or *CALD1* RNA above the cutoffs. In contrast, none of the STS expressed *TYR* RNA levels above the cutoff value, and only three STS expressed *SOX10* RNA above the cutoff (**Table 5**). The two STS cases with the highest *SOX10* RNA expression also labeled for SOX-10 with IHC. All except one STS expressed *CD34* RNA above the cutoff. *CALD1* and *DES* RNA levels above the cutoffs were detected in 12 and seven STS, respectively. There was a statistically significant difference in the RNA expression levels of select genes in OMM relative to STS (**Figure 6**) for *TYR* (192,538 ± 65,305 vs. 107.5 ± 106.7; $p < 0.05$), *SOX10* (405.5 ± 93.65 vs. 35.14 ± 27.53; $p < 0.05$), *CD34* (4.462 ± 0.9124 vs. 372.9 ± 159.2; $p < 0.05$), and *CALD1* (24.21 ± 5.784 vs. 166.7 ± 43.97; $p < 0.01$). No significant difference in RNA expression levels was identified

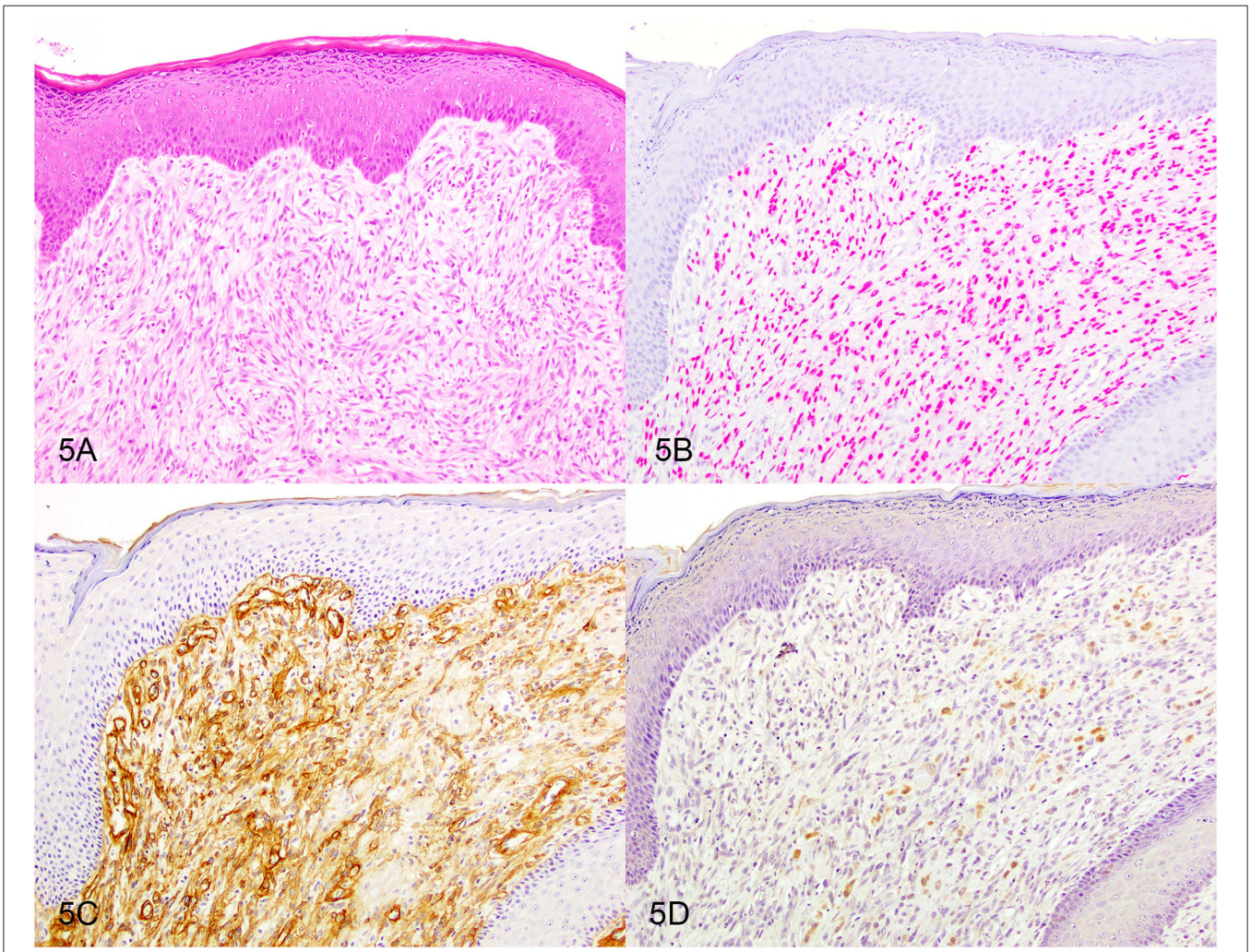

FIGURE 5 | Oral spindle cell tumor. Serial sections stained with **(A)** hematoxylin and eosin or immunohistochemically labeled for **(B)** SOX-10 showing red nuclear labeling or for **(C)** laminin showing brown basement membrane labeling or for **(D)** S100 showing brown cytoplasmic labeling. Magnification, ×20.

TABLE 2 | Number of cases with immunohistochemical expression of tested antibodies in oral spindle cell tumors (OSCT).

	MDX	SOX-10	Laminin	Desmin	S100
≤10%[a]	20	14	4	18	15
11–50%	0	1	10	0	2
51–80%	0	0	3	0	2
81–100%	0	5	3	2	1
Total number of positive	0	6	16	2	5

[a]*Neoplasms with ≤10% of cell labeling were considered negative.*

TABLE 3 | Cutoff values for RNA expression levels of each gene determined by receiver operating characteristic curve to establish sensitivity and specificity.

	Cutoff (arbitrary units)	Sensitivity (%)	Specificity (%)
SOX10[a]	19.4	100	85
TYR[a]	9,709	65	100
CD34[b]	15.9	95	100
CALD1[b]	90.0	60	100
DES[b]	0.065	35	95
LAMA1[b]	66.2	30	70

[a]*Sensitivity and specificity were calculated for oral malignant melanoma (OMM) relative to soft tissue sarcoma (STS).*
[b]*Sensitivity and specificity were calculated for STS relative to OMM.*

between OMMs and STS for *LAMA1* (241.8 ± 135.1 vs. 249.3 ± 149.4) and *DES* (0.1405 ± 0.1327 vs. 0.1040 ± 0.03911). Based on these data, the specificity and the sensitivity of *TYR* RNA levels for detecting OMM were 100 and 65%, respectively (**Table 3**). The specificity of *CD34* and *CALD1* RNA levels above the cutoff level for detecting STS was also 100%, and the sensitivity for these tests was 95 and 60%, respectively. In contrast, *SOX10* RNA levels had a specificity of only 85% for detecting OMM but a sensitivity of 100%. When analyzing the OSCT group by RT-qPCR, none of the cases expressed *TYR* RNA above the cutoff level, but 14 OSCTs expressed either *CD34* or *CALD1* RNA, consistent with a diagnosis of STS (**Table 6**). Of the remaining six OSCTs, three (#2, #18, and #19) expressed *SOX10* RNA above the cutoff level. Of these three OSCTs, one (#19) expressed *DES* RNA and was positive for laminin and negative for S100, desmin, and MDX by IHC. *SOX10* RNA was also expressed above the cutoff level in two OSCTs that had either *CD34* or *CALD1* RNA levels above the cutoffs (#1 and #20). In total, five OSCT cases expressed *DES* RNA and seven OSCT expressed *LAMA1* RNA above the cutoff. A final diagnosis for each OSCT was made based on the combined RNA expression and immunohistochemical labeling results. Fourteen OSCTs were diagnosed as STS based on RNA levels above the cutoffs for either *CD34* or *CALD1*, no expression of *TYR* RNA, and lack of labeling for MDX by IHC. Two of these 14 OSCTs labeled positively by IHC for desmin and five for S100, suggesting a differentiation toward pericytes or smooth muscle cells and nerve sheath cells, respectively. Two of the remaining six OSCTs were diagnosed as undifferentiated malignant neoplasms, as they lacked expression and labeling patterns that supported either an OMM or STS. The remaining four OSCTs were diagnosed as suspect STS. While three of those OSCTs had RNA expression levels of *SOX10* above the cutoff and immunohistochemically labeled for SOX-10, one OSCT also had RNA expression levels for *DES* above the cutoff, and all four OSCTs were immunohistochemically positive for laminin and negative for MDX and did not express *TYR* RNA, making a diagnosis of STS more likely.

DISCUSSION

Canine oral spindloid amelanotic malignant neoplasms present a diagnostic challenge for veterinary pathologists. As junctional activity and pigmentation are two of the most distinct diagnostic features of OMMs, tissues submitted for surgical biopsy that lack surface epithelium and pigmentation often require additional molecular testing for an accurate diagnosis. In this study, we were able to demonstrate that analysis of the expression of *TYR*, *CALD1*, and *CD34* RNA accurately differentiated STSs from OMMs. *TYR* encodes one of the most studied melanin enzymes that plays a crucial role in the early steps of melanin synthesis and has been detected in 100% of investigated human melanomas in some studies (8). The proteins encoded by *CD34* and *CALD1* have been shown to be expressed in various types of STS in dogs, but not melanocytic neoplasms (3, 7, 13). Furthermore, based on the results of this study, the immunodiagnostic MDX cocktail containing antibodies against Melan-A, PNL2, TRP-1, and TRP-2 remains the gold standard for accurately differentiating OMMs from STS and other undifferentiated malignant neoplasms in a routine diagnostic setting. While two of the cases in the OMM group could not be confirmed as OMM by IHC without inclusion of the overlying epithelium, the MDX cocktail was 100% specific and did not label any STS or OSCT in this study as defined by the selection criteria. We had hypothesized that some of the OSCTs could represent true OMMs that simply did not label with MDX IHC in the sections that were available for examination. Unexpectedly, this appears unlikely based on our results, especially the RNA expression analysis results, that any of the 20 OSCTs represent an OMM. A total of 14 of 20 OSCTs were instead confirmed as STS based on their RNA expression pattern, as these tumors had levels of *CALD1* or *CD34* RNA above the cutoff values and lacked the expression of *TYR* RNA. Of the remaining six OSCTs, all were negative for *TYR* RNA and lacked immunohistochemical labeling for S100, which has been reported to be commonly expressed in undifferentiated OMMs (7). One of these six OSCTs labeled strongly for laminin by IHC (in ≥81% of neoplastic cells), and a similar strong IHC labeling for laminin was not observed in OMMs in this study. Another one of these OSCTs expressed *DES* RNA levels above the cutoff, which is more consistent with a diagnosis of STS rather than OMM. Lastly, three of these six cases had *SOX10* RNA levels below the cutoff, making a diagnosis of OMM highly unlikely. Thereby, it is highly likely that 18/20 OSCTs in this study represented STS. Only two of the 20 OSCTs could not be classified as OMM or STS based on their RNA expression patterns and immunophenotyping and were diagnosed as undifferentiated malignant neoplasms. However, it

TABLE 4 | Relative RNA levels and immunoreactivity of oral malignant melanoma.

	RT-qPCR (relative RNA levels)						IHC (score *N*, 1, 2, 3)[a]		
	CALD1	**CD34**	**DES**	**LAMA1**	**SOX10**	**TYR**	**MDX**	**SOX-10**	**Laminin**
1	50	2.7	ND	15	585	1,014,163	3	3	*N*
2	42	11.2	0.06	239	1,788	29	1	3	2
3	6	0.8	ND	62	268	17,284	1	3	1
4	7	2.1	0.02	264	196	ND	3	3	*N*
5	12	2.9	ND	ND	421	372,535	2	3	*N*
6	24	13.0	ND	ND	279	467,252	2	3	*N*
7	23	11.4	ND	240	239	884,650	3	2	*N*
8	4	1.2	0.01	20	133	149,618	2	3	*N*
9	4	2.2	ND	66	314	235,907	2	3	*N*
10	14	3.2	ND	ND	29	ND	Epi	2	1
11	12	9.6	0.05	477	376	238,549	3	3	*N*
12	5	2.7	ND	8	154	ND	2	3	*N*
13	14	5.9	ND	2,691	545	103,153	2	3	1
14	41	9.7	ND	ND	176	49,260	3	3	*N*
15	22	1.5	2.66	664	964	191,577	3	3	*N*
16	89	1.8	ND	ND	318	ND	Epi	3	*N*
17	9	2.4	ND	ND	62	ND	1	3	*N*
18	89	1.8	ND	3	131	44,317	1	2	1
19	14	ND	ND	24	988	82,463	2	3	*N*
20[b]							1	3	2
20[b]	3	3.2	0.01	61	142	ND			

Gray background, RNA expression above cutoff or positive immunohistochemical labeling; ND, not detected; Epi, positive labeling was exclusively within the mucosal epithelium and thus precluded scoring.

[a] *Percentages of positive cells were assigned a score of N (≤10% positive labeling of neoplastic cells), 1 (11–50%), 2 (51–80%), or 3 (81–100%).*

[b] *Tissue in paraffin block was depleted, and another block from the same tumor was used for RT-qPCR.*

is highly unlikely that these neoplasms represent undifferentiated OMMs based on the absence of expression of both *TYR* and *SOX10* RNA as well as the lack of immunolabeling for MDX and SOX-10. These data clearly indicate that the incidence of STS among canine oral spindloid amelanotic neoplasms that lack junctional activity is significantly higher than assumed and that such cases require additional testing to avoid misdiagnosis as an OMM.

Intraepithelial nests of neoplastic melanocytes tend to represent the most differentiated stage of neoplastic melanocytes, and the inclusion of intact overlying epithelium in biopsies is essential for making an accurate diagnosis of spindloid amelanotic OMM (3, 15). While the MDX IHC cocktail has been shown to be highly sensitive and specific in detecting OMMs, we propose, based on the data presented here, that for OSCT cases that lack surface epithelium and that are MDX-negative, the relative expression levels of *TYR*, *CD34*, and *CALD1* RNA should be evaluated to discriminate between OMMs and STS.

SOX-10 is a transcription factor essential for neural crest and peripheral nervous system development and the formation of melanocytes (16). It has recently been used as a diagnostic IHC marker for human melanocytic neoplasms, as it is highly sensitive for detecting such tumors, including spindloid and desmoplastic subtypes (17). However, SOX-10 is essentially a pan-Schwannian and melanocytic marker (18). It commonly labels human peripheral nerve sheath tumors, including neurofibromas (95–98%), schwannomas (98–100%), and malignant peripheral nerve sheath tumors (29–50%), and has also been detected in myoepitheliomas, granular cell tumors, and mammary carcinomas, among others (19, 20). STS are much less common in humans than in dogs and, as such, are rarely considered as a differential for spindloid amelanotic melanomas. The use of SOX-10 IHC for the diagnosis of OMM in dogs had not yet been validated. In our study, SOX-10 IHC had 100% sensitivity but only 90% specificity, and, similarly, there was 100% sensitivity and only 85% specificity using relative *SOX10* RNA levels to discriminate between OMM and STS. The evaluation of SOX-10 expression by both IHC and quantitation of *SOX10* RNA expression in routine veterinary diagnostics is of limited use for diagnosing melanomas, similar to the evaluation of S100 or MITF expression (5). The lack of immunoreactivity for SOX-10 may be useful to exclude a melanocytic neoplasm, but labeling for SOX-10 should not be used as a single criterion for confirmation of a diagnosis of OMM. While in our study only 2/20 STS had immunoreactivity for SOX-10 and an additional tumor expressed *SOX10* RNA levels above the cutoff, five cases in the OSCT group had a strong immunoreactivity for SOX-10 and *SOX10* RNA levels above the cutoff. This difference in the percentage of cases in which SOX-10 expression was identified between STS and OSCT raises concern that our selection of STS may

TABLE 5 | Relative RNA levels and immunoreactivity of soft tissue sarcoma.

	RT-qPCR (relative RNA levels)						Immunohistochemistry (score *N*, 1, 2, 3)[a]			
	CALD1	**CD34**	**DES**	**LAMA1**	**SOX10**	**TYR**	**MDX**	**SOX-10**	**Laminin**	**Desmin**
1	91	18.7	0.01	8	ND	2,134	*N*	*N*	1	*N*
2	547	903.8	0.06	66	ND	ND	*N*	*N*	1	*N*
3	54	127.5	0.02	ND	1	ND	*N*	*N*	1	*N*
4	33	95.1	ND	64	ND	ND	*N*	*N*	1	*N*
5	727	895.5	0.07	ND	41	ND	*N*	*N*	1	*N*
6	118	254.8	0.03	ND	ND	ND	*N*	*N*	1	*N*
7	16	120.5	0.01	26	1	ND	*N*	*N*	1	*N*
8	279	137.6	ND	1,416	1	ND	*N*	*N*	2	*N*
9	415	3,192.8	0.36	36	10	ND	*N*	*N*	1	*N*
10	97	29.3	ND	ND	548	ND	*N*	3	3	*N*
11	51	117.6	0.48	121	ND	ND	*N*	*N*	1	*N*
12	15	5.8	ND	115	100	ND	*N*	2	3	*N*
13	153	85.9	0.08	2,762	ND	ND	*N*	*N*	*N*	*N*
14	124	68.7	ND	202	ND	16	*N*	*N*	*N*	*N*
15	40	318.2	0.11	23	ND	ND	*N*	*N*	1	*N*
16	342	304.7	0.02	125	ND	ND	*N*	*N*	2	*N*
17	106	396.0	0.19	3	ND	ND	*N*	*N*	2	*N*
18	40	303.4	0.61	7	ND	ND	*N*	*N*	2	1
19	28	25.0	0.02	8	ND	ND	*N*	*N*	2	*N*
20	58	57.2	0.01	2	ND	ND	*N*	*N*	2	*N*

Gray background, RNA expression above cutoff or positive immunohistochemical labeling; ND, not detected.

[a]*Percentages of positive cells were assigned a score of N (≤10% positive labeling of neoplastic cells), 1 (11–50%), 2 (51–80%), or 3 (81–100%).*

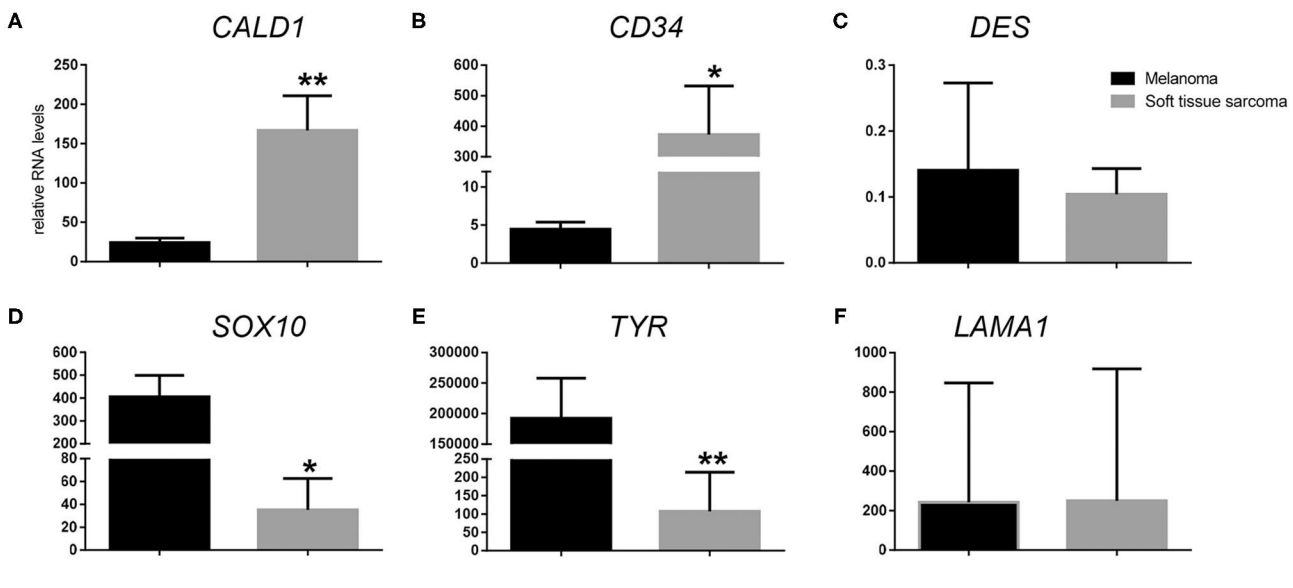

FIGURE 6 | Relative RNA expression levels of **(A)** *CALD1*, **(B)** *CD34*, **(C)** *DES*, **(D)** *SOX10*, **(E)** *TYR*, and **(F)** *LAMA1* in oral malignant melanoma vs. soft tissue sarcoma. $^{*}p < 0.05$, $^{**}p < 0.01$.

have been biased toward less differentiated tumors, leading to an underestimation of the number of SOX-10-positive STS. The reason for this speculation is that none of the five OSCTs with immunoreactivity for SOX-10 and *SOX10* RNA levels above the cutoff had expression levels of *TYR* RNA above the cutoff, which does not support a diagnosis of OMM. Furthermore, the RNA levels of *CALD1* and *CD34* were above the cutoffs for two of these five OSCT. There was 100% specificity for a

TABLE 6 | Relative RNA levels and immunoreactivity of oral spindle cell tumors.

	RT-qPCR (relative RNA levels)						Immunohistochemistry (score *N*, 1, 2, 3)[a]					Diagnosis[b]
	CALD1	CD34	DES	LAMA1	SOX10	TYR	MDX	SOX-10	Laminin	Desmin	S100	
1	219	7.5	0.01	38	236	ND	*N*	3	3	*N*	*N*	STS
2	53	4.1	0.03	44	704	ND	*N*	3	2	*N*	*N*	Suspect STS
3	125	98.7	ND	42	ND	231	*N*	*N*	2	*N*	2	STS
4	4	2.1	ND	77	ND	ND	*N*	*N*	*N*	*N*	*N*	UMN
5	219	3.7	0.46	6	ND	229	*N*	*N*	1	*N*	*N*	STS
6	57	16.4	0.73	571	ND	ND	*N*	*N*	1	3	*N*	STS
7	397	24.3	4.43	82	ND	7	*N*	*N*	1	3	*N*	STS
8	79	22.3	ND	ND	ND	ND	*N*	*N*	1	*N*	*N*	STS
9	168	24.6	0.01	16	2	9	*N*	*N*	1	*N*	*N*	STS
10	70	58.2	0.01	134	ND	ND	*N*	*N*	1	*N*	3	STS
11	9	37.3	ND	ND	ND	ND	*N*	*N*	*N*	*N*	*N*	STS
12	35	14.5	0.01	58	ND	ND	*N*	*N*	1	*N*	*N*	UMN
13	21	19.2	0.05	6	ND	82	*N*	*N*	*N*	*N*	*N*	STS
14	10	3.5	0.04	19	10	1	*N*	1	1	*N*	*N*	Suspect STS
15	34	21.0	0.16	71	ND	ND	*N*	*N*	1	*N*	*N*	STS
16	111	112.5	ND	530	ND	ND	*N*	*N*	2	*N*	1	STS
17	12	84.6	ND	ND	ND	ND	*N*	*N*	*N*	*N*	1	STS
18	7	4.0	ND	47	195	18	*N*	3	3	*N*	*N*	Suspect STS
19	12	10.1	0.11	ND	56	ND	*N*	3	1	*N*	*N*	Suspect STS
20	11	16.0	ND	114	79	ND	*N*	3	3	*N*	2	STS

Gray background indicates RNA expression above cutoff or positive immunohistochemical labeling.

ND, not detected; STS, soft tissue sarcoma; UMN, undifferentiated malignant neoplasm with no gene expression or immunohistochemical features of STS or oral malignant melanoma.

[a] *Percentages of positive cells were assigned a score of N (≤10% positive labeling of neoplastic cells), 1 (11–50%), 2 (51–80%), or 3 (81–100%).*

[b] *Final diagnosis based on RNA expression and immunohistochemical labeling.*

diagnosis of STS using an evaluation of *CALD1* and *CD34* RNA expression levels; following this paradigm, having the expression of *CALD1* and *CD34* RNA above the cutoff levels for detection excluded a diagnosis of OMM for these two cases. Lastly, one of the remaining three OSCTs with immunoreactivity for SOX-10 and *SOX10* RNA levels above the cutoff had RNA expression levels for *DES* RNA above the cutoff, and all three cases were immunohistochemically positive for laminin and negative for S100, which are features supportive of a diagnosis of STS.

To summarize, of the 20 OSCT cases, there were only five that could have been potentially diagnosed as OMMs based on the SOX-10 IHC and/or *SOX10* RNA data. Two of these were instead confirmed as STS based on *CALD1* and *CD34* RNA expression levels. For the remaining three cases, the lack of S100 labeling, expression of *DES* RNA above the cutoff in one case, and varying degrees of laminin labeling make a diagnosis of STS highly likely; therefore, these cases were diagnosed as suspect STS. One more case was diagnosed as a suspect STS, as it lacked an expression not only of *SOX10* and *TYR* RNA but also of *CALD1*, *CD34*, and *DES* RNA. In addition, it was negative for S100 and expressed low levels of laminin and low levels of SOX-10. An alternative classification of this case as an undifferentiated malignant neoplasm could be argued, considering the low numbers of IHC-positive cells.

Laminin is a key component of basement membranes and has been used as a marker for malignant peripheral nerve sheath tumors in dogs (9). Interestingly, in a minipig model of melanomas, neoplastic melanocytes were surrounded by the granular expression of laminin as evaluated by immunofluorescence (21). In humans, immunohistochemical labeling for laminin surrounding neoplastic melanocytes has been identified within the dermis (22). Neither RNA nor the protein expression of laminin has previously been investigated in canine OMMs. In our study, there was immunoreactivity for laminin in the pericellular matrix surrounding STS cells in 90% (18/20) of cases and surrounding neoplastic melanocytes in 30% (6/20) of cases. As such, the use of laminin IHC lacks the specificity needed to be a useful marker to discriminate between OMMs and STS. Additionally, the correlation between IHC and RNA levels for laminin was very poor, most likely due to sample heterogeneity within the analyzed portions of the mass. In general, sample heterogeneity may have a negative impact on both IHC results and RNA expression levels regardless of the target, especially when evaluating small punch biopsies. However, based on the consistency of RNA expression and IHC data in our study, the analysis of regular biopsy samples minimized the impact of sample heterogeneity for most evaluated targets, e.g., SOX-10.

Based on the combination of IHC and RNA expression results, OSCTs represent a highly heterogeneous group of neoplasms, with most representing soft tissue sarcomas. As determined by immunoreactivity for desmin and S100, two and five OSCT

cases had myopericyte and nerve sheath cell differentiation, respectively. Despite the limitations of using laminin IHC as a diagnostic criterion as discussed above, six OSCT cases had a widespread laminin immunoreactivity (score 2–3), and half of these cases were also positive for S100 by IHC, supporting nerve sheath cell differentiation. All five S100 IHC-positive OSCTs also had RNA expression levels for *CD34* above the cutoff, supporting a differentiation toward a perivascular wall tumor phenotype. Interestingly, while one of the OSCTs that was immunoreactive for desmin also had an RNA level of *CALD1* above the cutoff, supporting myopericyte differentiation, the other desmin IHC-positive case had low *CALD1* RNA levels. Of the 12 OSCTs with *CD34* RNA expression levels above the cutoff, four also had *CALD1* RNA levels above the cutoff, and three had *DES* RNA levels above the cutoff. Most surprisingly, two OSCTs had RNA levels above the cutoffs for both *CALD1* and *DES*, and one of these two cases also had *CD34* RNA levels above the cutoff. It is unclear whether these results simply reflect the heterogeneity of the analyzed samples (e.g., high vascularity) or if such diverse expression patterns are a reflection of the reactivation of genes that encode different lines of mesenchymal differentiation by malignant mesenchymal cells (9).

Tyrosinase is a copper-containing membrane glycoprotein that represents a key enzyme in the initiation of melanogenesis (23). The expression of *TYR* RNA has been detected in human melanoma cell lines (10). As expected, *TYR* RNA expression was highly specific for the detection of OMM in this study, and none of the STS or OSCT expressed *TYR* RNA above the cutoff. While the high specificity of *TYR* RNA expression for the detection of OMM could be exploited as a future diagnostic test, a sensitivity of 65% was an unexpectedly low result. In a study in human melanomas, only 74% of metastatic melanomas expressed *TYR* RNA (10). The lack of pigmentation in amelanotic or metastatic melanomas has been suggested as the cause for the inability to detect *TYR* RNA (24). All OMMs in this study were poorly pigmented, and RNA was extracted from large tumor areas rather than from sites where neoplastic cells were pigmented. This explanation is further supported by the negative *TYR* RNA results for the two OMMs that were only MDX-positive within intraepithelial nests, as the epithelium was excluded from RNA extraction in this study. Increased copy number gains in 8q24 at *MYC* have been detected in 90% of cutaneous melanomas in humans (24). The oncogene c-myc has been shown to have upstream regulatory effects on melanogenesis through suppression of the microphthalmia-associated transcription factor (MITF), which regulates the expression of tyrosinase (25). The downregulation of *TYR* has been observed in amelanotic melanomas with gains in 8q24 as a result of this mechanism (24). A similar genetic alteration may have caused the loss of expression of *TYR* RNA in our study. As the immunogenic tyrosinase has been found to be overexpressed in malignant melanocytes, as compared to normal cutaneous melanocytes (26), vaccines against tyrosinase that elicit a cytotoxic T cell immune response that targets melanocytes expressing tyrosinase have been used successfully in humans (27). Similarly, an important therapeutic option for OMMs is the xenogeneic human tyrosinase DNA-based vaccine, ONCEPT® (28). Interestingly, in our study, only 13 OMMs had extremely high relative levels of *TYR* RNA (to the order of 10^5), while seven cases had very low to imperceptible levels of relative RNA expression. An association between *TYR* RNA expression levels in canine OMM and responsiveness to the ONCEPT® vaccine has not been investigated. In a follow-up study, we intend to investigate a potential correlation between *TYR* RNA expression levels by OMMs and their responsiveness to the ONCEPT® canine melanoma vaccine.

CONCLUSION

In conclusion, this study determined that relative RNA expression levels of *TYR*, *CD34*, and *CALD1* discriminate between canine oral melanomas and soft tissue sarcomas. Moreover, RT-qPCR for the RNA expression of *TYR*, *CD34*, and *CALD* may be a useful diagnostic tool following a negative MDX result in suspected spindloid amelanotic OMM cases that lack an overlying epithelium.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MT generated, analyzed, interpreted data, and drafted the manuscript. TT created the study concept and design, generated, analyzed, interpreted data, and revised the manuscript. RS and MK created the study concept and design, interpreted data, and revised the manuscript. EN created the study concept and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.701457/full#supplementary-material

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H2AFZ: A Novel Prognostic Marker in Canine Melanoma and a Predictive Marker for Resistance to CDK4/6 Inhibitor Treatment

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Uncontrolled proliferation is a key feature of tumor progression and malignancy. This suggests that cell-cycle related factors could be exploited as cancer biomarkers and that pathways specifically involved in the cell cycle, such as the Rb-E2F pathway, could be targeted as an effective anti-tumor therapy. We investigated 34 formalin-fixed paraffin-embedded (FFPE) tissue samples of canine cutaneous melanocytoma, cutaneous melanoma, and oral melanoma. Corresponding clinical follow-up data were used to determine the prognostic value of the mRNA expression levels of several cell cycle regulated E2F target genes (E2F1, DHFR, CDC6, ATAD2, MCM2, H2AFZ, GINS2, and survivin/BIRC5). Moreover, using four canine melanoma cell lines, we explored the possibility of blocking the Rb-E2F pathway by using a CDK4/6 inhibitor (Palbociclib) as a potential anti-cancer therapy. We investigated the expression levels of the same E2F target gene transcripts before and after treatment to determine the potential utility of these molecules as predictive markers. The E2F target gene H2AFZ was expressed in 91.43% of the primary tumors and H2AFZ expression was significantly higher in cases with unfavorable clinical outcome. Among the other tested genes, survivin/BIRC5 showed as well-promising results as a prognostic marker in canine melanoma. Three of the four tested melanoma cell lines were sensitive to the CDK4/6 inhibitor. The resistant cell line displayed higher expression levels of H2AFZ before treatment compared to the CDK4/6 inhibitor-sensitive cell lines. The present results suggest that CDK4/6 inhibitors could potentially be used as a new anti-cancer treatment for canine melanoma and that H2AFZ could serve as a prognostic and predictive marker for patient selection.

Keywords: dog, melanoma, cancer biomarker, CDK4/6 inhibitor, E2F target genes

INTRODUCTION

Increased proliferation is an important characteristic of tumorigenesis. The proliferation rate is routinely estimated immunohistochemically using the Ki67 antibody (MIB-1). The level of Ki67 expression (as Ki67 index) is widely accepted as an indicator of prognosis and used as a prognostic marker in a number of human (1) and canine cancers (2–6). This indicates that a high degree

of proliferation is a common feature of tumor malignancies and their progression, suggesting the potential to target molecules and pathways specifically involved in cell proliferation and cell cycle as an effective anti-tumor therapy (7).

One of the most often deregulated pathways during cancer development and progression is the Rb-E2F pathway. This pathway is initiated by cyclin-dependent kinases (CDKs) that are able to phosphorylate the Rb protein. Once Rb is hyperphosphorylated, it detaches from the E2F proteins. E2F family members are transcription factors playing a crucial role in the regulation of cellular proliferation, apoptosis, and differentiation. The E2F family consists of activator E2Fs and repressor E2Fs that together provide a balanced regulation of the expression of E2F transcriptional target genes (8). E2F target genes are involved in cell cycle regulation, DNA replication, DNA repair, and mitosis (9), thus, their transcripts seem to represent valuable prognostic biomarkers in cancer. Indeed, to date several E2F target genes have been proposed as tissue prognostic markers in different human tumor types, including non-small cell lung cancer, lymphoblastic leukemia, and gastric cancer (10–16). However, data on E2F target genes in canine cancers are extremely limited. Survivin, a protein encoded by the E2F target gene *BIRC5*, has been suggested as a prognostic marker in several canine tumors, including cutaneous melanoma (17).

In dogs, melanocytic tumors can arise from the skin and from the mucosa, with the oral mucosa being the most frequent location of this type. While cutaneous melanocytic tumors in dog are frequently bening (called melanocytoma), canine oral malignant melanoma (MM) is in general a highly aggressive tumor, with high similarities with the human counterpart (18–20). Similar to human mucosal MM, canine oral MM is often associated with an aggressive malignant behavior with rapid invasion of surrounding normal tissues, frequent metastasis (21), and resistance to therapy (22).

Although numerous works have aimed to find new effective prognostic markers for canine MM, there is still a lack of reliable ones, and Ki67, beside specific histological features of malignancy, remains the only currently available established prognostic marker (2, 23, 24). However, there are still some limitations to its use, such as the presence of some cases with low Ki67 index but poor prognosis (2), the lack of consistency in Ki67 assessment on microscope, and inter-observer variations (25).

CDK4/6 inhibitors, namely palbociclib, ribociclib, and abemaciclib, represent novel effective therapies and are currently Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved for the treatment of breast cancers in humans, in combination with other therapeutics (26, 27). They share the same mechanism of action-based on the specific inhibition of the Rb-E2F pathway and consequent cell cycle arrest. A growing body of evidence indicates that these drugs could be used for other malignancies, such as those with poor prognoses, including metastatic melanoma (27). Cyclin-dependent kinases CDK4 and CDK6 represent promising therapeutic targets in human melanoma. Their inhibition affects cancer cell proliferation, blocking the progression from G1 to S phase, and inhibit the metastatic potential of melanoma cells, reducing their migration and angiogenesis (28). Furthermore, CDK4/6 inhibition is able to induce anti-tumor immunity by increasing tumor immunogenicity. This effect seems to be mediated by different mechanisms: enhancing tumor antigen presentation, suppressing the proliferation of regulatory T cells (Tregs) and, as a consequence, promoting cytotoxic T cell-mediated clearance of tumor cells (29). Specifically, the anti-proliferative effects of CDK4/6 inhibitors on both tumor cells and Tregs appear to be associated with reduced activity of DNA methyltransferase 1, that is encoded by the E2F target gene *DNMT1* (29). In dogs, CDK4/6 inhibitors have been recently proposed for the treatment of canine mammary tumors, based on promising *in vitro* investigations (30).

In order to select patients for this specific type of therapy, biomarkers are required. Loss of function of the Rb protein, as well as Cyclin E and E2F3 gene amplification have been identified as new putative markers of CDK4/6 inhibitor resistance (31). CDK4, CDK6, and cyclin D1 amplification, and CDKN2A loss are considered markers of CDK4/6 inhibitor sensitivity (31). Since the Rb-E2F pathway ends with the activation of E2F target genes, their expression levels could be investigated as potential sensitivity or resistance markers of CDK4/6 inhibition.

The aim of the present work was to investigate the prognostic utility of a pool of E2F target genes by correlating their levels of expression in primary melanocytic tumors with clinical follow-up data. Secondly, we wanted to investigate the effect of a CDK4/6 inhibitor in four oral melanoma cell lines to provide a valid scientific rationale for the use of this approach in the treatment of canine oral melanoma. Finally, the variations of E2F target gene expression in treated and untreated, resistant or sensitive cell lines were analyzed in order to understand the potential utility of these molecules as predictive biomarkers.

MATERIALS AND METHODS

Patients' Selection, Follow Up Data Collection

A retrospective study was performed on formalin-fixed, paraffin-embedded (FFPE) tissue samples submitted to the diagnostic laboratory or veterinary teaching hospitals of the Department of Veterinary Medicine of the University of Perugia in the period between 2009 and 2016. Cases were included in the present study only if they met the following inclusion criteria:

- good quality of the specimen (i.e., no artifacts due to incomplete fixation or fulguration artifacts);
- diagnosis of primary mucosal melanoma, cutaneous melanoma, or cutaneous melanocytoma, confirmed by immunohistochemistry (positivity for either Melan A or PNL2) for poorly pigmented or amelanotic cases;
- no antineoplastic therapy (i.e., chemotherapy, radiation) before surgery/biopsy;
- for incisional biopsies of large tumors, a surface area on cut section >1 cm;
- availability of follow-up information and a minimum follow-up period of 365 days after surgery.

From our sample series tumor relapses and metastases were excluded.

Thirty-four melanocytic tumors (14 oral melanomas, 16 cutaneous melanomas, 4 cutaneous melanocytomas) were selected. Follow-up information was collected for each case: clinicopathological information was retrieved through telephonic interviews with referring veterinarians or through the collection of medical records data from internal cases (**Supplementary Table 1**). The clinical outcome of dogs that died because of the tumor was considered "unfavourable," while the one of patients that survived or died due to causes unrelated to melanoma was considered "favourable." The overall survival (OS) was the time from first diagnosis/appearance to death for any cause; disease-free survival was the time from first diagnosis/appearance to the first event of recurrent disease or metastasis or death. The recurrence was considered as the reappearance of the tumor at the site of origin after removal.

Tumor Tissue Collection and Histologic Examination

Samples in this study were partially included in a previous study on the usefulness of tumor thickness and modified Clark level for the evaluation of canine melanocytic tumors (24). All samples were histologically evaluated for the parameters having the greater validity for prognostic use in canine melanocytic neoplasia according to the current literature (23, 24).

Sections (4 μm thick) were cut from the paraffin blocks and stained with H&E. The tumors were evaluated blindly and independently by two boarded pathologists (CB, IP). Tumors were reclassified using histologic parameters with the highest level of statistically supported validity for prognostic use in canine melanocytic neoplasia, based on the recent classification (23, 24). According to these criteria, all tumors that had concomitant lymph node/distant metastases had been correctly classified as malignant ("melanoma"). Mitotic count was performed for each case counting the number of mitotic figures in 10 contiguous non-overlapping HPF (40X objective and ocular of 22 mm), starting from the area of highest mitotic activity and avoiding areas with necrosis or severe inflammation. Poorly pigmented, amelanotic melanoma cases were confirmed by immunohistochemistry using anti-MelanA (mouse monoclonal, A103-M27C10-M29E3, Abcam, ab200544, antigen retrieval in Tris-EDTA pH 9.0, dilution 1:150) and anti-PNL2 antibodies (mouse monoclonal, Santa Cruz, sc-59306, antigen retrieval in Citrate buffer pH 6.0, dilution 1:150). Ki67 index was evaluated on slides stained by immunohistochemistry using anti-ki67 antibody (clone MIB-1, Code number: GA626, Agilent-Dako, Glostrup, Denmark, antigen retrieval in Tris-EDTA pH 9.0, dilution 1:150), and calculated by means of image analysis on a minimum of five photos (40x), acquired from intratumoural hotspots. Areas of necrosis, inflammation, and superficial ulceration were avoided. Pictures were taken from non-contiguous hot spot areas where Ki-67 seemed to be highly expressed. The minimum number of cells/cases to count was set to 1,000. The count was performed manually by one operator using the Multi-point tool of ImageJ software (NIH, Bethesda, MD, USA). Ki67 index was calculated as the number of positive cells on the total number of counted cells. Negative and positive controls (**Supplementary Figure 1**) were applied in the immunohistochemical experiments for the three antibodies.

Cell Lines

Four canine melanoma cell lines were used (**Supplementary Table 2**): LMCK, CMM10, and CMM12 were kindly provided by N. Sasaki, T. Nakagawa, K. Saeki, University of Tokyo, Japan (32); OLGA by Raffaella de Maria, University of Turin, Italy (33). The cell lines had been regularly tested and confirmed to be mycoplasma-free. LMCK, CMM10, and CMM12 had been in culture for >70 passages at the time of this study, and OLGA cells were at passage 20. LMCK, CMM10, and CMM12 cell lines were maintained in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F-12, Invitrogen, Waltham, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/streptomycin (P/S, Invitrogen). OLGA cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% P/S. The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. All cell lines grew as monolayer cultures and were maintained by passage when they reached over 90% confluence.

Genes and Primers Selection

To identify candidate biomarkers, we analyzed previously published gene expression signature data from tumor cell lines and tumor tissue samples (34–38) for E2F target genes that are highly expressed in tumors. The most abundantly expressed E2F target genes, with prognostic significance and that had been validated as classical E2F target genes using ChIP-sequencing (39) were selected: *E2F1, DHFR, CDC6, ATAD2, MCM2, H2AFZ, GINS2,* and survivin/BIRC5.

Canine primers for E2F target gene transcripts (**Supplementary Table 3**) were designed using the Primer3 design tool and were based on coding mRNA sequences. Canine primers for housekeeping genes GAPDH and RPS5 (**Supplementary Table 3**) were kindly provided by Louis Penning (Utrecht University, the Netherlands).

RNA Isolation and RT-qPCR

In order to isolate RNA from cells, cells pellets were lysed in RLT buffer (included in the RNeasy mini kit, Qiagen) containing 10% ß-Mercaptoethanol (Merck). RNA was isolated from cells using the RNeasy mini kit (Qiagen, cat. n. 74104) according to the manufacturer's procedure. The optional DNase digestion step was also included.

In order to isolate RNA from FFPE tumor tissue samples, five, 5 μm-thick paraffin sections were obtained for each sample. An RNA isolation commercial kit was used following the manufacturer's instructions (Purelink FFPE Total RNA Isolation Kit, Invitrogen, cat. n. K1621), where at first a deparaffinization step was included. Briefly, 300 μl Melting Buffer was added to FFPE samples, then the samples were centrifuged for 10–20 s at maximum speed (13,000 g) and incubated for 10 min at 72°C. Next, samples were incubated with 20 μl Proteinase K (20 mg/ml) with occasional mixing for 3 h at 60°C directly followed

by centrifugation for 1 min at maximum speed. The lysate below the formed thin paraffin layer was transferred into clean RNase-free tubes for the RNA isolation step. Both tissue and cellular RNA quantity and quality were assessed with a Nanodrop® ND-1000 spectrophotometer.

In order to perform reverse transcription and quantitative real-time PCR, cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Invitrogen) with random hexameric primers. In the qPCR reactions, an equivalent of 10 ng RNA was used with 3 μl 1.5 μM primermix (Biolegio, Nijmegen, the Netherlands), containing both forward and reverse primers, and 12.5 μl SYBR® Green mastermix (Bio-Rad, cat. n. 4364346, California, USA) in a 25 μl reaction. For all primer sets, optimum melting temperatures were determined. Additionally, PCR amplification efficiencies were tested using 10-fold dilution series of cDNA.

Using the BioRad CFX Connect real-time PCR detection system (Bio-Rad, cat. n. 1855201), an initial denaturation step was performed at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 61°C for 30 s. All reactions were performed in duplicate and negative controls were included.

The expression of each E2F target gene was normalized to two reference genes (GAPDH and RPS5). For RNA derived from the cultured cells the normalized expression was presented relative to the expression level of the same genes in the control (untreated) samples [ΔΔCt method, (40)]. For FFPE tumor tissue RNA the normalized expression was presented relative to the expression level of the same genes in the melanocytoma (benign tumor) samples.

CDK4/6 Inhibitor Treatment

In order to determine the effective dose of CDK4/6 inhibitor palbociclib (PD-0332991, Merck) on canine melanoma cell lines, 1.5×10^5 CMM10, CMM12, LMCK, and OLGA cells were first seeded on p60 dishes in regular cell culture medium without the drug. After 24 h, cell culture media were refreshed and indicated concentrations of the compound (1, 2.5, 5, and 10 μM; dissolved in 1% DMSO) were added. Our group has previously used Palbociclib in RPE cells, where a G1 phase arrest was induced using 1 μM Palbociclib (41). Since the effect of Palbociclib has not yet been investigated in CMM10, CMM12, LMCK, and OLGA cell lines, we used 1 μM as the starting dose to assess whether the canine melanoma cell lines were sensitive to Palbociclib treatment. Control cells were treated with 1% DMSO or received no treatment at all. Cells were harvested after 48 h of treatment to perform cell counts, flow cytometry, and RNA isolation. Cells were counted using a TC20TM automated cell counter.

For FACS analysis, cells were harvested, fixed in 70% ethanol and stored at 4°C for at least 24 h prior to DNA staining.

In the experiments for the evaluation of E2F target genes expression before and after CDK4/6 inhibitor treatment, p60 petri dishes were seeded with 1.5×10^5 cells for OLGA and 2.0×10^5 cells for LMCK, CMM10, and CMM12 in regular cell culture medium. After 24 h, cell culture media were removed and replaced with media containing 1 μM palbociclib dissolved in DMSO. For each cell line, an equal number of untreated cells was used as a control. After 48 h, cells were harvested and used for RNA isolation, cDNA synthesis, and quantification of E2F target gene transcripts by qPCR, as described in the sections "RNA isolation and RT-qPCR."

All the experiments were carried out in triplicates.

FACS Analysis

To enable flow cytometric analysis of DNA content, ethanol-fixed cells were mixed with a staining solution containing 2 μg/ml propidium iodide, 500 μg/ml RNase A, and 0.1% bovine serum albumin. Samples were analyzed on a BD FACSCanto II (Becton Dickinson) flow cytometer within 24 h after staining. Flow cytometry data were analyzed using FlowJo software.

Statistical Analysis

Data relative to gene expression were expressed as mean ± standard deviation (SD). For non-normally distributed variables, median, and interquartile range (IQR) were used. To test for equality of variances between groups, we used *F*-test or Levene's test. Depending on homoschedasticity or heteroscedasticity, we used the Student's *t*-test or Welch test, respectively, to perform comparisons. To compare cell growth between control and treated groups in *in vitro* experiments, we performed ANOVA followed by Dunnett's test with free-step down resampling. To compare non-normally distributed numeric variables, Kruskal-Wallis or Mann–Whitney U-test and Spearman correlation coefficient (ρ) were used. Rho (ρ) was interpreted according to Mukaka (42). For multiple comparisons, we reported Bonferroni-adjusted *P*-values. We performed the univariable Cox proportional-hazards regression to evaluate the association of the expression of the investigated gene transcripts with hazard of death and of developing recurrence/metastasis; results were reported as hazard ratio (HR) with the corresponding 95% confidence interval (95%CI). Statistical analysis was performed with the software R (R version 3.5.2); P-values ≤ 0.05 were considered statistically significant.

RESULTS

E2F Target Genes H2AFZ and Survivin/BIRC5 Are More Highly Expressed in Melanomas With Unfavorable Clinical Outcome

Before including all the selected canine melanoma cases in the study, we confirmed the melanocytic origin of the neoplastic cells of the poorly pigmented, amelanotic melanoma cases by immunohistochemistry using anti-MelanA and anti-PNL2 antibodies. In the tested cases, we observed multifocal, moderate to marked, finely granular, cytoplasmic Melan A (**Figures 1A,D**), and PNL2 (**Figures 1B,E**) immunolabeling.

In order to investigate the prognostic significance of the selected E2F target genes in canine melanoma samples, we analyzed RNA obtained from FFPE tumor samples of different types of canine melanocytic tumors (melanocytoma, cutaneous, or oral MM) and we compared different groups of patients, considering the following signs of tumor malignancy and

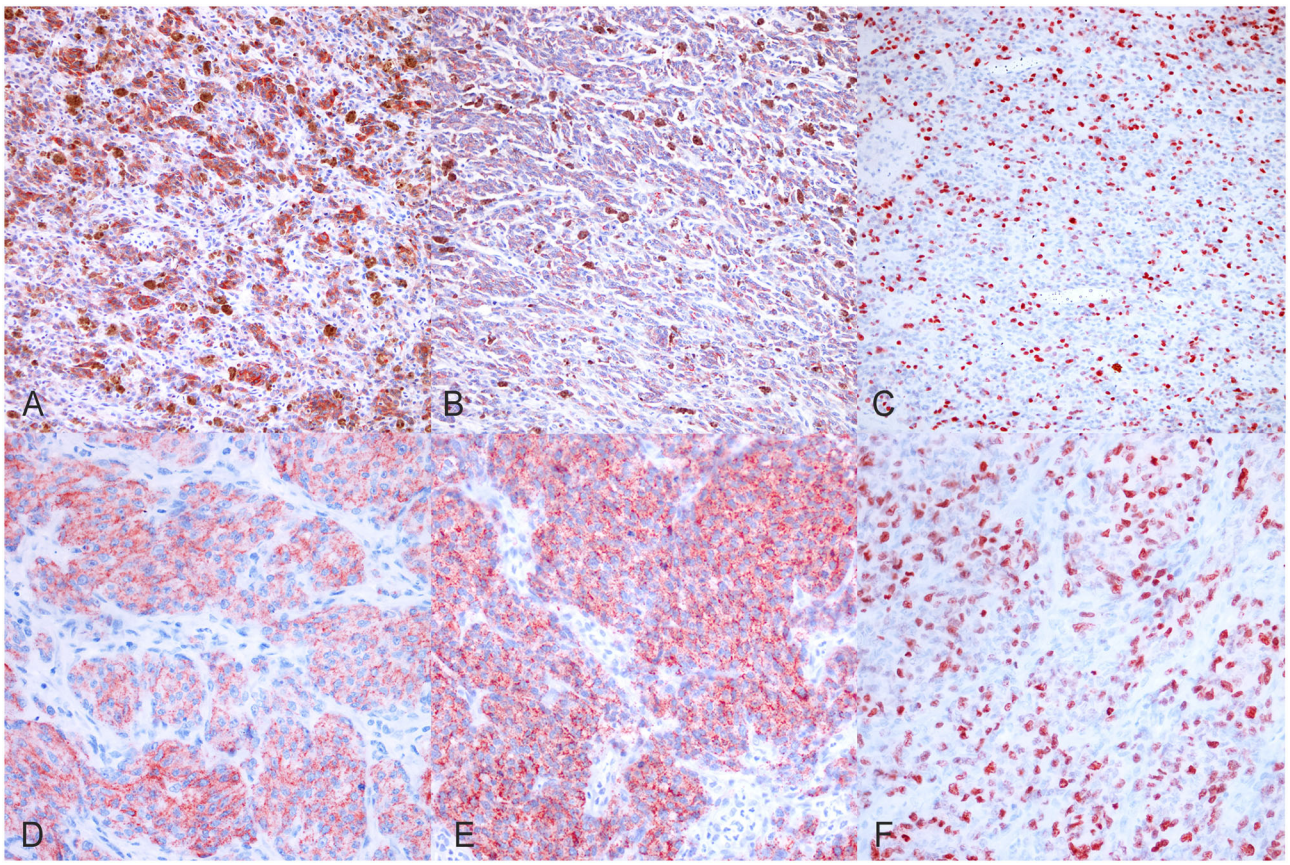


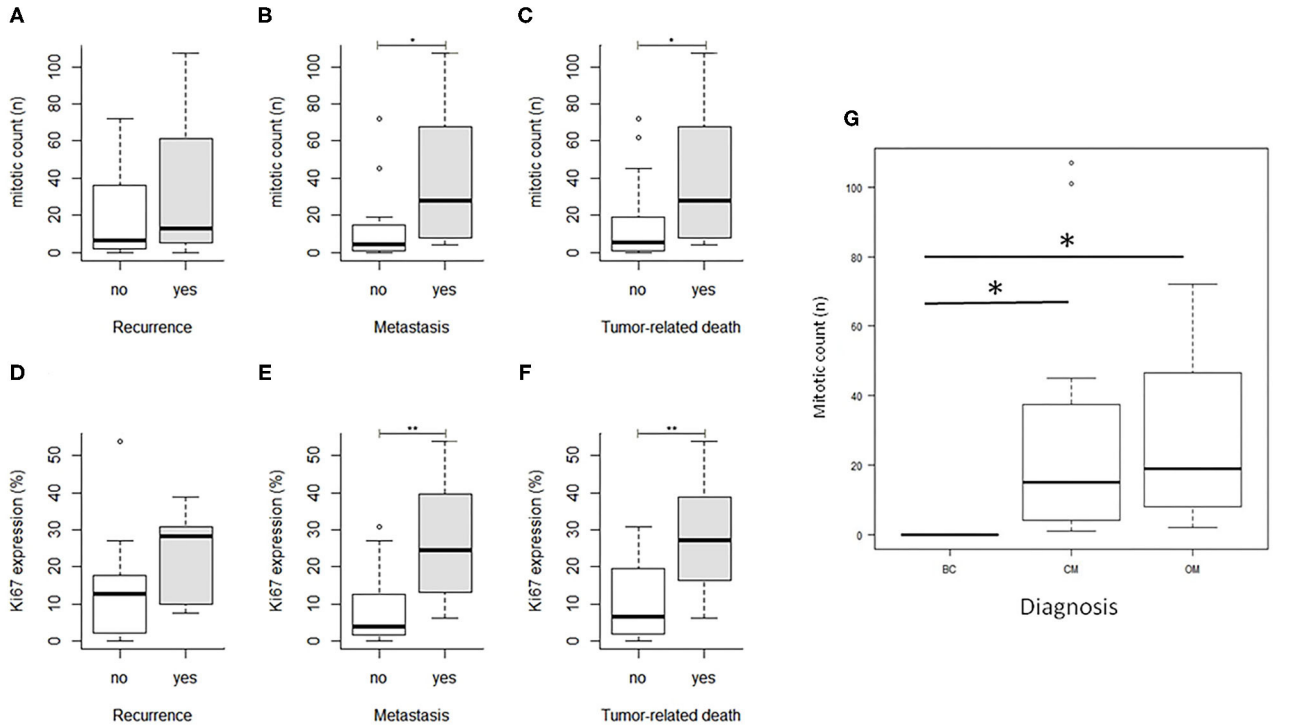
FIGURE 1 | Immunohistochemical results. **(A)** Canine, Oral melanoma (palate). Multifocal, moderate, finely granular, cytoplasmic Melan A immunolabeling (200x, AEC and hematoxylin). **(B)** Canine, Oral melanoma (palate). Multifocal, moderate, finely granular, cytoplasmic PNL2 immunolabeling (200x, AEC and hematoxylin). **(C)** Canine, Oral melanoma (palate). Nuclear immunolabeling of Ki-67 (200x, AEC and hematoxylin). **(D)** Canine, Oral melanoma (gingiva). Diffuse, finely granular, marked, cytoplasmic Melan A immunolabeling (400x, AEC and hematoxylin). **(E)** Canine, Oral melanoma (gingiva). Diffuse, finely granular, marked, cytoplasmic PNL2 immunolabeling (400x, AEC and hematoxylin). **(F)** Canine, Oral melanoma (gingiva). Nuclear immunolabeling of Ki-67 (400x, AEC and hematoxylin).

progression: mitotic count, presence of recurrence, metastasis, death due to melanoma, disease free interval (DF), OS. Since cutaneous and oral melanoma normally have different clinical behavior, we first investigated the association of diagnosis with malignancy indicators (recurrence, metastasis, and death due to melanoma) as well as with OS time and disease-free time. Additionally, we compared the expression of all genes of interest among the three group. Since no relevant results emerged in our sample population, we considered the diagnosis not influent for the purposes of our study and performed the analysis grouping all tumors (**Supplementary Figures 2A–C**). Levels of expression of the investigated genes were also correlated with mitotic count and Ki67 index evaluated on immunohistochemical stained slides (**Figures 1C,F**). Mitotic count and Ki67 index were both significantly higher in cases with metastasis and unfavorable clinical outcome; in line with previous studies, they were associated with diagnosis and an increased risk of death or to develop recurrence/metastasis (**Figures 2A–I**).

Gene expression was normalized to the level of reference genes GAPDH and RPS5 and fold change expression of the PCR product of each gene was calculated using the mean value of benign melanocytoma cases as reference, based on the ΔΔCt method (40). Six cases (two melanocytoma, three cutaneous, and one oral melanoma cases), in which all genes, including reference genes, were not detectable, were excluded from the analyses.

From the data analysis (**Figures 3A,B**), *H2AFZ* was the most frequently and highly expressed gene in all three groups of melanoma cases, with the highest levels of expression in the cutaneous melanoma.

H2AFZ mRNA was detectable in 91% of the total cases (**Figure 3A**), and in all, except one (94.7%) of the cutaneous cases (both benign and malignant). The levels of H2AFZ expression were higher in cases with recurrence (**Figure 3C**) or metastasis (**Figure 3D**), but no statistically significant association was found. However, *H2AFZ* expression was significantly higher in cases with unfavorable clinical outcome compared to cases with favorable clinical outcome (**Figure 3E**). Additionally, *H2AFZ* expression showed a significant moderate positive correlation with the mitotic count (**Figure 4A**) and the Ki67 index (**Figure 4B**), and it showed a significant association



H

	HR	95%CI	P VALUE
Overall survival time			
mititic count	1.023	1.004-1.042	**0.020**
Ki67	1.066	1.023-1.112	**0.002**
Disease-free time			
mititic count	1.031	1.009-1.054	**0.006**
Ki67	1.062	1.023-1.102	**0.001**

I

		Median	IQR	P VALUE
Mitotic count(n)				
Recurrence	yes	12.5	7.5-38.0	0.837
	no	6.0	2.0-36.0	
Metastasis	yes	27.5	9.0-50.75	**0.014**
	no	4.0	1.0-14.25	
Tumor-related death	yes	27.5	9.0-50.75	**0.035**
	no	5.0	1.0-19.0	
Ki67(%)				
Recurrence	yes	28.1	9.9-30.8	0.101
	no	12.7	2.3-53.7	
Metastasis	yes	24.3	14.7-39.2	**0.004**
	no	3.7	1.7-12.7	
Tumor-related death	yes	27.2	16.3-38.8	**0.008**
	no	6.5	2.1-18.6	

FIGURE 2 | Mitotic count and Ki67 index are higher in melanoma cases with recurrence, metastasis, and unfavorable outcome. Correlation of Ki67 index and mitotic count with clinical follow up data **(A–F)**; correlation of mitotic count with diagnosis (BC, benign cutaneous melanoma; CM, cutaneous malignant melanoma; OM, oral melanoma); *$P < 0.05$ **(G). (H)** The table shows the results of survival analysis of mitotic count and Ki67 (Cox proportional regression analysis; HR: hazard ratio). **(I)** the table shows the association between mitotic count and Ki67 expression with clinical data (IQR: interquartile range).

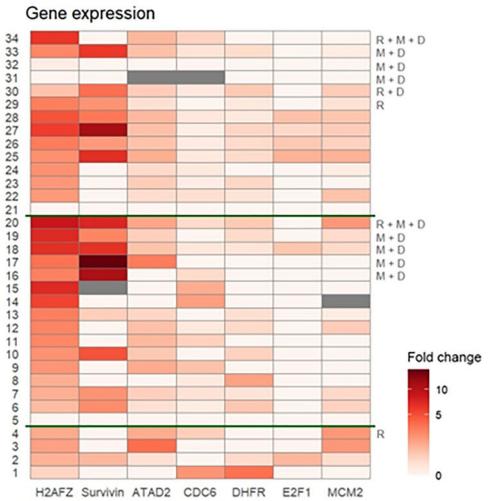
with increased hazard of death and with the development of recurrence/metastasis ($P < 0.05$) (**Figures 3I–K**).

Although survivin/*BIRC5* mRNA was expressed at low levels in these samples (**Figure 3B**), resulting in a lower percentage of cases expressing this gene (51.43%, 18/35) (**Figure 3A**), differences were observed comparing groups with or without metastasis (**Figure 3F**), and a significant higher expression was seen in cases with unfavorable clinical outcome compared to those with favorable clinical outcome (**Figure 3H**). Furthermore, survivin/BIRC5 was significantly associated with increased

A

	Cutaneous Melanocytoma		Cutaneous Melanoma		Oral Melanoma		Melanocytic Tumours	
Case N.	4	%	17	%	16	%	35	%
H2AFZ	4	100	16	94,1	12	75	32	91,4
ATAD2	3	75	12	70,6	11	68,8	26	74,3
CDC6	2	50	12	70,6	10	62,5	24	68,6
DHFR	2	50	10	58,8	9	56,3	21	60
MCM2	3	75	8	47,1	9	56,3	20	57,1
BIRC5	1	25	10	58,8	7	43,8	18	51,4
E2F1	1	25	1	5,9	4	25	6	17,1

B



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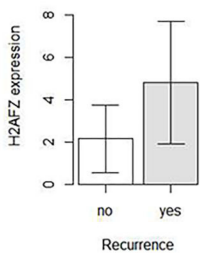

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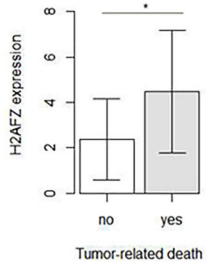

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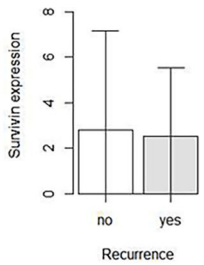

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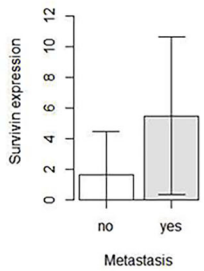

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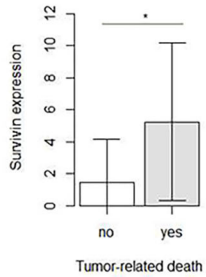

I

		Mean	SD	P VALUE*
H2AFZ ($2^{-\Delta\Delta CT}$)				
Recurrence	yes	4.8	2.9	0.109
	no	2.1	1.6	
Metastasis	yes	4.4	2.8	0.053
	no	2.2	1.8	
Tumor-related death	yes	4.5	2.7	**0.043**
	no	2.3	1.8	
BIRC5/Survivin ($2^{-\Delta\Delta CT}$)				
Recurrence	yes	2.5	3.0	0.853
	no	2.8	4.3	
Metastasis	yes	5.5	5.2	0.062
	no	1.6	2.8	
Tumor-related death	yes	5.2	4.9	**0.043**
	no	1.4	2.7	

J

GENE	HR	95%CI	P VALUE
H2AFZ	1.38	1.08-1.76	**0.011**
SURVIVIN	1.20	1.06-1.36	**0.004**
ATAD2	1.20	0.61-2.35	0.600
CDC6	0.30	0.02-4.13	0.371
DHFR	0.36	0.03-4.40	0.425
MCM2	0.84	0.27-2.61	0.761
E2F1	1.84	0.29-11.76	0.520

K

GENE	HR	95%CI	P VALUE
H2AFZ	1.36	1.06-1.72	**0.015**
SURVIVIN	1.11	1.00-1.24	0.060
ATAD2	1.10	0.60-2.04	0.754
CDC6	0.45	0.07-2.93	0.407
DHFR	0.30	0.02-4.59	0.390
MCM2	1.49	0.62-3.58	0.374
E2F1	1.17	0.09-15.87	0.906

FIGURE 3 | H2AFZ and survivin/BIRC5 mRNA expressions represent prognostic factors in canine melanocytic tumors. **(A)** Table indicating number and percentage of positive cases for all genes in all the melanocytic tumors (right columns) and on specific types. **(B)** Heat map showing gene expression levels for all genes ordered

(Continued)

FIGURE 3 | from the bottom to the top: diagnosis (1–4, cutaneous melanocytomas; 5–20, cutaneous melanomas; 21–34, oral melanomas); R, recurrence, M, metastasis, D, tumor-related death; the heatmap was generated by a square root transformation of RT-qPCR data presented as fold change (ΔΔCt method). **(C–H)** Comparison of H2AFZ **(C–E)** and survivin/BIRC5 **(F–H)** transcript expression levels between patient groups. Groups are based on clinical follow-data on recurrence, metastasis, death due to melanoma (unfavorable outcome). Error bars show SDs for each group. *$P < 0.05$. **(I)** Table shows the association between H2AFZ and survivin/BIRC5 expression with clinical data. *t-test or Welch test, depending on homogeneity of variances. **(J)** Table shows the results of survival analysis of gene expression depending on overall survival time (Cox proportional regression analysis). **(K)** Table shows the results of survival analysis of gene expression depending on disease-free time (Cox proportional regression analysis).

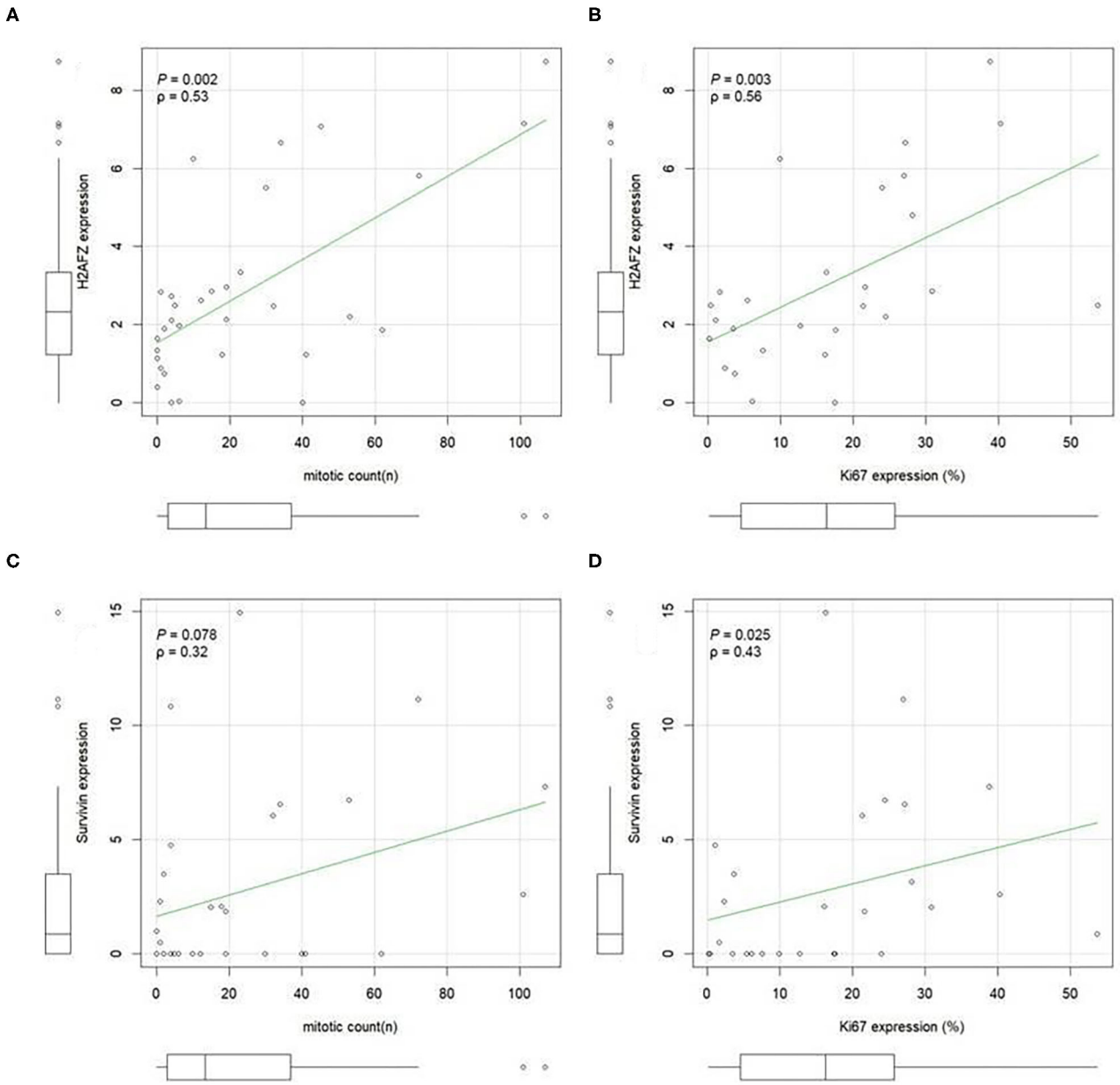

FIGURE 4 | H2AFZ and survivin/BIRC5 mRNA expressions in tumor tissue correlate with histological proliferation markers. A positive correlation was evident between H2AFZ **(A)**, but not survivin/BIRC5 **(C)**, expression levels and mitotic count (MI); while both gene expression levels positively correlated with Ki67 index **(B,D)**. Spearman correlation coefficient. Ki67 was calculated as the number of positive cells on the total number of counted cells (mean 1,090 cells, ranging from 900 to 1,376 cells) using Image J analysis system.

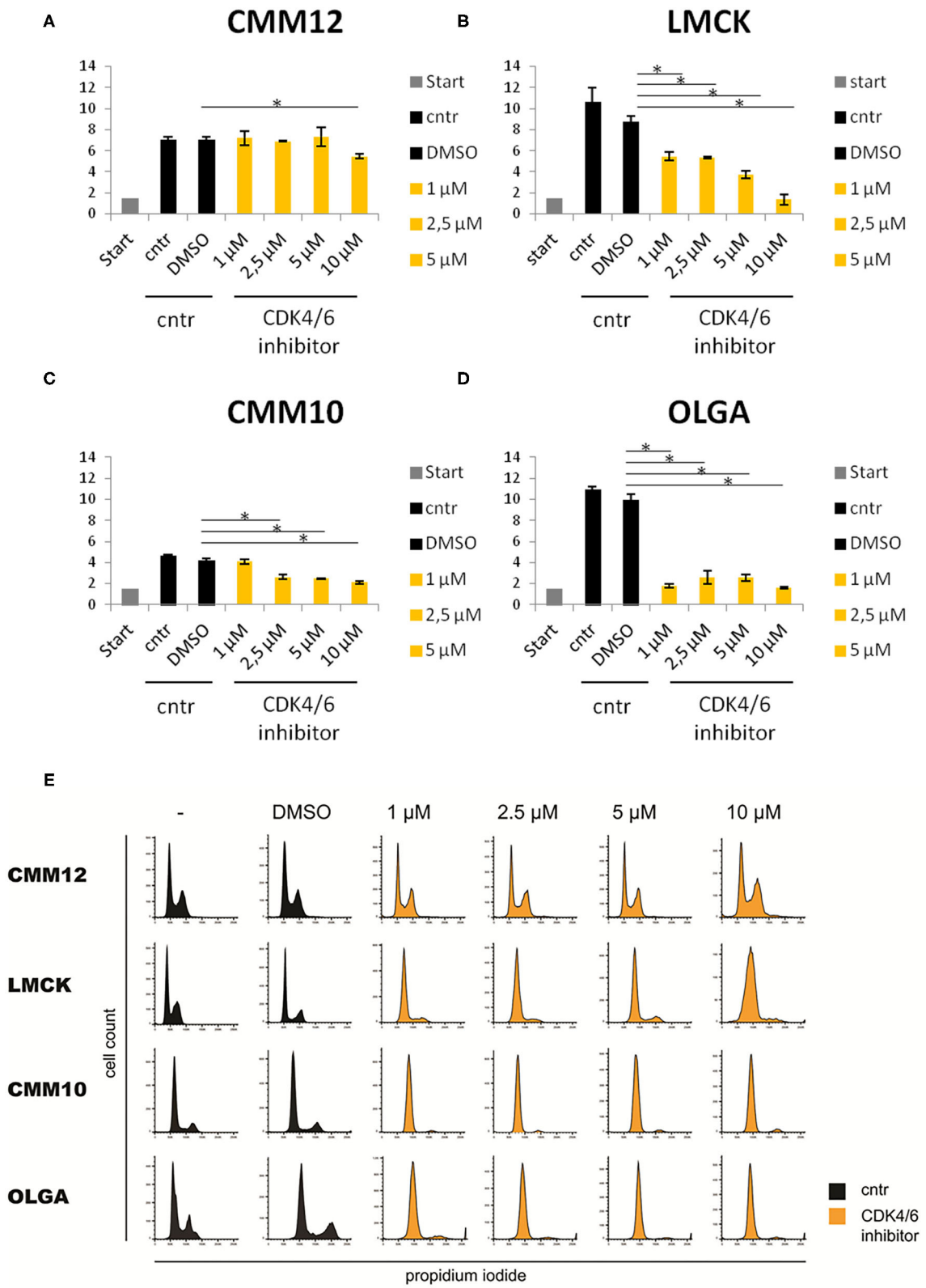

FIGURE 5 | Three of the four tested melanoma cell lines were sensitive to CDK4/6 inhibitor (Palbociclib). Sensitivity of CMM12, CMM10, LMCK, and OLGA melanoma cell lines toward CDK4/6 inhibitor palbociclib. **(A–D)** Cell numbers of melanoma cells treated with different doses of palbociclib for 48 h. Gray bars represent the cell

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FIGURE 5 | numbers that were seeded at the start of the experiment. Black bars represent the cell numbers of control cells at the end of the experiment. Orange bars represent cell numbers of CDK4/6 inhibitor treated cells at the end of the experiment. Error bars represent the standard error from the mean based on three biological triplicates. *$P < 0.05$. **(E)** FACS cell cycle analysis of propidium iodide stained melanoma cells treated with 1, 2.5, 5, or 10 μM palbociclib, and untreated or 1% DMSO treated controls. Black graphs represent the cell numbers of control cells at the end of the experiment. Orange graphs represent cell numbers of CDK4/6 inhibitor treated cells at the end of the experiment.

hazard of death ($P < 0.01$) (**Figures 3I–K**). A significant low correlation was observed between survivin/BIRC5 gene expression and Ki67 index (**Figures 4C,D**).

All the other investigated genes were expressed in similar percentages of cases (**Figure 3A**), but with lower expression compared to H2AFZ and survivin/BIRC5 (**Figure 3B**). No statistically relevant differences in their expression were found comparing the different groups of patients, nor associations with mitotic count, hazard of developing recurrence/metastasis or death (**Supplementary Figure 3**).

Data obtained in the present work demonstrate a prognostic significance of *H2AFZ* and survivin/BIRC5 in the cohort of investigated samples of canine melanocytic tumors.

LMCK, OLGA, and CMM10 Melanoma Cells Are Sensitive, While CMM12 Melanoma Cells Are Resistant to Treatment With a CDK4/6 Inhibitor

Due to the high expression of E2F target genes in canine melanoma tissue samples, we further investigated the possibility of targeting the Rb-E2F pathway, as a new potential anticancer therapeutic approach for canine oral melanoma using a CDK4/6 inhibitor. In LMCK and OLGA cells, a clear and significant reduction in cell numbers was observed after treatment with all concentrations of the CDK4/6 inhibitor (**Figures 5A,D**). In addition, a minor inhibiting effect of DMSO on cellular proliferation was observed when comparing cell numbers of DMSO-treated control cells to untreated control cells. In CMM10 melanoma cells, the differences between CDK4/6 inhibitor treated cells and control cells were less obvious, although with palbociclib concentrations ≥2.5 μM cell numbers slowly but steadily decreased with increasing concentrations of the compound (**Figure 5C**). In CMM12 cells, cell numbers remained relatively constant with increasing concentrations of the CDK4/6 inhibitor and a significant reduction of cell proliferation was seen only at the maximum palbociclib concentration tested (**Figure 5B**; **Supplementary Table 4**).

FACS cell cycle profiles confirmed the cell number data for CMM12 cells, as cells in all conditions displayed a similar cell cycle profile and no sign of a G1 cell cycle arrest was observed (**Figure 5E**). In contrast, the cell cycle profiles of palbociclib-treated CMM10, LMCK, and OLGA cells showed a marked decrease in the S-phase population of cells compared to controls, indicating a G1 cell cycle arrest.

CDK4/6 Inhibitor Decreases E2F Target Gene Expression in Oral Melanoma Cells

In order to explore how the levels of expression of the investigated cell-cycle related genes change during CDK4/6 inhibitor treatment, we analyzed the expression levels of the E2F target genes E2F1, CDC6, DHFR, H2AFZ, MCM2, GINS2, and ATAD2 and compared them between CDK4/6 inhibitor treated cells and controls (**Figures 6A–D**).

In CMM10, LMCK, and OLGA cells, treatment with 1 μM of palbociclib resulted in a reduced expression of the E2F target genes compared to untreated control cells. This reduction was significant in CMM10 for all E2F target genes, with the exception of DHFR; for LMCK it was significant for MCM2 and GINS2. In CMM12 cells, in contrast, the expression of all the genes tended to be slightly higher compared to the control, and only the difference in ATAD2 expression was statistically significant (**Supplementary Table 5/1, 5/2**). In line with the cell number and FACS data, CDK4/6 inhibitor treatment caused no clear reduction in E2F target gene expression in CMM12 cells.

In order to assess if E2F target gene transcripts expression varies among the different cell lines under normal conditions, we compared the expression of the selected genes in the four melanoma cell lines. Without CDK4/6 inhibitor treatment, in CMM12 cells H2AFZ and MCM2 appeared to be more highly expressed compared to the other melanoma cell lines (**Figure 6E**).

DISCUSSION

The results showed in the present work clearly indicate that some E2F target genes, namely H2AFZ and survivin/BIRC5, have potential prognostic value in canine melanocytic tumors. Furthermore, our results demonstrate that pathways regulating the expression of these genes can be targeted to arrest cell proliferation using a CDK4/6 inhibitor. Since resistant cells showed higher levels of expression of some of the same E2F target genes compared to sensitive ones, we suggest that they can be potentially useful for patient selection as predictive markers.

We observed that H2AFZ showed significant differences in its mRNA expression comparing different groups of patients, showing associations with unfavorable outcome (death due to melanoma), increased proliferation rate (mitotic count and Ki67 index), increased hazard of death and developing recurrence/metastasis. H2AFZ together with H2AFV represent two allelic genes encoding for two isoforms of the highly conserved H2A variant H2A.Z, namely H2A.Z.1 and H2A.Z.2, which has a well-established role in transcriptional regulation (43). Histone H2A variants have previously been demonstrated as a biomarker of tumor progression in several types of human cancers (44). In particular, H2A.Z is overexpressed in multiple malignant tumors, such as breast cancer (45), prostate cancer (46), bladder cancer (47), liver cancer (48, 49), as well as malignant melanoma (50, 51). Both H2A.Z isoforms are highly expressed in human melanoma and high expression levels correlate with poor prognosis (52). In line with this data, our

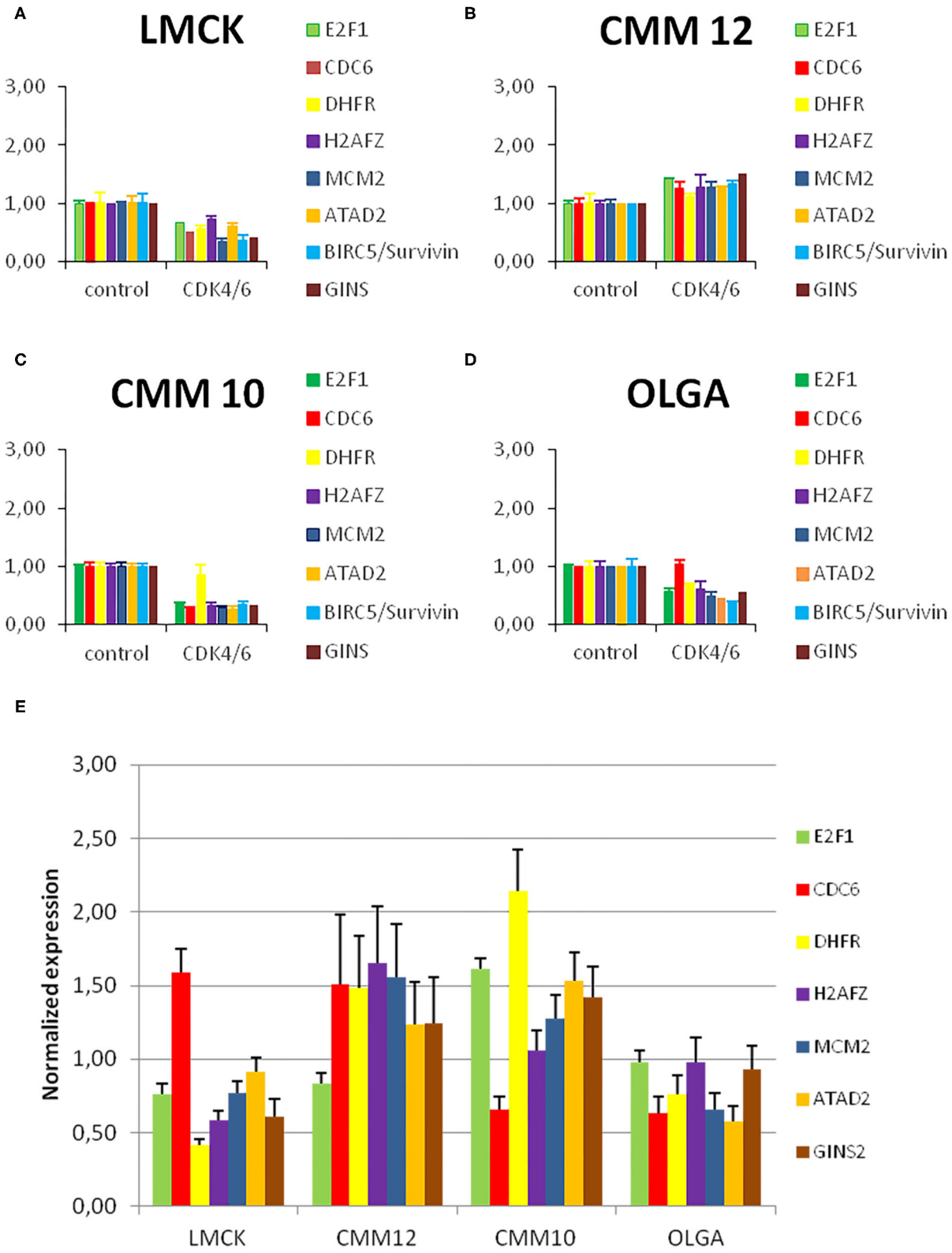

FIGURE 6 | Resistant melanoma cell line showed high levels of H2FZ before and after treatment with CDK4/6 inhibitor. **(A–D)** E2F target gene expression in melanoma cells before and after treatment with a CDK4/6 inhibitor. Normalized E2F target gene transcript levels in melanoma cells treated with 1 μM palbociclib

(Continued)

FIGURE 6 | compared to untreated control cells. Gene expression was normalized to reference genes GAPDH and RPS5. Bars represent the fold change of the PCR product of indicated genes in CDK4/6 inhibitor treated cells relative to untreated control cells. Error bars represent the standard error from the mean based on two technical replicates and three biological triplicates. **(E)** Normalized E2F target gene transcript levels in melanoma cells under normal conditions compared to the average level of expression of each gene in all cell lines. Gene expression was normalized to reference genes GAPDH and RPS5. Bars represent the fold change of the PCR product of indicated genes in CMM12, OLGA, and LMCK cells relative to the average of all cell lines as a control. Error bars represent the standard error from the mean based on two technical replicates.

results indicate that H2AFZ is an abundantly expressed E2F target gene and a useful prognostic marker to be used in canine melanoma, also in FFPE tissue samples. Results obtained concerning BIRC5, encoding the survivin protein, the smallest member of the IAP (inhibitor of apoptosis) protein family, are in line with previous results we observed in canine cutaneous melanocytic tumors (17), confirming its utility as a prognostic marker in this type of cancer. Besides (wild-type) survivin, BIRC5 encodes four other survivin variants generated through alternative splicing. Survivin is the more intensively studied and widely acknowledged as an orchestrator of cell division and inhibitor of apoptotic pathways (53). Both protein and mRNA survivin expression has been correlated with patient survival in human melanoma (53), in line with our results. The relatively low percentage of samples expressing the gene, however, would suggest that its mRNA levels are not as high as H2AFZ, resulting in several negative FFPE cases. Therefore, we think that this type of marker would be more suitable for fresh tumor samples, instead of formalin fixed cases. All the other investigated genes were detected in a variable percentage of cases and did not show any significant association with clinical data. It would be worth to confirm these results with a larger sample size and performing multivariable analysis to establish the independent prognostic significance of the two markers. Furthermore, considering that the type of surgery applied can deeply impact on the prognosis, further studies recording these data should be performed in order to better understand the prognostic value of the investigated markers.

Based on the high expression of E2F target genes in the investigated tumors, we hypothesized that pathways regulating their expression can be targeted to arrest cell proliferation as a potential new anti-cancer therapy for canine oral melanoma. We selected a CDK4/6 inhibitor, palbociclib, that is currently approved in clinical practice and most advanced in clinical trials (54) and we treated four melanoma cell lines. Palbociclib is a third-generation CDK4/6 inhibitor, orally administered, acting through a competitive and reversible bound to the ATP pocket of the inactive kinase, thus strongly inhibiting both CDK4 and CDK6. As a consequence, RB hyperphosphorilation is prevented, E2F family members are not released, thus the cell will be in a cell cycle arrest phase (55). To our knowledge, this is the first study testing the sensitivity of canine melanoma cell lines to treatment with CDK4/6 inhibitor palbociclib. Using cell number data, FACS cell cycle profiles, and E2F target gene expression profiles, we demonstrated that three of the investigated canine melanoma cell lines, including two primary tumor-derived (CMM10, LMCK) and one metastatic (OLGA) cell lines, were sensitive to the treatment, suggesting a potential use of this drug for the treatment of canine oral melanoma. These data can pave the way for the start of clinical trials in dogs with oral melanoma with a potential important impact for the cure of canine patients as well as for humans with a similar, but extremely rare, form of mucosal melanoma. However, one canine melanoma cell line (CMM12) was resistant to CDK4/6 inhibition of proliferation and G1 cell cycle arrest. In this work, we did not further investigate the mechanisms mediating CDK4/6 inhibitor resistance in CMM12 cells. As a first step in order to understand if the selected E2F target genes could function as predictor markers of CDK4/6 inhibition resistance, we compared the expression profiles of E2F1, CDC6, DHFR, H2AFZ, MCM2, and ATAD2 among the four canine melanoma cell lines. We observed that H2AFZ and MCM2 were expressed at relatively high levels in CMM12 melanoma cells compared to the other cell lines, suggesting a possible utility of E2F target gene expression in tumor cells as markers to predict response to treatment with a CDK4/6 inhibitor. Although further investigations are needed to confirm this hypothesis, we can speculate that increased E2F target gene expression could represent a consequence of Rb mutation, responsible for CDK4/6 inhibitor resistance. Indeed, from previously published works we know that loss of Rb function is the main mechanism that is described to confer resistance to CDK4/6 inhibitors (31). One study showed that Rb immunostaining was detected in most of the canine melanoma cases investigated and associated with a very low or absent p16 immunolabelling (56). However, the applied method could not give any information about the actual functional activity of the detected RB protein in the tissue. In human melanoma cell lines, loss of expression of the endogenous cyclin-dependent kinase inhibitor (CDKN2A), encoding for two distinct potent tumor suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$, has shown to correlate with sensitivity to palbociclib; while Rb loss correlated with resistance (57). In a mouse model (patient derived xenograft) of human mucosal melanoma carrying CDK4 amplification, a robust anti-tumor effect of palbociclib has been reported (58). Studies on the characterization of the histone variant H2A.Z.2 as a driver of malignant melanoma showed that H2A.Z.2 exerts a key role in promoting cell cycle progression by controlling the transcriptional output of E2F target genes. H2A.Z.2 deficiency sensitizes melanoma cells to chemotherapy and to MEK (also known as mitogen-activated protein kinase (MAPK) kinase or MAP2K) inhibitors, indicating it as an important mediator of cell proliferation and drug sensitivity in malignant melanoma (52). These data, along with our results, strongly suggest that H2AFZ could potentially be used as a predictive marker to estimate melanoma treatment response.

Conclusions

In the present study, we identified H2AFZ as a novel promising prognostic marker and we highlighted the possible utility

of survivin as a prognostic marker also at the mRNA level for both cutaneous and mucosal canine melanocytic tumors. Furthermore, we demonstrated the sensitivity of three oral melanoma cell lines, including a metastatic one, to treatment with a CDK4/6 inhibitor, suggesting this could be a new effective therapeutic approach for canine oral melanoma. Finally, we showed that H2AFZ might be a marker for predicting resistance against CDK4/6 inhibitor treatment.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this study involve client-owned animals, retrospectively or prospectively and a client consent of the biopsy material has been collected for each included case. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LB and AB conceived of the presented idea. AB supervised the findings of this work. LB performed the experiments on tumor tissues and wrote the manuscript. AA performed the *in vitro* experiments. SS performed statistics. CB and IP classified and selected the tumor cases and samples included in the work. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.705359/full#supplementary-material

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OPINION
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Current Status of Canine Melanoma Diagnosis and Therapy: Report From a Colloquium on Canine Melanoma Organized by ABROVET (Brazilian Association of Veterinary Oncology)

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INTRODUCTION

Melanoma is a prevalent, aggressive form of cancer in dogs. New treatment or preventive modalities are necessary to control this disease in dogs. On December 03, 2020, the Brazilian Association of Veterinary Oncology, ABROVET, organized the "Colloquium on Canine Melanoma" to present the newest achievements for the treatment of this disease.

Invited talks included fundamental aspects of canine melanoma, and conventional and innovative therapies. The talks were delivered online and more than 100 attendees joined the transmission. This report aims to present the most important information about canine melanoma discussed at the Colloquium.

Canine Melanoma: Fundamental Aspects

Melanocytic Neoplasms in Dogs: A Pandora's Box, by Carlos Eduardo Fonseca Alves

Canine oral melanoma is one of the most diffuse tumors in dogs worldwide and it is related to a high metastatic rate and poor prognosis. The first publication regarding melanocytic tumors was in

1949 (1), and the first publication of a canine melanoma from oral mucosa was in 1950 (2). After the publication of the first oral melanoma case report (2), several other studies have been performed in order to establish diagnostic, prognostic, and predictive markers.

The first important approach for treating dogs with oral melanoma is to establish the prognosis (3). Although several studies have tried to standardize new prognostic molecular factors, the histological features remain pivotal. Recently, the Oncology Pathology Working Group (OPWG), a Veterinary Cancer Society and American College of Veterinary Pathologists initiative, proposed a consensus on the diagnosis of and histopathologic prognostication for canine melanocytic neoplasms (3). Among the histological prognostication criteria proposed in the consensus, nuclear atypia, vascular invasion, and mitotic index were considered in addition to other histological criteria (**Figure 1**). Interestingly, the degree of pigmentation presented a direct relation with overall survival. Thus, dogs with amelanotic melanoma had a shorter lifespan (4). However, the outcome is not predictable in melanomas with moderate, low, or no pigmentation (3).

Owing to the increasing use of big data in cancer research, recent studies have focused on different molecular prognostic and predictive markers for canine oral melanoma. Among them, somatic focal amplifications on chromosome 30 have been associated with poor patient outcomes. Chromosomal imbalances and transcriptome dysregulations seem to be frequent in canine oral melanoma (5–8). To summarize genomic data associated with melanoma mutations, an integrative *in silico* analysis was performed with the two previous canine oral melanoma studies (9) and the human data available for human melanoma in "The Cancer Genome Atlas" (10). This analysis identified 60 commonly mutated genes among human and canine melanomas (**Figure 2**).

Most of the 60 dysregulated genes were related to immune response and tyrosine kinase terms, such as negative regulation of T-helper cell differentiation, SCF complex assembly, and activation of protein kinase A activity. For a better visualization, the biological process terms related with the 60 genes are shown in **Figure 3** (11, 12).

Among the 60 dysregulated genes in canine and human melanoma, protein-to-protein interaction (PPI) analysis was performed for better visualization of the protein interaction network. In this analysis, the disconnected nodes were excluded, which revealed four independent networks (**Figure 4**). The genes *RPL8*, *POLR2B*, *FBXO32,* and *SOCS5* showed the highest interactions. *RPL8* gene is a ribosomal protein responsible for protein synthesis and *POLR2B* is responsible for encoding the second largest subunit of RNA polymerase II. The *FBXO32* gene is related to mediation of ubiquitination and degradation of target proteins and *SOCS5* belongs to the suppressor of cytokine signaling family, recognized as a STAT inhibitor protein family (13). This analysis identified genes that could be related to both canine and human melanomas.

Although canine oral melanoma has been studied for the past 70 years, the improvement of genomic and transcriptome analysis in the recent years allow for identification of markers that can be used with predictive or prognostic value.

Histological Aspects and Molecular Markers Associated With Aggressiveness in Melanomas, by Enio Ferreira

Regardless of the animal species affected, melanomas (malignant neoplasms of melanocytes) are aggressive neoplasms because of their high metastatic potential. In dogs, melanomas represent ∼7% of all malignant tumors (14). The most common primary sites are the oral cavity, skin, mucocutaneous junctions, paws (nail junction and cushions), and, more rarely, the eyeball and meninges (15). Histopathological evaluation of melanomas can assist in both: the differential diagnosis of benign lesions and the prognostic determination. The mitotic index, nuclear atypia, and the degree of pigmentation stand out as relevant prognostic markers for melanomas. In addition to these, analysis of vascular invasion, junctional activity, and ulceration are also cited as determining prognostic factors in both cutaneous and oral melanomas by some authors (16, 17). There is no evidence that the histological type may be a relevant prognostic factor. The use of molecular markers (MELAN-A, PNL-2, HMB45, TRP-1, and TRP-2) is suggested for assisting the diagnosis of amelanotic melanomas, as these proteins are expressed in most of such tumors. Moreover, the analysis of Ki-67 expression is decisive in the prognostic definition of melanomas, but its analysis is dependent on the primary site of involvement (18). The expression of COX-2, KIT, metalloproteinases, EGFR, SOX, and proteins related to the epithelium-mesenchymal transition has been well-explored in canine melanomas (19–21). These proteins have a direct relationship with a more aggressive histological behavior, determined by the histopathological characteristics mentioned above. The analysis of gene mutations in melanomas in veterinary medicine is still not well-understood. Some changes have already been identified in the NRAS gene, PTEN KIT, and ERK1/2; however, their relevance is still uncertain (22). Clinical studies are necessary to determine the prognostic and predictive value of protein expressions and mutations in canine melanomas.

Conventional Therapies for Canine Melanoma

Oncological Surgery and Its Limitations on the Treatment of Melanoma in Dogs, by Jorge Castro

Surgery is the gold standard of treatment and the most common one for the local management of all melanomas, including oral (23), cutaneous, and digital melanomas (24). Surgical resection with wide margins has always been one of the recommended approaches for oral melanoma in dogs, associated with regional lymphadenectomy. The more aggressive resections involving the maxillary bone (maxillectomies) can be associated with temporary ligation of the carotid artery. This is an adjuvant surgical technique that the literature contemplates as a surgical option with a lower loss of blood. Although surgical treatment is the main choice, Liptak and Withrow (25) and Boston et al. (23) recommend that because of its high metastatic potential, systemic therapy should also be considered as a therapeutic option for melanomas.

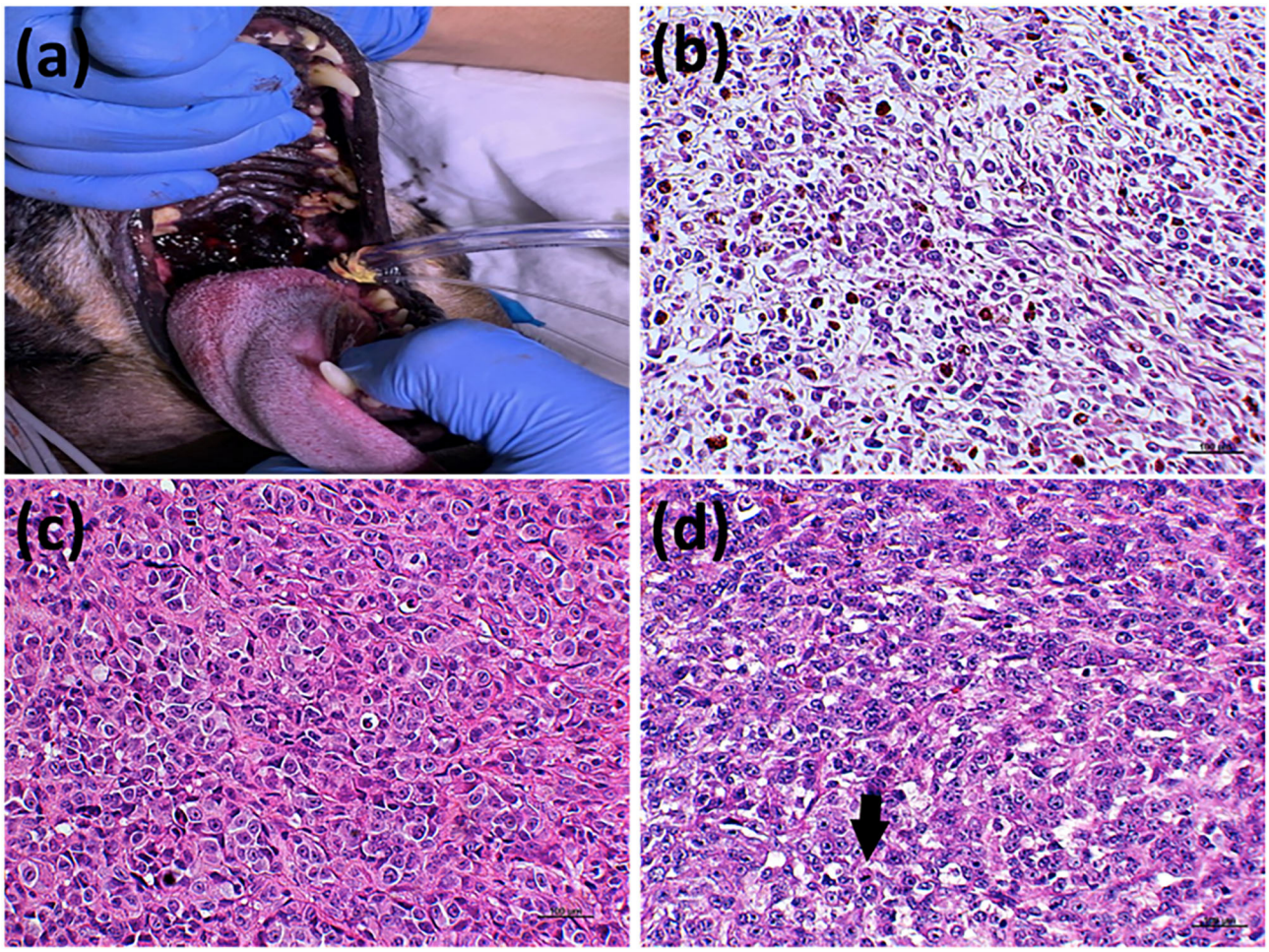

FIGURE 1 | Prognostic criteria for canine oral melanomas. **(a)** Patient with an oral melanoma in the palate with high degree of pigmentation. The degree of pigmentation has a direct 661 relation with patient's prognosis. Patients with heavily pigmented tumors show better outcomes. **(b)** Histological evaluation of a canine oral melanoma with <50% of pigmented cells. **(c)** Histological section of a canine oral melanoma with a marked nuclear atypia, also considered a poor prognostic factor. **(d)** Canine oral melanoma histological section evidencing a mitosis (arrows), one of the most important prognostic factors. Hematoxylin and eosin counterstaining, 40x.

Surgical resections with wide margins, including 2–3 cm of bone margins and 1 cm of soft tissue margins, were performed in 70 cases of canine oral malignant melanomas (26). Histopathological analysis showed that 72.9% of the tumors were completely excised, with 10% of these patients showing local tumor recurrence. In this study, dogs that had surgery as the only treatment option had a progression-free interval >567 days and a mean survival time (MST) of 874 days. In the same year, Boston et al. (23) obtained 79.3% (73/92) success rate in complete excisions between surgeries performed with wide margin, based on histological evaluation, and the recurrence rate was 8.3% (6/73) in this group (23). The average survival time for these dogs was 354 days.

The resection of regional lymph node neoplasms in the face and maxilla region has been studied and performed since the last century (27) and a more recent study has mapped these lymphatic centers (28). The analysis of sentinel lymph nodes provides essential information about the clinical stage of the disease and helps to determine the most appropriate treatment plan (29, 30). In a survey conducted by Williams and Packer with 100 dogs with oral malignant melanoma, 53% had cytological or histopathological evidence of metastasis in mandibular lymph nodes, even in normal-sized lymph nodes (31). Therefore, lymphatic toilet (removal of regional lymph nodes) is recommended, especially in dogs with oral melanomas. In digital melanomas, lymphatic drainage in the limbs must also be evaluated and the removal of the regional lymph node in surgical planning is advocated (30, 31).

Radiation Therapy in Dogs With Oral Melanomas, by Simone C. S. Cunha; Natália Oyafuso Da Cruz

Radiation therapy (RT) is a localized cancer treatment modality, which acts on proliferating cells that fall inside the radiation field. In cases of canine oral melanoma, RT can be used as an adjuvant

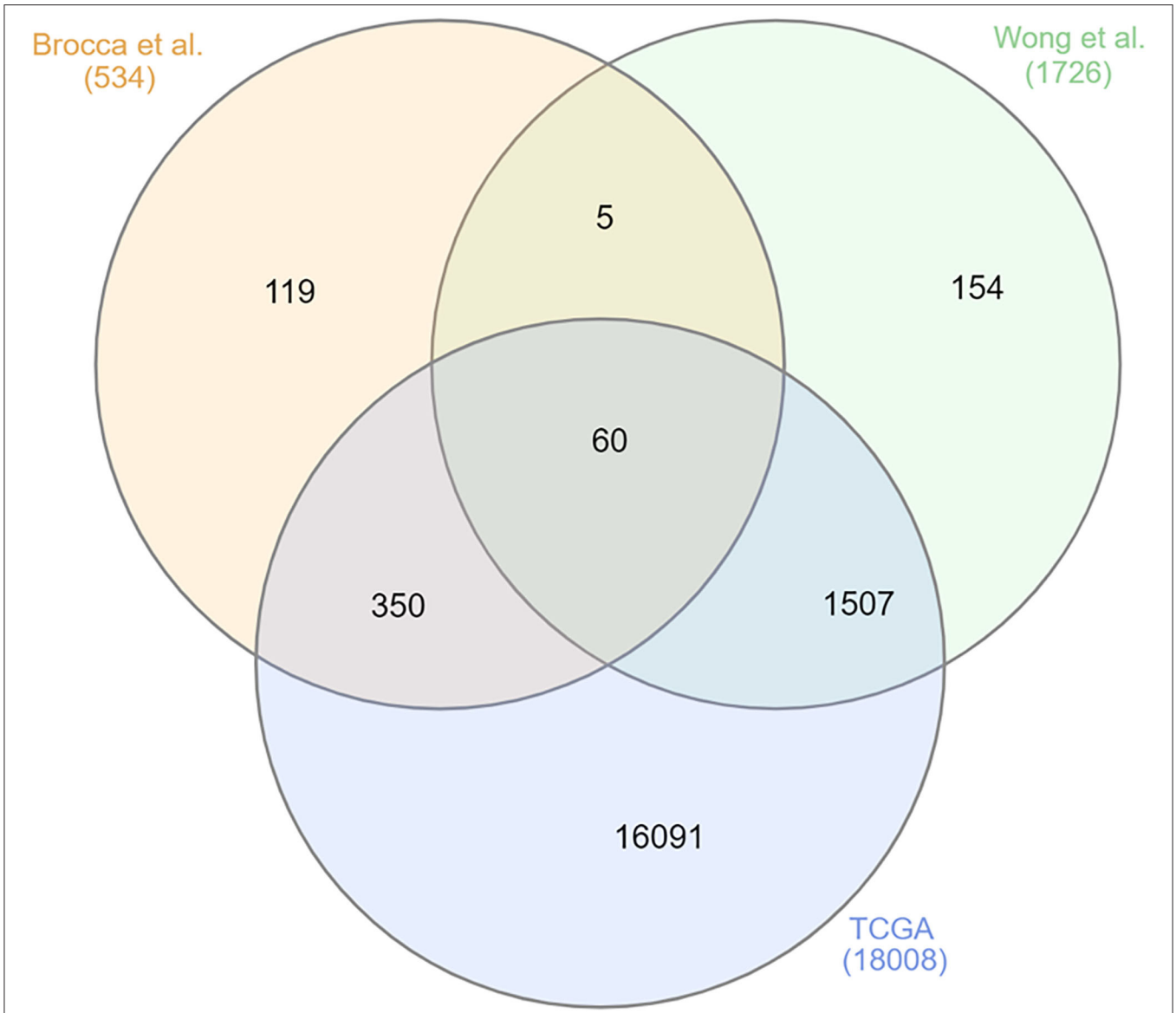

FIGURE 2 | Venn diagram demonstrating the most commonly mutated genes among the three datasets, two canine oral melanoma (8, 9) and one human melanoma dataset (The Cancer Genome Atlas—TCGA 10). Here, 60 commonly mutated genes were identified. Figure generated online (http://www.interactivenn.net/).

post-operative or a palliative therapy. Radiation field should, whenever possible, include regional lymph nodes, regardless of whether they have macroscopic alterations suggestive of regional metastasis. The main radiation protocols for melanoma are based on hypofractionation (3–6 RT fractions once or twice weekly), due the alpha/beta ratio range of 0.5–2.5 Gy. These ratios are similar to those of late-responding normal tissues, which are preferentially damaged by radiation delivered in large doses per fraction (32, 33). Side effects are usually mild, self- limiting, and confined to radiation field, and may include alopecia, skin hypo- or hyperpigmentation, dry radiodermatitis, and oral mucositis. The overall response rate of oral melanoma in previous literature is reported to be 75–85%, including complete and partial tumor responses, and the mean survival time is 230–363 days (34–36). In the first author's (SCSC) experience, radiation therapy is routinely used as an adjunctive post-operative therapy (in cases of incomplete surgical resection) or, more commonly, as a palliative treatment (in unresectable tumors or surgery declined by the dog owners). The high rate of distant metastasis (44–58%) remains the significant limiting factor for curative intention treatment of canine oral melanoma (34, 37, 38). The author Cunha et al. (39) evaluated the survival of 24 dogs with oral melanoma treated with orthovoltage radiotherapy. The mean age and standard deviation was 13 $\pm$ 2.6 years and the overall response rate was 93%, including 64% partial (one stage I, one stage II, seven stage III, and one stage IV) and 29% complete tumor responses (one stage

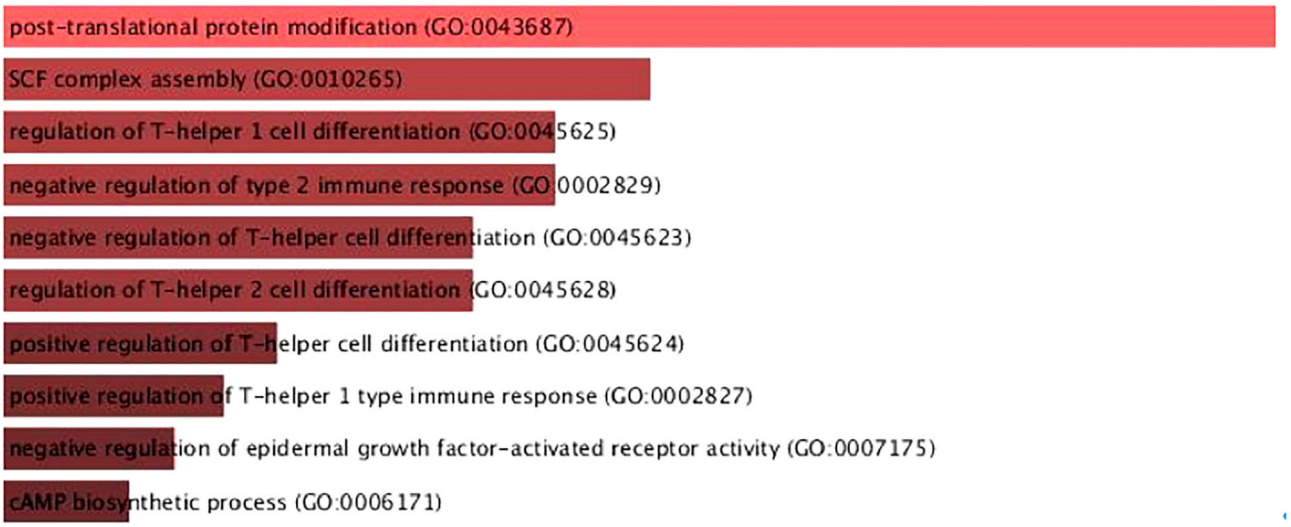

FIGURE 3 | Biological process terms of the 60 dysregulates genes in canine and human melanoma. It is possible to observe differences in processes related to immune system response. Figure generated using the online tool Enrichr [https://maayanlab.cloud/Enrichr/enrich#; (11, 12)].

III and three stage IV). Mean survival time was 390 days in stage I, 286 days in stage II, 159 days in stage III, and 90 days in stage IV. There was no influence of sex and age in RT responses. In conclusion, Radiation Therapy can be an interesting alternative treatment for canine oral melanoma, leading to reduction of tumor volume and pain relief, with mild side effects.

Innovative Therapies for Canine Melanoma

Viral Vectors in Melanoma, by Bryan Strauss

Currently, immunotherapy has emerged as the most successful usage of viral vectors applied for gene therapy of melanoma, including IMLYGIC and chimeric antigen receptor T cells (CAR-T cells) (40, 41). In either case, the goal of the gene transfer approach clearly involves oncolysis (therapeutic destruction of tumor cells) associated with or due to the activity of immune cells. The work of the Viral Vector Laboratory (Instituto do Câncer do Estado de São Paulo, Faculdade de Medicina, Universidade de São Paulo) shares this objective and has developed a non-replicating adenoviral vector, AdRGD-PG, with improved mechanisms of transduction because of the inclusion of the RGD peptide in the virus fiber protein and high-level transcription of the therapeutic genes under the command of a p53-responsive promoter, termed PG (42, 43). Since melanoma cells frequently retain wild-type p53 (44), this can be exploited to drive transgene expression and act as a tumor suppressor protein, when re-activated (45).

We used the AdRGD-PG platform for the transfer of p14ARF (alternate reading frame of the CDKN2a locus, p19Arf in mice, p14ARF in humans, and canines) to activate p53 and promote cell death. In addition, transfer of the interferon-β gene not only acts as an immune modulator, but also contributes to the activation of p53 (46). We used the B16F10 mouse model of melanoma to show that the combination of p19Arf and IFNβ gene transfer resulted in cooperative cell killing *in vitro* accompanied by the expression of immunogenic cell death (ICD) markers and *in vivo* where C57BL/6 mice revealed activation of a Th1 immune response (43–46). However, IFNβ is known to function in a species-specific manner (47, 48); therefore, it is imperative to test our approach in relevant models. Recent work using human cDNAs and established human melanoma cell lines produced a similar result, where cell killing was even more efficient and continued to be associated with the expression of ICD markers (49, 50). Strikingly, an *ex vivo* model using human SK-MEL-147 cells was used to show that cell death induced by our treatment activated human dendritic cells that caused priming of T cells to induce a cytolytic response on encountering naïve tumor cells (50). Thus, we have shown that our gene transfer approach promotes oncolysis and immune activation and can be considered as an immunotherapy. Currently, we are gearing up to examine the efficacy of our approach in veterinary cases of melanoma because of their relevance and similarity to human cases as well as the demand for efficient therapies for canines. For this, a set of AdRGD-PG vectors has been constructed encoding the canine cDNAs of p14ARF and IFNβ. In parallel, three cell lines were isolated from canine oral melanomas (unpublished results), characterized for their ability to grow in tissue culture as well as form subcutaneous tumors in BALB/c nude mice. These cell lines were shown to harbor wild-type p53 and show permissive transduction and reporter gene expression with our AdRGD-PG vectors (51). This model system is currently being tested and will serve as proof of concept before embarking on experimentation in spontaneous cases of canine oral melanoma. These critical steps have been taken to demonstrate the potential of our immunotherapy in a relevant model.

Immuno-Oncology in Canine Oral Melanoma, by Cristina De Oliveira Massoco

In the last 20 years, immunotherapy has gained prominence since this therapeutic modality has significantly increased the effectiveness of treatments and the survival rates of cancer

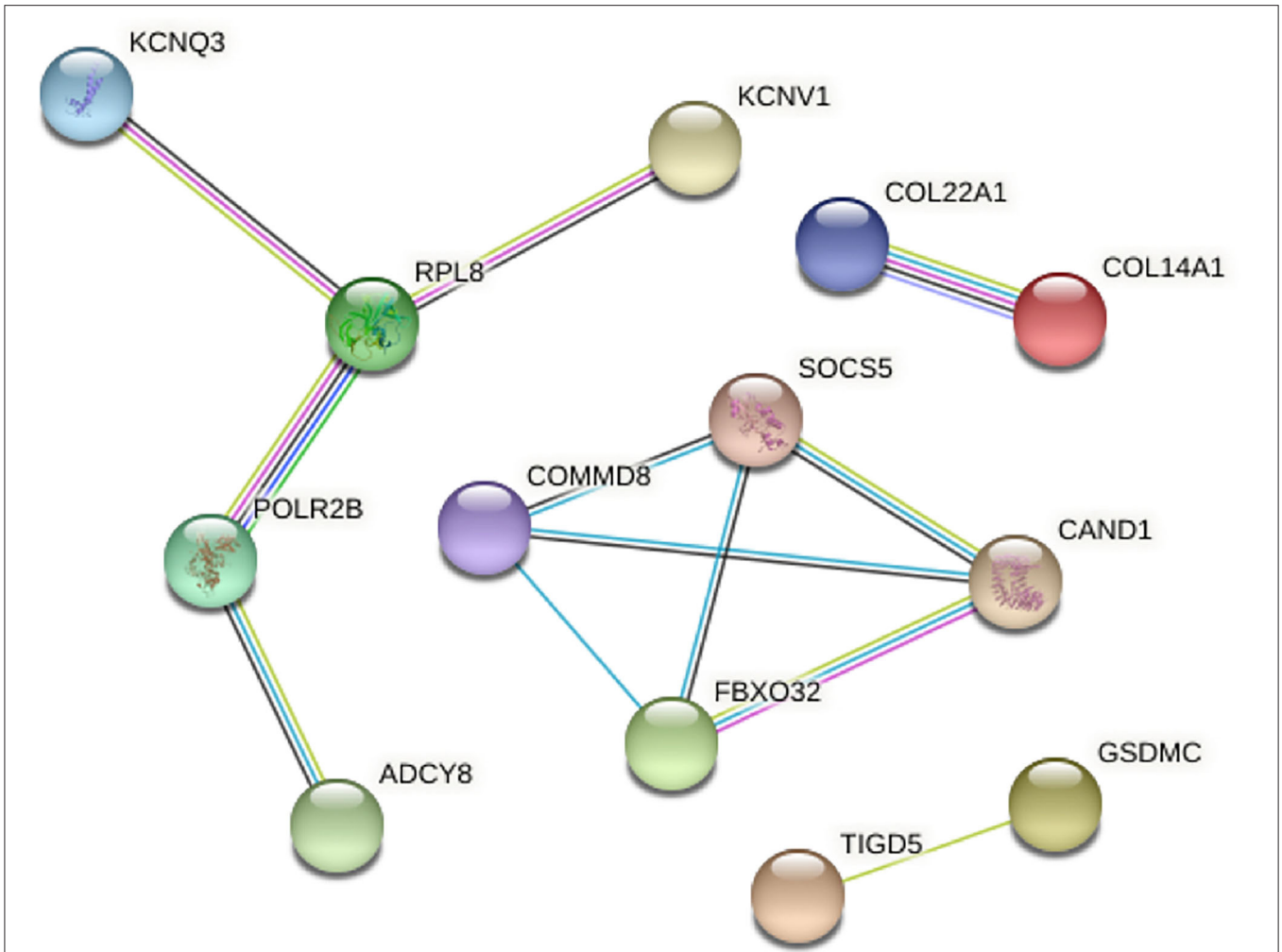

FIGURE 4 | Protein-to-protein interaction analysis with the commonly mutated genes between canine and human melanoma. The disconnected nodes were excluded and four different networks were identified. Figure generated using the online tool STRING (https://string-db.org/).

patients. The discovery that cells of the immune system interact with tumor cells and eliminate them is not a totally new concept and was born in the middle of the nineteenth century with the experiments carried out by Coley on human patients (52). Several clinical observations about spontaneous tumor regression and evidence showing a positive correlation between the presence of lymphocyte in a tumor microenvironment and increased survival rate reveal an increasing interest in the cancer immunosurveillance theory.

Especially for malignant melanoma, control of the disease with classical therapies and clinical management remains challenging, and yet immunotherapy modalities seem to be promising. Such strategies include DNA-based canine melanoma vaccine, immunotherapy methods using dendritic cells, and (non) viral vectors to deliver gene products, among others that have been studied (53). Even though responding melanoma-bearing dogs may have a partial or complete regression, some patients who are treated with immunotherapy showed varying response rates within cohorts with the same malignancy (54). Studies have shown that the efficacy of the immune system, in an anti-tumor context, is limited by the characteristics of immune cells present in the tumor microenvironment. These are reprogrammed to an immunosuppressive profile determined mainly by tumor cells, thus generating fewer effective responses against tumors (55, 56).

Cancer cells actively employ various tactics to delay, alter, or even stop antitumor immunity. These mechanisms develop continuously during the progression of cancer and become more diverse and complex in late-stage cancers (57). Therefore, it is critical to identify tumor immunity in the microenvironment to determine the outcomes and responses to immunotherapy. Identifying specific defects in the antitumor immune response as infiltrated immune cell population analysis associated with other immune signatures in dogs could serve as a basis for future studies to choose patients who will benefit from immunotherapy for oral melanoma.

Immunotherapy in the Treatment of Patients With Melanoma: OncoTherad® Nano-Immunotherapy, by Wagner Fávaro

Canine oral melanoma (COM) is a highly aggressive and metastatic cancer. Many studies have demonstrated that post-treatment medium survival time ranges from 4.8 to 12 months (58). Conventional treatments have not shown suitable disease control partly because surgery rarely achieves complete resection, and chemotherapies require low tumor loads to be effective. Immunotherapy has become a promising cancer therapy, improving the prognosis of patients with many different types of cancer, and offering the possibility for long-term cancer remission. In this scenario, a new perspective is represented by OncoTherad® nano-immunotherapy. OncoTherad® is a nanostructured inorganic phosphate complex associated to glycosidic protein, developed by University of Campinas (UNICAMP), Brazil, which exhibits immunomodulatory and antitumor properties (59). OncoTherad® leads to a distinct stimulation of the innate immune system mediated by Toll-like receptors (TLRs) 2 and 4, resulting in an increased activation of the interferon (IFN) signaling pathway (60, 61). OncoTherad®-induced stimulation of the immune system via TLR2 and TLR4 occurs through the phosphorylation of hydroxylated amino acids, such as tyrosine, threonine, and serine by compounds that contain phosphate salts; this results in the activation of stimulator of interferon genes, with a consequent increase in the production of IFN-α and IFN-γ (62). The increase in the production of IFNs mediated by TLRs-2 and 4 promotes the activation of $TCD8^+$ cells, dendritic cells, natural killer cells, and M1 macrophages, culminating in the induction of immunogenic cell death (62). Furthermore, OncoTherad® decreases the expression of receptor activator of nuclear factor-κB (RANK) and receptor activator of nuclear factor-κB ligand (RANK-L) system and, as a result, prevents or inhibits metastases progression (63). Thus, the aim of this study was to evaluate the efficacy of OncoTherad® nano-immunotherapy for first-line chemotherapy-relapse high-grade COM (with or without metastasis). We carried out a prospective, multicenter study in 19 (8 men, 11 women) consecutive patients with COM-relapse (≥1 previous course of first-line chemotherapy). OncoTherad® treatment (G1 group, $n = 10$) was initiated with its intramuscular (22 mg/mL) application twice per week for 3 months, followed by application, once every other week, until 1 year of treatment (**Figure 5**). OncoTherad® was associated (G2 group, $n = 09$) with chemotherapy (Carboplatin: 250—300 mg/m^2—intravenous) or electrochemotherapy (bleomycin: 15,000 IU/m^2—intravenous) (**Figure 5**). All canine patients received follow-up exams for 2 years. The Ethics Committee for Animal Experimentation (CEUA)/UNICAMP approved the animal procedures (protocol number 4861-1/2018). The median age of the 19 patients was 12.5 years (range 09–16) and follow-up time was 24 months. Based on iRECIST criteria, the overall complete response rate was 31.6% (G1 group−40.0%; G2 group−22.2%), overall partial response rate was 42.1% (G1 group−30.0%; G2 group−55.6%), and overall stable disease rate was 10.5% (G1 group−20.0%; G2 group−0.0%). Only 15.8% of total patients (G1 group−10.0%; G2 group−22.2%) presented progressive disease. The median overall progression-free survival was 640 days (G1 group−676.5 days, G2 group−599.4 days). In conclusion, OncoTherad® nano-immunotherapy seems an effective treatment option for chemotherapy-relapse COM patients and may provide benefits for preventing tumor progression.

Electrochemotherapy in the Treatment of Melanomas, by Marcelo Monte Mór Rangel

Electrochemotherapy is a new modality of local cancer treatment, which is increasingly gaining space, both in human and veterinary medicine. Its high index of objective response to different histological types, and its selectivity to cancerous tissue has encouraged its application in neoplasms that are more resistant to standard treatments. The main objective of surgery in the treatment of melanomas is the complete resection of the tumor and this usually occurs in wide surgeries. Large surgeries can be challenging in oral cavity melanomas and the need for adjuvant therapies aiming at better local control may be necessary. Electrochemotherapy is a local control technique with a high rate of objective response, selectivity to neoplastic tissue. Its use has been proposed in oral malignant melanomas that are difficult to resect, either as an adjunct to surgery or as a single treatment. The prior studies have shown promising results with singular use of the technique and its use as an adjunct with other therapies. These results corroborate the need for further studies to prove that the technique can be an alternative treatment for malignant melanomas in dogs (64–80).

Electrochemotherapy in the Treatment of Canine Oral Malignant mela noma, by Carlos Brunner

From July to December 2018, 58 melanomas in dogs were treated with electrochemotherapy with bleomycin and electroporation with BK100 electoporator, at the Universidade Paulista, UNIP, in São Paulo, SP, Brazil. The samples were composed as follows: a total of 54% of them were males, with an average age of 11.4 years. There was a higher prevalence of the following breeds: Yorkshire (10.7%), Golden Retriever (8.9%), Poodle (7.4%), Teckel (5.3%), and SRD (5.3%), similar to data found in literature. The incidence of oral melanomas was higher (75%), which was in contrast to the results of the literature review (62%). Bleomycin was used intra-lesionally at a dose of 1 U/cm^3 or intravenously at a dose of 15 Y/m^2, followed by electroporation with a BK100 pulse generator with a series of 8 pulses of 1 KV in 100 μs square waves each. This protocol has resulted in satisfactory survival rates after 2 years, including some complete remissions, in grade 1 and 2 melanomas. As described in classic literature, the anatomical location influences the biological behavior of melanomas; patients who underwent multimodal therapy, including surgery, conventional chemotherapy, and electrochemotherapy were the ones who obtained the best results.

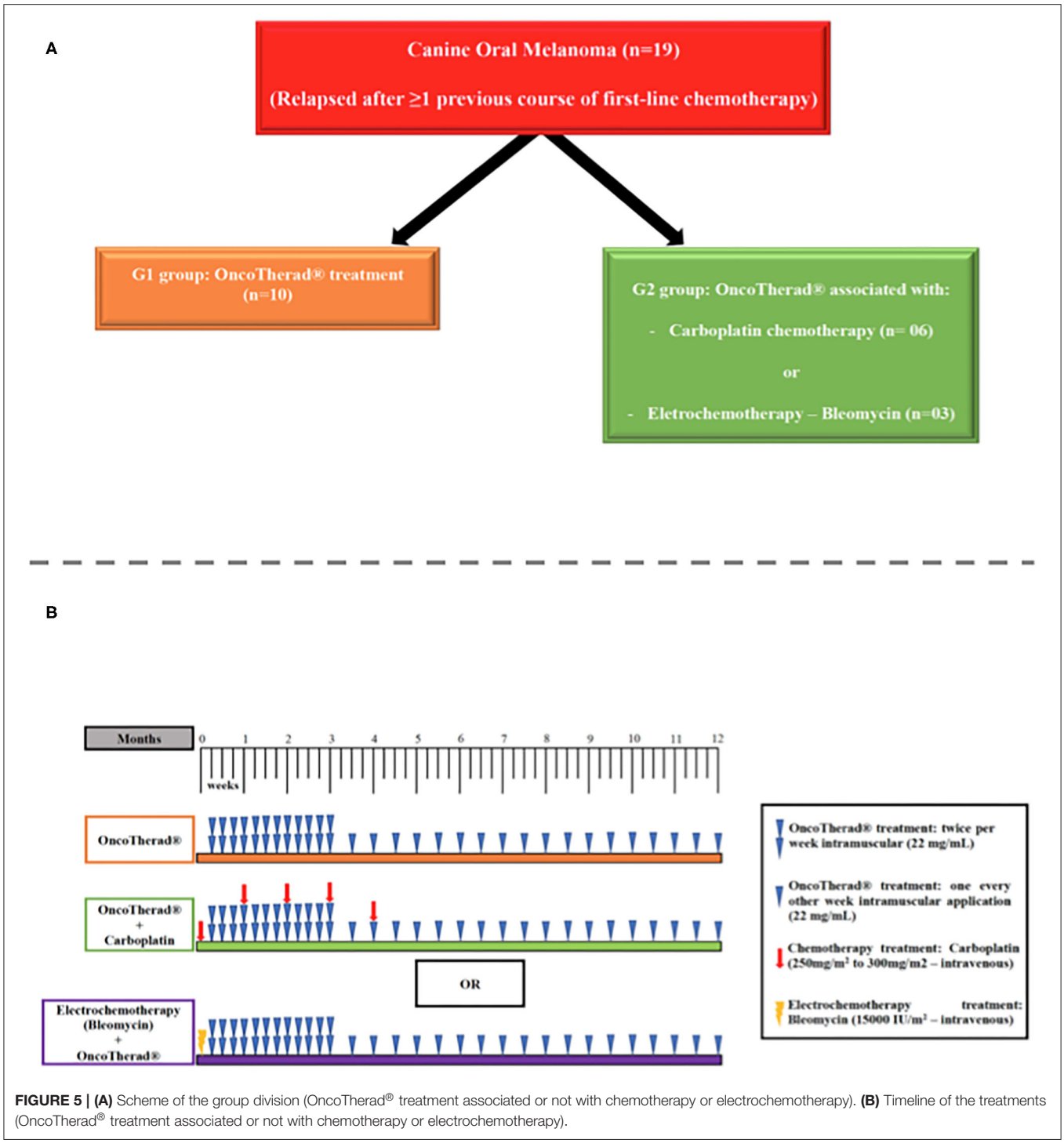

FIGURE 5 | (A) Scheme of the group division (OncoTherad® treatment associated or not with chemotherapy or electrochemotherapy). **(B)** Timeline of the treatments (OncoTherad® treatment associated or not with chemotherapy or electrochemotherapy).

Electrochemotherapy for the Treatment of Canine Melanoma: the Experience of Argentina, by Matías Tellado

For this round table, two cases of melanoma were presented. The results were evaluated according to the prognostic factors reported in previous work (81). The first patient was an 8-year-old, male cross-bred (mongrel) dog with stage I oral malignant melanoma located in the soft palate. No bone involvement was seen as determined by computed tomography scan. It was treated with BIOTEX EPV-200 (BIOTEX SRL, Buenos Aires, Argentina) electroporator, using 20 G disposable needles electrode. The device delivered 8,100 μs long square pulses of 1,000 V/cm at 5 kHz according to ESOPE 2018 standards (82). A month after the electrochemotherapy session, a complete response was

obtained, and the patient remained disease free 11 months later and until the writing of this work. The second case was a 9 year-old female boxer with a stage IV oral malignant melanoma located in the right angle of the mandible, that extended to the soft palate and mucosa. No bone involvement was seen as assessed by CT scan. The patient had a small single pulmonary metastasis determined by chest radiograph. The treatment consisted of surgical excision of the oral mass, followed by ECT in the tumoral bed and margins. A month after the ECT treatment, the patient presented a complete local response. The local response and the lung metastasis remained unchanged until 5 months later. The results obtained were in accordance with previous published results regarding predictive factors of response to ECT in oral melanoma (80, 81). Early stages and absence of bone involvement displayed good response rates. In the second case, even if it was in an advanced stage, the combination with surgery to reduce the tumoral burden and the absence of bone involvement allowed us to expect a good outcome of the treatment.

Clinical Reports of Melanoma Undergoing Electrochemotherapy or Calcium Electroporation, by Denner Santos Dos Anjos

Multimodal therapies have been used in melanomas, such as surgery, radiotherapy, chemotherapy, immunotherapy, electrochemotherapy, and electro gene therapy. ECT is an effective local treatment that combines the administration of chemotherapeutic drugs, such as bleomycin or cisplatin, followed by the delivery of permeabilizing electrical pulses. In veterinary medicine, electrochemotherapy has been widely used because of its high efficacy in all solid tumors, such as cutaneous and subcutaneous tumors, skin metastasis, melanoma, sarcomas, and visceral tumors, such as thymoma and bladder cancer (83–89). In addition to bleomycin and cisplatin being the chemotherapeutic drugs mainly used in electrical pulses, carboplatin may also be a good candidate for electric pulses based on our clinical experience (Dr. Denner dos Anjos). Moreover, calcium electroporation (CaEP) is a novel therapeutic perspective for patients with cutaneous metastasis of MM and remission of metastatic melanoma foci. Its approach leads to supraphysiological calcium influx into neoplastic cells leading to acute ATP depletion killing cancer cells by necrosis and release of dangerous cellular signals boosting immunologic system locally. We have observed some patients diagnosed with MM stage III (tumor size more than 4 cm, negative lymph node) who underwent ECT with carboplatin plus CaEP had a disease free-interval of >720 days after one session or ECT with carboplatin and locally administered cisplatin (1 m/cm^3) had a disease free-interval of >365 days. Furthermore, neoadjuvant ECT can also be used for partial remission in order to perform a better surgical approach for patients with advanced disease. To conclude, ECT may be an option for local control in regions where anatomic limitation is a challenge for wide excision.

DISCUSSION AND CONCLUSION

The goal of scientific meetings and workshops is to call attention to new achievements in the field to ensure advancement of science and applications. In conclusion, this colloquium provided important information about canine melanomas. The Brazilian Association of Veterinary Oncology, ABROVET, hopes that this report can help veterinary oncologists from all over the world to better diagnose and treat these aggressive neoplasms in dogs.

AUTHOR'S NOTE

The Brazilian Association of Veterinary Oncology, ABROVET, is a non-profit organization that was created in 2004 by veterinary oncologists from Brazil (https://abrovet.org.br/). ABROVET frequently organizes scientific and cultural events and seeks to develop the field of Veterinary Oncology in Brazil. In 2016, ABROVET organized the 3rd. World Veterinary Cancer Congress in the city of Foz do Iguaçu, Brazil. The Colloquium on Canine Melanoma was an online event held in December 2020, and had around 200 participants.

AUTHOR CONTRIBUTIONS

CF-A, ÊF, CO, BS, WF, ND, NO, SC, JC, MR, CB, MT, DA, and SF: writing and editing the manuscript. AB and LB: mentoring and editing the manuscript. MLZD: mentoring, writing, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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Quantification of Global DNA Methylation in Canine Melanotic and Amelanotic Oral Mucosal Melanomas and Peripheral Blood Leukocytes From the Same Patients With OMM: First Study

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Oral mucosal melanomas (OMMs) are aggressive and resistant cancers of high importance in veterinary oncology. Amelanotic OMM produces comparatively less melanin and is considered to be more aggressive than melanotic OMM. Global DNA methylation profiles with hypomethylated or hypermethylated patterns have both been associated with aggressive neoplasms; however, global DNA hypomethylation seems to correlate to higher aggressiveness. Accordingly, global DNA methylation in peripheral blood leukocytes has been investigated to understand the role of systemic or environmental factors in cancer development. This study aimed to quantify global DNA methylation in canine melanotic and amelanotic OMM samples and in the peripheral blood leukocytes of the same dogs. Tumor tissue samples were collected from 38 dogs, of which 19 were melanotic and 19 were amelanotic OMM. These were submitted to immunohistochemistry (IHC) with anti-5-methylcytosine (5mC) and anti-Ki67 primary antibodies. Ki67- and 5mC-positive nuclei were manually scored with the help of an image analysis system. Peripheral blood samples were collected from 18 among the 38 OMM-bearing dogs and from 7 additional healthy control dogs. Peripheral blood leukocytes were isolated from the 25 dogs, and DNA was extracted and analyzed by high-performance liquid chromatography (HPLC) for global DNA methylation. The pattern of global DNA methylation in both canine melanotic and amelanotic OMM indicated higher percentages of weakly or negatively stained nuclei in most of the OMM cells, presuming predominant global DNA hypomethylation. In addition, Ki67 counts in amelanotic OMM were significantly higher than those in melanotic OMM ($p < 0.001$). Global DNA methylation different immunostaining patterns (strong, weak or negative)

correlated with Ki67 scores. Global DNA methylation in circulating leukocytes did not differ between the 9 melanotic and 9 amelanotic OMM or between the 18 OMM-bearing dogs and the 7 healthy dogs. This study provides new information on canine melanotic and amelanotic OMM based on global DNA methylation and cell proliferation.

Keywords: cancer, DNA methylation, melanoma, dog, cell proliferation

INTRODUCTION

Melanoma is the most common malignant tumor in the oral cavity of dogs, accounting for 30–40% of all oral cavity cancers in dogs and 7% of all types of cancer in this species (1, 2). Breeds with higher mucosal pigmentation have a higher risk of developing oral melanoma, particularly the Scottish Terrier, Golden Retriever, Dachshund, Cocker Spaniels, and Poodles. An epidemiological study involving 2,350 affected dogs revealed that poodles are at a high risk of developing oral melanomas (3). Melanomas generally occur in middle-aged to older dogs, with average age of 9 years, regardless of gender. In dogs, 40% of OMM cases without lymphadenomegaly already have lymph node metastasis. In addition, the presence of metastatic lymph nodes at the time of diagnosis accounts for ∼25% (2, 4). Goldschmidt et al. (5) reported that melanomas in canines could be classified in melanotic (or melanocytic), when they kept the capacity to produce melanin, and amelanotic (or amelanocytic) when this capacity was totally or partially lost.

Melanotic and amelanotic OMMs are considered to be aggressive cancers in dogs. A first study from our group revealed, for the first time, differences between melanotic and amelanotic OMM, so that amelanotic OMM had more mitotic cells than melanotic counterparts, differed in the expression of connexins (i.e., the gap junction proteins) and dogs bearing amelanotic OMM presented a shorter lifespan in comparison to those with melanotic OMM (6).

Canine OMM can be considered a good model for human OMM (2, 3, 7–9) as both harbor NRAS mutations. However, according to Brocca et al. (10), single nucleotide polymorphysms are not a frequent event in *KIT* activation in canine OMM, and these differ from human OMM in this aspect.

In a study involving 95 dogs, canine cDNA sequencing data for six genes relevant to human melanoma classification detected somatic mutations in the NRAS and PTEN genes at the human hotspot sites, except in BRAF in oral melanomas (3). Wong et al. (11) performed a cross-species analysis by sequencing tumor-germline pairs from 46 primary human muscosal, 65 primary canine oral and 28 primary equine melanoma cases from mucosal sites.The data revealed recurrently mutated driver genes shared between species such as *NRAS*, *FAT4, PTPRJ, TP53* and *PTEN*, and pathogenic germline alleles of *BRCA1, BRCA2* and *TP53*.

Investigations into epigenetic characteristics of canine OMM have been scarce in recent years.

Cancer-associated epigenetic changes include global hypomethylation, site-specific hypomethylation and hypermethylation, and modification of chromatin linked to tumor suppressor gene silencing and oncogene activation (12). In several studies, reductions in global DNA methylation and hypermethylation of tumor suppressor genes are associated with carcinogenesis (13, 14). Although these patterns are independent, they may coexist in neoplasms and have been considered as biomarkers associated with cancer (2, 15, 16).

The study of global DNA methylation by using antibodies anti- 5mC started in the 1990's by Dr Alain Niveleau, from the Faculté de Médecine Claude Bernard Lyon I in France, who initially produced the antibody against 5mC. Piyathilake et al. (17), compared the results of global DNA methylation evaluated by radiolabeled methyl incorporation (CPM/microg of DNA) with immunohistochemical staining of the same tissue sections with a monoclonal antibody developed against 5-methylcytosine (5mC). The results suggested that both radiolabeled methyl incorporation assay and immunostaining for 5mC could be used to demonstrate hypomethylation of DNA in squamous cell carcinoma tissues compared to matched uninvolved tissues.

Based on our previous studies using anti-5-methylcytosine (5mC) immunostaining, global DNA methylation patterns was found to differ in mast cell tumor grades (18): grade 3, high-grade mast cell tumors, which present worse prognosis, showed predominant weak immunostaining for 5mC. Moreover, canine mammary cancers (19) were identified to develop a higher aggressiveness in hypomethylated (predominantly 5mC-weakly immunostained) tumors.

The evaluation of peripheral blood leukocyte global DNA methylation has been an interesting approach in epigenetics research (20, 21), as it allows us to investigate the influence of the environment on cancer development. Global hypomethylation in peripheral blood cell DNA has been associated with increased risk and prognosis of various cancers in humans (22). Alterations in DNA methylation in peripheral blood cells may not necessarily correlate with epigenetic changes in tumors; however, global DNA hypomethylation in peripheral blood leukocytes may reflect the genomic instability of an individual, which may lead to cancer development (23, 24). Recently, Epiphanio et al. (25) sought to determine whether peripheral blood global DNA methylation was associated with canine multicentric lymphomas. Based on their findings, dogs with non-Hodgkin lymphomas presented global DNA hypomethylation of circulating leukocytes compared to healthy dogs.

No reports regarding global DNA methylation in canine OMM, nor reports linking the global DNA methylation of peripheral blood leukocytes with oral melanoma in dogs, have been published in the field of veterinary medicine. Therefore, the aim of this study was to evaluate global DNA methylation by 5mC immunostaining and cellular proliferation in melanotic and

amelanotic OMM, and quantify global DNA methylation in the peripheral leukocytes of dogs with OMM.

MATERIALS AND METHODS

Ethics and OMM Samples

The study protocol was approved by the Committee of Ethics on the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science (Process number: 9522230517) and the School of Medicine of the University of São Paulo (Process number: 997/18). All dog owners signed informed consent forms.

For this study, OMM samples were acquired from two sources.

The first *set of 18 OMM* was obtained from dogs enrolled at the Provet Veterinary Hospital and Pet Life Care Hospital in São Paulo. Amelanotic and melanotic OMM samples (9 samples of each) were obtained from the animals during the surgical procedures for removal of tumors, or electrochemotherapy procedures, and blood samples from the same dogs were obtained for the quantification of leukocyte global DNA methylation. Blood was also collected from seven healthy dogs with similar ages to serve as the control. The groups were composed of male and female dogs, with or without defined breeds. The exclusion criteria were: history of other neoplasms, use of corticosteroids and chemotherapy before sample collection, and other concomitant serious diseases. The second *set of OMM* was composed of 20 melanotic and amelanotic OMM samples (10 samples of each) in paraffin blocks, that were kindly provided by Dr Cristina de Oliveira Massoco, from the Laboratory of Pharmacology and Toxicology at the SVMAS – USP.

All OMM samples were fixed in 10% formalin and routinelly embedded in paraffin wax. The 5μm sections were stained with Hematoxylin and Eosin to confirm the diagnosis before being submitted to immunohistochemistry. All amelanotic OMM had <50% pigmented cells (26), and their diagnosis was confirmed by immunostaining with the anti-Melan A antibody (27).

Blood Sampling for the Analysis of Leukocyte Global DNA Methylation

Four mL of peripheral blood, obtained via venipuncture, was collected from 18 melanoma-bearing and the 7 control dogs in EDTA tubes. Blood was centrifuged at 3,000 × g for 10 min at 4 °C, and the buffy coat containing leukocytes was separated. Samples were stored at −80 °C for subsequent DNA extraction.

Immunohistochemistry

All melanoma samples were obtained immediately after surgery, fixed in 10% formalin, and embedded in paraffin wax. The obtained 5 μm sections were subjected to immunohistochemistry with the following antibodies: anti-Melan A, anti-5-methyl cytosine (5mC), and anti-Ki-67 (**Table 1**). Additional slides representing samples from the negative control were subjected to the same immunohistochemical protocol, except the treatment with the primary antibodies.

After deparaffinization, the slides were gradually hydrated in ethanol, and the peroxidase was blocked for 40 min at 37°C in a solution containing hydrogen peroxide. The cells were then washed with phosphate buffer (PBS) and subjected to antigenic recovery in a Pascal pressure cooker (DAKO®) for 15 min with citrate buffer (pH 6.0). Thereafter, the slides were washed with PBS and incubated overnight at 4°C with specific primary antibodies.

TABLE 1 | Primary antibodies used for immunohistochemistry and their dilutions.

Antibody	Species	Clone	Dilution	Antigen recovery
Anti-MelanA (Dako®)	Mouse (monoclonal)	A103	1:50	Citrate buffer
Anti-Ki67 (Dako®)	Mouse (monoclonal)	MIB-1	1:50	Citrate buffer
Anti-5MeCyt (Abcam®)	Mouse (monoclonal)	33D3	1:100	Citrate buffer

Following overnight incubation, the slides were washed with PBS and incubated with Polymer EnVision ™ + Dual Link System-HRP® polymer for 30 min in the oven, according to the manufacturer's recommendations. After washing with PBS, the reactions were revealed with 3-amino-9-ethylcarbazole (AEC) for melanotic OMM or diaminobenzidine (DAB) for amelanotic OMM (DAKO®). The slides were counterstained with hematoxylin, washed with 0.5% ammoniacal water, washed under running water, subjected to diaphanization (protocol of ethyl alcohol and xylol), and finally assembled. The bleaching of melanotic melanomas was not performed in this study as bleaching modifies the immunogenicity of the tissue antigens, which may interfere with the final results of immunostaining.

Immunostaining with anti-5mC and anti-Ki67 was performed in separated batteries, with all samples from the same set placed in each battery to enable uniformity of the exposure time and quantification of the staining intensity.

Quantification and Scoring of 5mC- and Ki67-Positive Nuclei

An optical microscope (NIKON, Tokyo, Japan) linked to the Image-Pro Plus system (v.4.5.0.29; Media Cybernetics, Rockville, MD, USA) was used to quantify the 5mC- or Ki67-positive nuclei in the canine OMM. Digital photomicrographs were captured under the same light conditions, and 5 to 8 microscopic fields (40 × objective) of each sample were analyzed (counting and classifying at least 500 melanoma cells per sample) by the author NVS.

OMM cells were considered positive for 5mC if they presented nuclei with brown/gold (diaminobenzidine, DAB) or red (AEC) coloration. Nuclear staining intensity was classified as 0 (no staining), 1+ (weak staining), or 2+ (strong staining). The percentage of positive (2+, 1+) or negative nuclei was obtained for each photomicrograph, and then the percentage of 5mC 2+, 1+ or 0 was obtained for each tumor. The mean (for parametric tests) or median (for non-parametric tests) of the values for melanotic or amelanotic OMM was then obtained, and used for the statistical comparisons.

To quantify Ki67-positive cells, nuclei were scored as positive or negative on the Ki67 immunostained slides. At least 500 cells in 5 to 10 high-power fields (40 × objective) were counted per slide. The percentage of positive cells in each slide was calculated, and then a mean was obtained with the sum of the percentage of

positive nuclei in each group (amelanotic or melanotic OMM), divided by 19.

DNA Extraction and High Performance Liquid Chromatography for Quantifying Global DNA Methylation in Peripheral Blood Leukocytes

DNA extraction and purification were carried out according to the protocol provided in the Gentra Puregene® DNA extraction kit (QIAGEN Sciences, Maryland, USA). Briefly, the previously collected blood leukocyte fractions from 25 dogs (3 groups, 25 samples) were added to 600 μL of erythrocyte lysis solution (*RBC Lysis Solution*, Gentra Puregene® kit) after 10 min of incubation at 25°C room temperature. After the samples were centrifuged at 2,000 × g for 5 min at 4°C, the supernatant was carefully discarded. The samples were then added to 500 μL of cell lysis solution (*Cell Lysis Solution,* Gentra Puregene® kit) and 25 μL of ribonuclease A (15 mg/mL). Samples were subsequently incubated at 37°C for 15 min, and the protein was precipitated by the addition of 300 μL of protein precipitation solution (*Protein Precipitation Solution,* Gentra Puregene® kit) and centrifuged at 3,000 × g for 10 min at 4°C. The supernatant was transferred to a tube containing 1 mL of cold 2-isopropanol, and the precipitated DNA was collected via centrifugation at 2,000 × g for 5 min at 4°C. The DNA was washed with 1 mL of 70% ethanol, dried, and resuspended in 20 μL of 0.1 mM deferoxamine solution. The DNA concentration was determined by measuring the UV absorption at λmax: 260 nm (Libra S12 Spectrophotometer, Biochrom, Cambridge, UK), and DNA purity was assessed using the UV absorbance ratio at λmax: 260/λmax: 280 nm. Aliquots of 10-μg DNA samples in 0.1 mM deferoxamine solution were added to 2.5 μL of Tris-HCl/$MgCl_2$ buffer (200 mM, pH 7.4) and 1 unit of DNase I. Samples were incubated at 37°C for 60 min. After the addition of phosphodiesterase I (0.001 units) and alkaline phosphatase (1.2 unit), incubation was continued for another 60 min at 37°C. At the end of incubation, the samples were centrifuged at 9,300 × g for 10 min. Aliquots (10 μL) were injected into the HPLC-UV analytical system (*Shimadzu Corporation*®, Kyoto, Japan) to quantify global DNA methylation, expressed as percent 5-methyl-2'-deoxycytidine (5MeCyt), using the following equation:

$$5\text{mC}\ (\%) = \frac{5\text{mC} \times 100}{5\text{mC} + \text{dC}*_{(\text{nmol})}}$$

*deoxycytidine (dC).

The chromatographic condition consisted of a 250 × 4.6 mm i.d., 5 μm, Shim-pack VP-ODS column (Shimadzu, Kyoto, Japan) with a C18 4.0 × 3.0 mm pre-column (Phenomenex, Torrance, CA) eluted with a gradient of 0.1% formic acid in water (solution A) and methanol:water (1:1) with 0.1% formic acid (solution B) at a flow rate of 1 mL/min and 35°C. The following conditions were employed: from 0 to 25 min, 0 to 40% B; from 25 to 26 min, 40 to 0% B; and from 26 to 40 min, 0% B. The PDA detector was set at 286 nm. Calibration curves were constructed at intervals of 0.01 to 1.2 nmol of 2′-deoxycytidine (dC) and 0.002–0.04 nmol of 5mC.

TABLE 2 | Characteristics of the dogs included in the study.

Canine patient	Gender	Age (yrs)	Breed	WHO stage
Melanotic OMM				
M1	M	15	Mongrel	II
M2	F	14	Mongrel	II
M3	M	14	Poodle	II
M4	F	14	Beagle	II
M5	F	12	Poodle	II
M6	F	16	Mongrel	II
M7	M	12	Mongrel	II
M8	M	13	Lhasa apso	II
M9	M	12	Mongrel	II
M10	M	9	Golden retriever	III
M11	F	12	Yorkshire	II
M12	F	11	Schnauzer	II
M13	M	10	Poodle	III
M14	F	15	Mongrel	III
M15	M	13	Mongrel	III
M16	M	15	Poodle	III
M17	M	13	Mongrel	II
M18	M	12	Golden retriever	III
M19	M	11	Schnauzer	II
Amelanotic OMM				
A1	F	11	Mongrel	II
A2	F	12	Mongrel	II
A3	F	13	Mongrel	II
A4	F	12	Yorkshire	II
A5	F	12	Mongrel	II
A6	F	14	Yorkshire	II
A7	F	14	Mongrel	II
A8	F	12	Mongrel	II
A9	M	13	Mongrel	II
A10	M	13	Pinscher	II
A11	F	16	Mongrel	III
A12	F	12	Pug	III
A13	M	8	Teckel	I
A14	M	14	Mongrel	I
A15	F	14	Cocker Spaniel	II
A16	M	15	Teckel	III
A17	F	16	Mongrel	III
A18	M	9	Teckel	III
A19	F	8	Mongrel	III
Clinically Healthy (controls)				
C 1	F	12	Mongrel	Clinically Healthy
C 2	M	11	Mongrel	Clinically Healthy
C3	F	10	German Shepherd	Clinically Healthy
C4	M	12	Mongrel	Clinically Healthy
C5	M	10	German Shepherd	Clinically Healthy
C6	M	14	Mongrel	Clinically Healthy
C7	F	14	Mongrel	Clinically Healthy

Statistical Analysis

GraphPad Prism® software was used for the statistical analyses. After the normality test, 5mC- or Ki67- positive nuclei quantifications were compared between melanotic and amelanotic OMM. The Kruskal Wallis non-parametric test, with the Dunn's *post-test*, was used to compare 5mC-positive cell medians between melanotic and amelanotic OMM (considering $p < 0.05$). The Student's *t*-test was employed to compare the percentages of Ki67-positive nuclei between melanotic and amelanotic OMM; $p < 0.05$ was set as the significance limit. The Student's *t*-test and ANOVA were employed as the statistical tests to quantify the global DNA methylation in blood leukocyte. The Spearman's rank correlation coefficient was used to define the level of correlation between 5mC and Ki67 scores.

RESULTS

Subjects Characteristics

In this study, samples from 19 dogs with melanotic OMM and 19 with amelanotic OMM (ages: 8–16 years-old; sex ratio: 52.64% females and 47.36% males) were included. The dog breeds with melanotic OMM were Mongrels (8/19), Poodles (4/19), Beagles (1/19), Lhasa Apsos (1/19), Golden Retriever (2/19), Schnauzer (2/19) and Yorkshier (1/19), while those with amelanotic OMM were Mongrels (11/19), and Yorkshires (2/19), Pischer (1/19), Pug (1/19), Teckel (2/18), Cocker Spaniel (1/18). The control group was composed of seven healthy dogs (age: 10–14 years-old, sex: 57.14% males and 42.85% females), where 5/7 and 2/7 were Mongrels and German shepherd dogs, respectively. All OMM bearing dogs were taken to the veterinary hospital to undertake electrochemotherapy, surgery and or dendritic cell vaccination. Most dogs were in WHO stage II (4), and none of them had metastasis at the moment of diagnosis and therapeutic procedures. The characteristics of the dogs enrolled in the study are shown in **Table 2**.

Scoring of 5mC and Ki67-Positive Nuclei

Melanotic and amelanotic OMM samples were analyzed to derive their immunoreactivity to 5mC to investigate global DNA methylation.

Nuclear immunostaining for 5mC was classified as strong (2+), weak (1+), or negative (0). Most cells from both melanotic and amelanotic OMM samples presented weak or negative staining for 5mC (see **Table 3**). In addition, no differences between melanotic and amelanotic OMMs regarding the strong, weak, and negative nuclei percentages was observed, as shown in **Table 3** and **Figure 1**.

Figure 2 presents example photomicrographs of the immunostainings for 5mC and Ki67 in melanotic and amelanotic OMM.

The percentage of Ki67-positive nuclei was significantly higher in amelanotic OMM than in melanotic OMM, as shown in **Table 3** and **Figure 3**.

TABLE 3 | Summary data of the Ki67 and 5mC quantifications in melanotic and amelanotic OMM.

	Ki67	5mC 2+	5mC 1+	5mC -negative
Melanotic OMM ($n = 19$)	21.12 ± 9.03	1.71 ± 1.57	40.64 ± 16.41	57.65 ± 16.61
Amelanotic OMM ($n = 19$)	57.32 ± 12.04*	2.83 ± 2.37	58.15 ± 18.76	39.02 ± 18.41

Data represent the mean of the percentages (mean ± standard deviation).
**Statistically significant, Student t-test ($p < 0.001$) so that amelanotic OMM have significantly higher positive nuclei.*
Kruskal Wallis and Dunn's test ($p > 0.05$) were used to compare the 5mC scores for strong, weak, and negative nuclei immunoreactivity between melanotic and amelanotic OMMs.

Correlation Between 5mC and Ki67 Scorings

The correlation between 5mC score in melanotic and/or amelanotic OMM and the Ki67 score was evaluated by the Spearman's correlation coefficient, a non-parametric measure of rank correlation. It assesses how well the relationship between two variables can be described using a monotonic function. This coefficient assumes values between−1 and 1. **Table 4** shows the degrees of correlations obtained. Significant low to moderate correlations were obtained when the 5mC and Ki67 from 38 samples were tested. No significant correlations were observed when either melanotic or amelanotic 5mC scores were compared with the respective Ki67 score.

Quantification of the Global DNA Methylation in Canine Blood Leukocytes by HPLC

Quantification of global DNA methylation in leukocytes collected from melanotic OMM, amelanotic OMM, and control dogs was performed according to the area obtained in the

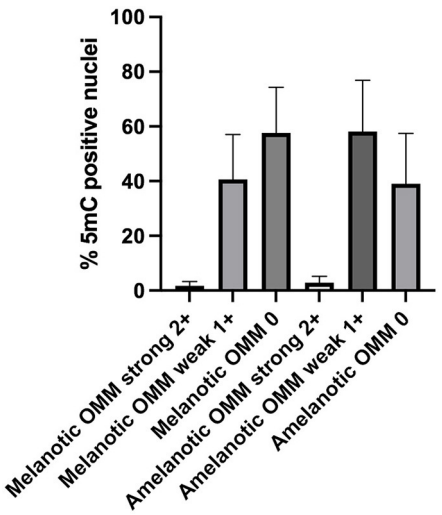

FIGURE 1 | Scores of the 5mC-positive nuclei in samples of canine melanotic and amelanotic OMMs. The 5mC-positive cells were divided into strong (2+), weak (1+), or negative (0) staining. Medians were compared by Kruskal Wallis, with the Dunn's *post-test*. No significant differences were obtained. However, more than 90% of the cells in both melanotic and amelanotic OMM samples were either weakly or negatively stained for 5mC.

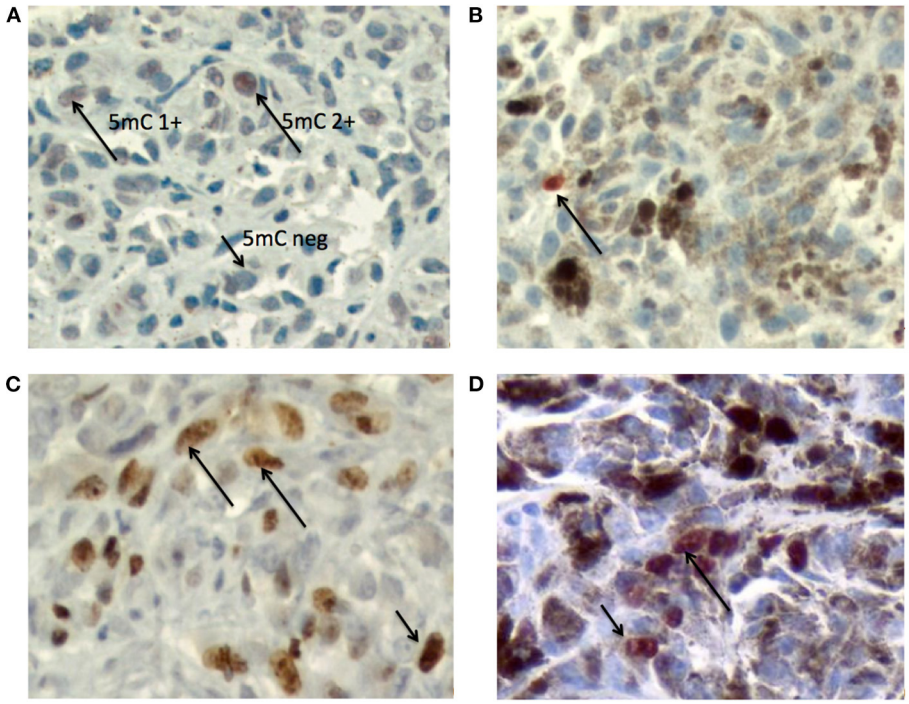

FIGURE 2 | Photomicrographs of the immunostainings for 5mC and Ki67 in melanotic and amelanotic OMM. **(A)** Immunostaining of 5mC in amelanotic OMM. Arrows indicate the patterns 5mC2+ and 5mC 1+ and 5mC 0 (DAB + hematoxylin). **(B)** Immunostaining of 5mC in melanotic OMM. Arrow indicates the 5mC 2+ nucleus (AEC + hematoxylin). **(C)** Immunostaining of Ki67 in amelanotic OMM. Arrows indicate a positive nuclei (DAB + hematoxylin). **(D)** Immunostaining of Ki67 in melanotic OMM. Arrows indicate positive nuclei (AEC + hematoxylin). Objective = 40x.

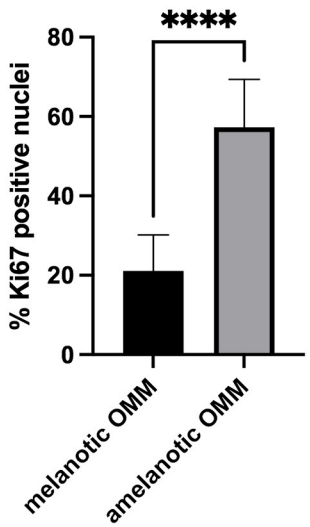

FIGURE 3 | Scores of the Ki67-positive nuclei in samples of canine melanotic and amelanotic OMM. Means were compared using the Student's *t*-test. The percentage of Ki67-positive cells was significantly higher (****) in amelanotic OMM than melanotic OMM ($p < 0.05$).

TABLE 4 | Summary of the Spearman's correlation analysis of the 5mC data and Ki67 positive nuclei in melanotic and amelanotic OMM.

Data used for correlation	Spearman r	Correlation	*P*-value	Significant?
Melanotic + amelanotic OMM (*n* = 38) × Ki67 (*n* = 38)				
5mC 2+ × Ki67	0.3166	Positive, low	0.0528	*
5mC 1+ × Ki67	0.4439	Positive, moderate	0.0052	**
5mC negative × Ki67	−0.4426	Negative, moderate	0.0054	**
Melanotic OMM (*n* = 19) × Ki67 (same samples, *n* = 19)				
5mC 2+ × Ki67	0.4368	Positive, moderate	0.0615	ns
5mC 1+ × Ki67	0.04561	Positive, very low	0.8529	ns
5mC neg × Ki67	−0.02807	Negative, very low	0.9092	ns
Amelanotic OMM (*n* = 19) × Ki67 (same samples, *n* = 19)				
5mC 2+ × Ki67	0.1255	Positive, low	0.6087	ns
5mC 1+ × Ki67	0.2386	Positive, low	0.3253	ns
5mC negative × Ki67	−0.1719	Negative, low	0.4815	ns

** and ** = significance level, ns = non significant.*

chromatograms that refer to dC and 5mC. By analyzing the chromatograms, peripheral blood leukocytes from melanotic OMM and amelanotic OMM patients and controls were found to have a similar global DNA methylation content (5.26 ± 0.64, melanotic OMM; 4.64 ± 1.05, amelanotic OMM; and 5.06 ± 0.46, controls, *P*-value ≥0.05) (**Figure 4**).

When leukocyte global DNA methylation in melanotic OMM and amelanotic OMM were combined and compared to the controls, no significant difference was obtained by the Student

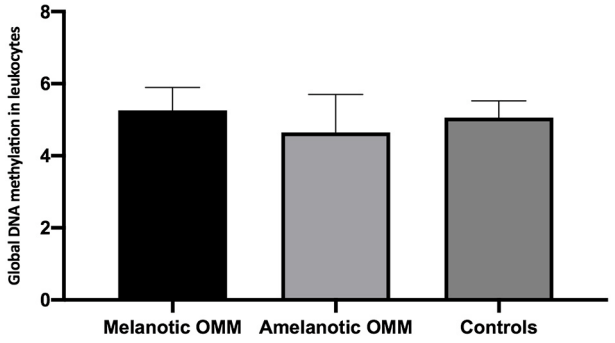

FIGURE 4 | Quantification of global DNA methylation in peripheral blood leukocytes from dogs bearing melanotic OMM, amelanotic OMM, or control dogs. No statistical differences were obtained when the means were compared by ANOVA ($p > 0.05$).

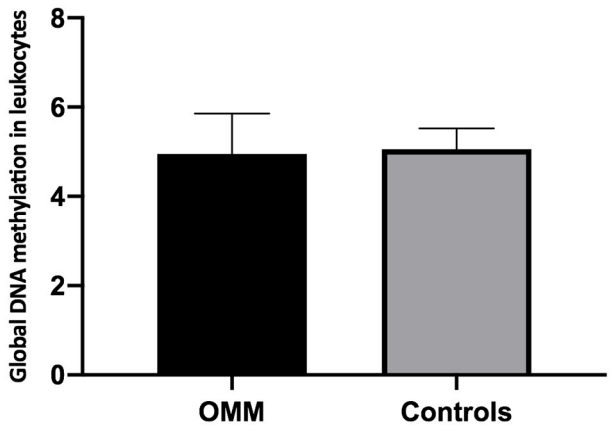

FIGURE 5 | Quantification of global DNA methylation in peripheral blood leukocytes from dogs bearing melanotic and amelanotic OMM or control dogs. No statistical differences were obtained when means were compared by the Student's *t*-test ($p > 0.05$).

t-test (4.95 +0.90, OMM and 5.06 ± 0.46, controls) ($p > 0.05$) (**Figure 5**).

DISCUSSION

OMM is a very prevalent neoplasm in dogs. A report from our group have revealed differences in cell proliferation (mitosis countings), expression of connexins, and outcome between melanotic OMM and amelanotic OMM (6). Although several parameters have been identified to establish the behavior and prognosis of OMM (2, 26), only few studies have investigated the differences between melanotic and amelanotic OMM (6, 9, 28). To our knowledge, studies evaluating the global DNA methylation landscape in canine OMM and in blood leukocytes of OMM bearing dogs have not been reported in the literature, which motivated us to carry out the present study.

This study included OMM from both female and male dogs, aged 10–16 years old. Dogs with melanotic OMM, amelanotic OMM, and clinically healthy (blood leukocyte control) were enrolled in the cohort. OMM is reported to mainly affect animals aged 8–11 years (1) or middle-aged to older dogs, with average age being 9 years, and no gender predilections (21). This study included a homogeneous population of older dogs, and most of the dogs enrolled herein were females (52.64% females and 47.36% males).

Several histopathological parameters have been studied to predict the prognosis of OMM in dogs, including morphological classification, nuclear atypia, mitoses, cellular pleomorphism, margin evaluation, pigmentation, and cell proliferation (29). In the present study, tumor global DNA methylation and growth fraction were assessed to compare melanotic OMM to amelanotic OMM.

Initially melanotic OMM were differentiated from amelanotic OMM via gross observation of the tumor mass and morphological characteristics based on histopathological examination, as well as by estimating the number of cells that expressed melanin, according to the criteria of the Oncology Pathology Working Group OPWG (26). All amelanotic melanomas included in this study were whitish upon gross examination and had <50% of melanin-expressing cells based on the histopathological assessment. In addition, all amelanotic OMMs were subjected to immunohistochemistry with Melan A as the primary antibody, showing positivity. Such finding assured that amelanotic OMM was diagnosed with certainty.

Both genetic and epigenetic events are involved in carcinogenesis and cancer (14–16). Epigenetic events include DNA methylation, histone acetylation, and micro-RNA. Because epigenetic studies in canine melanomas are scarce, this study aimed to compare global DNA methylation in melanotic OMM to that in amelanotic OMM.

We performed immunohistochemical analysis of melanotic and amelanotic OMMs using 5mC as the primary antibody. All 38 samples, separated in 2 sets of 18 and 20 samples, respectively, were subjected to IHC in the same batch to avoid differences in DAB (for amelanotic OMM) or AEC (for melanotic OMM) revealing time as well the potential differential intensity that may be induced. The OMM slides were analyzed in an image analysis system to quantify strong, weak, or negative nuclei (at least 500 per case). The same quantification approach was used in other studies (18, 19, 30). The results of these quantifications showed that both melanotic and amelanotic OMMs presented higher percentages of weakly stained and negative 5mC nuclei. Such finding may presume global DNA hypomethylation in the OMM cells, according to the pioneer studies from Piyathilake et al. (17). The predominance of global DNA hypomethylation is in general associated with highly aggressive neoplasms. The negative nuclei have been interpreted as bearing a very low level of 5mC, which was impossible to detect using the immunohistochemical technique.

Although gene specific methylation was not evaluated, hypermethylation of cancer-related genes is known to play a fundamental role in tumorigenesis and may lead to increased invasion and possibly higher aggressiveness. Koroknai et al. (31) identified several methylation changes that can play a role in human melanoma progression, including hypermethylation

of the promoter regions of the ARHGAP22 and NAV2 genes that are commonly altered in locally invasive primary melanomas as well as during metastasis. In addition, Yamamoto et al. (32) investigated the role of epigenetic aberrations in human melanomas by genome-wide DNA methylation analysis of 51 clinical malignant melanoma samples. Hierarchical clustering analysis revealed two DNA methylation epigenotypes: high- and low-methylation subgroups. Tumor thickness was significantly greater in cases of high-methylation tumors than low-methylation tumors. Further, the prognosis was significantly worse in high-methylation cases.

In the OMM samples, hypermethylated and hypomethylated nuclei were found, with higher numbers in hypomethylation cells. New studies isolating these cells may be performed to evaluate gene-specific DNA methylation in these cells.

In contrast (18, 19, 30), demonstrated that the most aggressive tumor subtypes have shown predominant weakly stained nuclei that presumably revealed global DNA hypomethylation. Unfortunately, in this study, we could not evaluate the normal methylation pattern of canine mucosal melanocytes, since these cells are roughly seen in histological sections of oral mucosa in clinically healthy dogs.

Many studies have revealed a decrease in general methylation with aging, and epidemiological analyses have used leukocyte methylation as a risk marker, suggesting a predisposition to cancer by DNA hypomethylation (21, 22). In our study, we investigated the level of global DNA methylation in aged dogs only, since the study protocol has not included the analysis of blood leukocytes of dogs from other ages. However, this can be approached in future studies.

Some studies have reported aberrant DNA methylation profiles in canine melanomas. By employing genome-wide next-generation sequencing to analyze the DNA methylation patterns of canine melanomas, studies found increased methylation at thousands of normally unmethylated CpG sites in CGIs and decreased methylation at normally methylated CpG sites in non-CGIs. Six genes with "sequence-specific DNA binding" annotation were significantly enriched, including three homeobox genes (HMX2, TLX2, and HOXA9). As a result, the researchers concluded that these genes may be involved in the tumorigenesis of canine malignant melanoma (33). Another recent study examined the DNA methylation status of long interspersed nucleotide element-1 (LINE-1) as a surrogate marker of genome-wide methylation changes in this disease. These studies compared 41 canine melanoma patient samples and six cell lines to normal mucosae. Based on their findings, LINE-1 showed remarkable hypomethylation in melanomas compared to normal mucosae (34).

Cell proliferation assessed via immunostaining and quantification of Ki67 can be considered an important biomarker of tumor aggressiveness and prognosis. Therefore, we sought to quantify the Ki67-positive nuclei in the same melanotic and amelanotic OMM samples according to the guidelines of Meuten et al. (29). Amelanotic OMM has a significantly higher Ki67 index than melanotic OMM; this is despite both tumors displaying Ki67 indexes higher than 19.5 cells/high power field (hpf), which indicates poor prognosis (29).

Smedley et al. (28) revealed that the value of the Ki67 index is useful as a prognostic factor among other histological features. Sevastre et al. (35) also analyzed cell proliferation (Ki67) in canine melanoma samples and found a positive correlation between Ki67 index value, necrosis, and mitosis. Therefore, by considering cell proliferation, we suggest that amelanotic OMM has a higher growth fraction (36) than melanotic OMM in dogs.

Interstingly, the 5mC scores in melanotic and amelanotic OMM revealed a low to moderate positive correlation with Ki67 scores, while the negative characteristic (absence of 5mC staining) was negatively and moderately correlated with the Ki67 score. This may indicate that if a neoplasm shows many negative 5mC nuclei, it may show a higher growth fraction. This may be in accordance with the presumed relationship between the global DNA hypomethylation and the higher cell proliferation and possibly higher aggressiveness.

Epigenetic alterations may be affected by environmental modifications. The assessment of global DNA methylation in circulating leukocytes is thus being carried out to understand the environmental influences on cancer (23, 24). The role of global DNA methylation in blood leukocytes is relevant to the determination of possible etiological factors and pathogenesis of melanoma; this is because it is affected by lifestyle and environmental factors and is potentially a molecular link mediating the association between unhealthy lifestyle/environmental carcinogens and cancer risk (23).

DNA methylation has been intensively investigated as an epigenetic factor with potential biomarkers for cancer and carcinogenesis. According to the results of this study, the pattern of circulating leukocyte global DNA methylation in dogs bearing melanotic and amelanotic OMM was similar based on the HPLC methods employed. However, a tendency for hypomethylation was noted in the amelanotic melanomas. Further, leukocyte global DNA methylation in combined melanotic and amelanotic OMMs did not differ from that observed in control animals. Such findings indicate that canine OMM may not be associated with environmental or systemic factors. Thus, local etiological factors may be considered. Local factors related to OMM pathogenesis may include consanguinity, trauma, chemical exposure, hormones, genetic susceptibility, the presence of pigmented cells, or even the oral microbiota. Inflammation may also be associated with the etiology of these tumors (2).

In conclusion, this study revealed the presence of higher 5mC weakly stained nuclei, presumably global DNA hypomethylation, in both canine melanotic and amelanotic OMM, and significantly higher growth fraction in amelanotic OMM than melanotic OMM detected by Ki67 scoring. No alterations were found when the leukocyte global DNA methylation was compared among melanotic, amelanotic, and healthy dogs of similar ages. The study's main limitation is its relatively low number of melanoma samples. However, to our knowledge, this is the first report to evaluate global DNA methylation in canine melanomas and peripheral blood leukocytes. Canine OMM is considered to be a good model for human OMM (3, 7–9, 37). Notably, in present day clinics, there has been translation of new cancer treatments from pet dogs to humans (38). Further, findings regarding tumors from one species may influence the prevention and treatment in

another. As the differentiation between melanotic and amelanotic melanomas can be important to understanding the prognosis and new possibilities of intervention, a study involving a higher number of OMM cases is ongoing.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee of Ethics on the Use of Animals (CEUA) of the School of Veterinary Medicine and Animal Science (Process number: 9522230517) and the School of Medicine of the University of São Paulo (Process number: 997/18). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

NS: conceptualization, methodology, conducted the study, and writing the manuscript. TE, FH-B, and AL: methodology and editing the manuscript. KC: supplied animal materials for the study and editing the manuscript. JF: methodology and data analysis. CM: supplied materials for the study, methodology, and editing the manuscript. MD: conceptualizadion, funds, writing the manuscript, supervision, and editing the manuscript. All authors contributed to the article and approved the submitted version.

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COX-2 Silencing in Canine Malignant Melanoma Inhibits Malignant Behaviour

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Metastatic melanoma is a very aggressive form of cancer in both humans and dogs. Dogs primarily develop oral melanoma of mucosal origin. Although oral melanoma in humans is rare, both diseases are highly aggressive with frequent metastases. This disease represents a "One Health" opportunity to improve molecular and mechanistic understanding of melanoma progression. Accumulating evidence suggests that cyclooxygenase-2 (COX-2) may play a critical role in the malignant behaviour of melanoma. In this study we analysed 85 histologically confirmed melanomas from canine patients and showed that COX-2 is overexpressed in both oral and cutaneous melanomas and that COX-2 expression correlates with established markers of poor prognosis. To determine the role of COX-2 in melanoma we developed two melanoma cell lines with stable integration of an inducible doxycycline-regulated expression vector containing a COX-2 targeted micro-RNA (miRNA). Using this system, we showed that cellular proliferation, migration and invasion are COX-2 dependent, establishing a direct relationship between COX-2 expression and malignant behaviour in canine melanoma. We have also developed a powerful molecular tool to aid further dissection of the mechanisms by which COX-2 regulates melanoma progression.

Keywords: melanoma, COX-2, canine, malignant, one health

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INTRODUCTION

In humans, melanoma is one of the most aggressive types of cancer, every year killing ∼50,000 people worldwide (1). Cutaneous forms represent the most common cases and are responsible for 65% of all skin malignancy-related deaths (2). Melanomas of the oral cavity are rarer but are associated with aggressive behaviour, including a tendency to repeated relapse and to metastasise, and poor patient outcomes (3). In contrast, the majority of melanomas forming in dogs are of mucosal origin, and most commonly seen in Scottish terriers, golden retrievers, poodles and dachshunds indicating a genetic predisposition to this disease. Canine oral melanoma is very aggressive and highly metastatic, with frequent metastases to local lymph nodes and lungs (4). Therefore, there is potential to adopt a "One Health" approach to improve molecular and mechanistic understanding of melanoma progression. Accumulating evidence suggests that cyclooxygenase-2 (COX-2) expression may be considered as a prognostic biomarker and as a potential therapeutic target in both human melanoma (5, 6) and canine melanocytic neoplasms (7).

COX-2, also known as prostaglandin-endoperoxide synthase 2 (PTGS2), is an inducible form of the rate-limiting enzyme in the metabolic conversion of arachidonic acid to prostaglandins, including prostaglandin E2 (PGE_2), a significant mediator of inflammation and angiogenesis. Previously, COX-2 overexpression has been described in multiple human cancers including skin, bone, oesophageal, breast, lung, pancreatic, colon, cervical, prostate and bladder cancer, and is inversely associated with patient survival (8). At the molecular level the COX-2/PGE_2 axis modulates a number of signal transduction pathways in human cancer cell lines that affect tumour cell proliferation, apoptosis, immune evasion, angiogenesis, cellular adhesion, differentiation and invasion (9). In canine osteosarcoma, we have previously shown that COX-2 expression is 141-fold higher in the cancer stem cell population compared to the non-cancer stem cell population, and that COX-2 plays a major role in tumour initiation (10). Consistently, inhibition of COX-2 with either non-steroidal anti-inflammatory drugs (NSAIDs) or with specific COX-2 inhibitors (COXIBs) can reduce the mortality of several types of cancer (11) and can switch the immune response from a tumour-promoting profile to a tumour-destructive one, re-ordering the tumour microenvironment (12). However, despite ample epidemiological evidence supporting an inverse relationship between NSAID use and the incidence and progression of cancer the clinical application of these drugs for cancer prevention remains controversial. NSAIDs and COXIBs have been shown to have off-target effects and to supress the biosynthesis of other physiologically relevant prostaglandins that are associated with the adverse side-effects of these drugs on the GI tract and cardiovascular system, including elevated risk of myocardial infarction and stroke (13, 14). We have previously shown that mavacoxib (Trocoxil™), a clinically relevant COXIB in veterinary medicine, which is currently licenced to treat pain and inflammation in canine osteosarcoma, can exert anti-tumourigenic effects on a panel of canine cancer cell lines with low COX-2 expression indicating that this drug may act in a COX-2 independent manner (15).

In the context of canine melanoma, the exact role of COX-2 in the progression of the disease has not been fully elucidated. Previous studies have examined the association between COX-2 expression and prognostic factors, such as Ki-67 proliferation index and survival time after diagnosis, in melanocytic tumours and concluded that COX-2 may be associated with malignancy and may be a potential prognostic marker (7, 16, 17). *In vitro*, COX-2 inhibition by celecoxib in two canine melanoma cell lines has been shown to supress cell growth and have anti-tumour effects (18). One of the limitations of veterinary research is a lack of molecular tools to dissect the contribution of COX-2 to tumour progression. A model system in which reversible loss of COX-2 function could be controlled in space and time would be of significant value. In this study we have correlated the expression level of COX-2 in samples from canine patients with established markers linked to poor prognosis, and we describe the development of two melanoma cell lines with stable integration of a doxycycline-regulated expression vector containing a COX-2 targeted micro-RNA (miRNA), to enable us to reversibly suppress endogenous *COX-2* gene expression. We utilised this system to show that COX-2 is an essential driver of cellular proliferation, migration and invasion of canine melanoma cells.

MATERIALS AND METHODS

Retrospective Case Material and Histologic Classification

Eighty-five cases from canine patients in which melanoma was diagnosed were retrieved from the archive of the Comparative Pathology Laboratory, Department of Pathology, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Informed client consent was obtained for all patient samples used in this study. Of these, 29 cases were oral melanomas and 56 were cutaneous melanomas. To confirm the primary diagnosis each sample was re-examined independently by two pathologists (E.F. and T.S.) according to the criteria, established by the World Health Organisation (WHO), of the International Histological Classification of Tumours of Domestic Animals (19), using a Olympus BX41 microscope. Formalin-fixed and paraffin-embedded (FFPE) tissues were cut into sections (4 μm) using a Leica RM2245 microtome. Deparaffinised samples were used for Hematoxylin and Eosin (H&E) staining. Clinicopathological features were evaluated: (1) presence of ulceration; (2) degree of pigmentation; (3) morphology of neoplastic cells (spindle, epithelioid or mixed); (4) mitotic index (MI); and (5) tumoural vascular invasion. The MI was determined by counting all the mitoses within 10 random, non-overlapping high-power fields (HPFs) (×400), FN 22/40x objective (2.37 mm^2) (20). A MI was attributed according to the number of mitoses: low MI (<4 mitoses per 10 HPFs); high MI (≥4 mitoses per HPFs) (21). The degree of pigmentation was estimated using a subjective scale from 0 (no pigmentation), 1 (pigmentation in 25% of neoplastic cells), 2 (pigmentation in 25–50% of neoplastic cells) to 3 (pigmentation in >50% of neoplastic cells).

Immunohistochemical Analysis

FFPE tissue samples were cut into 4 μm sections and processed for immunohistochemical (IHC) analysis of S-100, melan-A, PNL-2, COX-2 and Ki-67. All immunostainings utilised the streptavidin-biotin-peroxidase complex method with a commercial detection anti-mouse/anti-rabbit system (Novolink Polymer Detection Sistem; Leica Biosystems, Newcastle upon Tyne, UK) according the manufacturer's instructions. Antigen retrieval was performed by Pascal® in citrate buffer pH 6.0 (DakoCytomation Target Retrieval Solution) at 125°C for 2 min, followed immediately by 20 min at room temperature (RT). All sections were incubated with the primary specific antibody: S-100 (1:100 dilution, Clone S100, Dako, Glostrup, Denmark), melan-A (1:100 dilution, Clone A103, Dako, Glostrup, Denmark), Melanoma Antigen (1:100 dilution, Clone PNL-2, Santa Cruz Biotechnology, Dallas, Texas, USA), COX-2 (1:80 dilution, Clone SP21, Thermo Fisher Scientific, Walthan, MA, USA) or Ki-67 (1:50 dilution, Clone MIB-1, Dako, Glostrup, Denmark) for 18 h at 4°C. The antibody reactions were visualised with chromogen 3,3′ -diaminobenzidine tetrachloride (DAB) diluent (Dako, Code K3468), incubated for 3 min at RT. The samples were washed 3x times in distilled water for 5 min at RT, then counterstained with

Giemsa for 30 min at RT, dehydrated and mounted (22). Sections treated with isotype-matched primary antibodies mouse anti-human IgG were used as negative controls and this study also included adequate positive controls: canine mammary tumour for COX-2; and canine epidermis for melan-A, PNL-2, and Ki-67. Images were captured on BX41 Olympus microscope. A subjective scale was then used to establish the degree of positive staining for S-100, melan-A and PNL-2 (23, 24). For COX-2 staining a distribution score and intensity were multiplied to obtain a total score, which ranged from 0 to 12. Distribution was estimated as the percentage of positive cells in 10 HPFs (x400): 0 (absent); 1 (< 10% stained cells); 2 (10–30% stained cells); 3 (>30–60% stained cells); and 4 (≥60% stained cells). For staining intensity: 0 (absent); 1 (weak); 2 (moderate); and 3 (strong). The samples with a final score 0–5 were considered to have COX-2 low expression, and samples with a score of 6–12 were considered to have high COX-2 expression (24). The Ki-67 index was evaluated by counting 500 tumour cells within a microscopic grid at high magnification (×400) and the index was expressed as a percentage.

RNA Extraction and Real-Time Quantitative PCR From FFPE Samples

RNA was extracted from FFPE tissue samples using the RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, Walthan, MA, USA, Code AM1975), according to the manufacturer's instructions. Extracted RNAs were quantified and 260/280 nm absorbance determined by NanoDrop Nucleic Acid Quantification (Thermo Fisher Scientific, Walthan, MA, USA). Total RNA was reverse transcribed using the M-MLV kit (Invitrogen, CA, USA, Code 280250-13) according to the manufacturer's instructions. The RNA integrity was evaluated by conventional RT-PCR analysis with the amplification of a 120 base pair fragment of the dog GAPDH reference gene, using the forward 5′-TTCCACGGCACAGTCAAG-3 and reverse 5′-ACT CAGCACCAGCATCAC-3′ primers. For quantitative real-time PCR 90 ng of cDNA was used in a final qPCR reaction with SYBR Green® PCR Master Mix Kit according to manufacturer's instructions (Invitrogen, CA, USA) and ABI PRISM® 7500 Sequence Detection System (Carlsbad, CA, USA). For the RT-qPCR the cycling conditions used were: (stage 1) 1 cycle of 50°C for 2 min; (stage 2) 1 cycle at 95°C for 10 min.; (stage 3) 40 cycles of 95°C for 15 s then 55°C for 1 min. After completion of the amplification reaction, melt-curve analysis was carried out at 95°C for 15 s to produce a dissociation curve. Relative gene expression levels were obtained by normalisation to the expression level of a housekeeping gene (HPRT) (25). Primer sequences are shown in **Table 1**.

Cell Culture

The panel of canine melanoma cell lines used in this study included CMGD2, CMGD-5, CML-10 and TLM1 (Kerafast, Boston, MA, USA), cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 μg/ml streptomycin (ThermoFisher Scientific) and 5% HEPES (ThermoFisher Scientific). Canine transitional cell carcinoma of the urinary bladder cell line (K9TCC) (a kind gift from Deborah Knapp and Jane Stewart, Purdue University) was grown in DMEM/F-12 (ThermoFisher Scientific) containing HEPES and L-glutamine and supplemented with 10% FBS and 100 μg/mL penicillin/streptomycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

TABLE 1 | Primer sequences for the amplification of qRT-PCR products from canine FFPE tissue samples.

Gene	Forward primer (5′-3′)	Reverse Primer (5′-3′)
COX-2	CCTGACACCTTGCAAATAG	ATTCCACAAACTGGGTAAGG
HPRT	CCTTGGTCAAGGAGCATAATC	GTCAAGGGCATATCCTACAAC

TABLE 2 | Primer sequences for the amplification of qRT-PCR products from canine cell lines.

Gene	Forward primer (5′-3′)	Reverse Primer (5′-3′)
COX-2	GCCCTATACATCATTCGAAGAAC	CACCATAAAGGGCCTCCAA
GAPDH	GGGAAGATGTGGCGTGAC	GAAGGCCATGCCAGTGAG

Real-Time Quantitative PCR

RNA was extracted from cells using the RNeasy Kit (Qiagen, CA USA) according to the manufacturer's instructions. Real-time PCR was executed using 50 ng of amplified RNA and the Stratagene Mx3000p qPCR system (Aligent, CA, USA), using the Platinum® SYBR® Green qPCR SuperMix-UDG according to manufacturer's instructions (Invitrogen, CA, USA). For the RT-qPCR the cycling conditions used were: (stage 1) 1 cycle of 95°C for 2 min; (stage 2) 40 cycles of 95°C for 5 s followed by 60°C for 10 s. After completion of the amplification reaction, melt-curve analysis was carried out according to the instrument's instructions. Relative gene expression levels of COX-2 were obtained by normalisation to the expression level of a housekeeping gene (GAPDH) (25). Primer sequences are shown in **Table 2**.

Construction of a Canine Inducible COX-2 miRNA Expression Vector

The generation of inducible vectors expressing COX-2 miRNAs were constructed using custom designed complementary canine COX2 oligos (Invitrogen's RNAi Designer, USA), with each containing 4 nucleotide overhangs necessary for directional cloning. The complementary COX2 oligos were annealed and the resulting double-stranded oligo was cloned into pcDNA™6.2-GW/EmGFP-miR vector (pcDNAGFPmiRNACOX2-346) according to the Block-iT ™ PolII miR RNAi expression vector kit manual (Invitrogen). A negative control plasmid, pcDNATM6.2-GW/EmGFP-miRneg (included in the kit), containing a miRNA insert predicted not to target any known vertebrate gene, was also used for the subsequent cloning steps.

Custom designed canine double-strand COX2-346 oligo, with 4 nucleotide overhangs (bold), cloned into pcDNA™6.2-GW/EmGFP-miR vector:

TGCTGTGGACTCTCAATCAAATGTGAGTTTTGGCCACT
GACTGACTCACATTTTTGAGAGTCCA
CACCTGAGAGTTAGTTTACACTCAAAACCGGTGACTG
ACTGAGTGTAAAAACTCTCAGGTGTCC

The pcDNAGFPmiRNACOX2-346 and pcDNA™6.2-GW/EmGFP-miRNeg vectors are Gateway® compatible due to the presence of attB sites flanking the COX-2 miRNA insert and can be transferred to other Gateway® adapted destination vectors that contain attR sites. After removal of emGFP, canine COX-2 miRNAs and miRNeg expression cassettes were transferred to the pTRIPZdest destination vector (a kind gift from Dr Peter Hohenstein, Roslin Institute) using the rapid Invitrogen BP/LR protocol (Invitrogen). The destination vector pTRIPZdest is a lentiviral inducible vector engineered to be Tet-On. Following transfer of the COX2miRNA in the pTRIPZdest, the tetracycline response element (TRE), placed upstream of a minimal promoter, will drive the co-expression of a TurboRFP reporter gene and the COX2miRNA if the transactivator (rtTA3) binds to TRE which only occurs in the presence of doxycycline (DOX). The induced expression of TurboRFP allows tracking of transfection efficiency, TRE promoter activity and COX2miRNA expression. The pTRIPZdestmiRCOX2 vectors contain the puromycin resistance gene (puro) which allows for puromycin selection of any mammalian cell lines that are stably transfected with the vectors.

Transfection of Inducible COX-2 miRNA Expression Vector and the Generation of Stable Cell Lines

CMGD-2, CMGD-5, CML-10 and TLM-1 cells were seeded at 2 $\times$ 10^5 in 6-well plates and incubated for 24 h. At 80% confluency cells were transfected with 2.5 μg DNA (pTRIPZdestmiRCOX2-346 expression vector) per well at a final concentration of 1 μg/μl using 12.5 μl (CMGD-2 and CMGD-5), 9 μl (CLM-10) and 6 μl (TLM-1) Lipofectamine® 2000 reagent (ThermoFisher Scientific, USA) according to the manufacturer's instructions. pTRIPZdestmiRNeg plasmid negative control plasmid was used as a negative control in subsequent experiments. To establish stable transfection, cells were exposed to 2 μg/ml of puromycin 48 h post-transfection. After 10–14 days of selection, puromycin-resistant colonies were pooled and expanded.

Proliferation Assay

Transfected cells were seeded in triplicate in opaque 96-well plates (Corning, CA, USA) at 2,000 cells/well. Cells were incubated 37°C, 5% CO_2 and cell viability was assayed 48 h later using the CellTiterGlo® Luminescent Cell Viability Assay (Promega, Madison, USA, Code G7570) according to the manufacturer's instructions. Luminescence was recorded using a Viktor3 luminometer (PerkinElmer, Massachusetts, USA). Data was averaged, and then normalised against the average signal of untreated/negative control treated samples.

Colony Formation Assay

Transfected cells were seeded at 500 cells/10 cm plate and immediately treated with the indicated dose of DOX. Plates were incubated until colonies were visible at 37°C in a humidified CO_2 incubator. Growth media was changed approximately once a week. The colonies were fixed by incubating for 5 min with ice-cold methanol at RT. Colonies were air dried and then stained with Giemsa (Invitrogen, Paisley, UK) for 20 min at RT. The total number of colonies were counted.

Invasion Assay

Invasion assays were performed using transwell system with two compartments separated by 8 μm pore polycarbonate philtres coated with Matrigel (BD Biosciences). In the upper compartment $\sim$2 $\times$ 10^4 cells were seeded in serum-free media, and the lower compartment contained media spiked with 5% FBS. Cells were allowed to transmigrate for 24 h. Non-migrated cells on the upper side of the transwell were removed. Cells on the underside of the transwell were fixed with methanol and stained with 0.1% crystal violet. These represented cells that had migrated through the philtre. Ten different fields (x200) were counted in triplicate experiments.

Cellular Migration Assay

Transfected cells were incubated at 37°C in humidified 5% CO_2 incubator grown until 80–90% confluent in 6-well plates. A scratch was made in the confluent monolayer of cells with a pipette tip. The gap was visualised using the Axiovert 40 CFL microscope coupled with an AxioCAM HRm camera (Carl Zeiss Ltd, Cambridge, UK) and measured at 6 points/gap and an average taken. Measurements were made over the indicated time course. Scratch width was converted to "relative migration distance" of cells, where the distance is a percentage of the initial wound width.

Statistical Analysis

Data were expressed as a mean ± S.D. Statistical analysis was performed with GraphPad Prism version 5.0 for Windows (GraphPad software, CA USA) using Spearman's correlation for non-parametric analysis test, analysis of variance and Student's *t*-test or mann-whitney test. The criterion for significance was $p < 0.05$ for all comparisons.

RESULTS

Immunohistochemical Analysis of Canine Melanoma Samples

A total of 85 melanoma tumours (29 orals and 56 cutaneous) were included in this study and classified accordingly: 23 spindle cell, 53 epithelioid cell and nine mixed. High pigmentation level is inversely correlated with overall survival time in human patients and is an important marker of the disease (26). Here the degree of pigmentation was estimated using a subjective scale from 0 (no pigmentation) to 3 (pigmentation in >50% of neoplastic cells). Of the 85 samples: 20 had no pigmentation; 42 scored 1; 7 scored 2; and 16 scored 3. Regarding the mitotic index (MI), 74 cases were evaluated (24 orals and 50 cutaneous), of those, 14 oral and 20 cutaneous melanomas had a high MI (≥4 mitoses per 10 HPF) (**Table 3**). The MI could not be evaluated on 11 other cases due to high pigmentation.

TABLE 3 | Clinicopathological parameters of the oral and cutaneous melanomas.

	Oral	Cutaneous
Histological type		
Epithelioid cells	23	30
Spindle cells	3	20
Mixed	3	6
Pigmentation		
0 (no pigmentation)	12	8
1 (25% of cells)	12	30
2 (25–50%)	1	6
3 (>50%)	4	12
Mitotic index		
<4 mitoses	10	30
≥4 mitosis	14	20

TABLE 4 | Immunohistochemical positivity of oral and cutaneous melanomas by S-100, melan-A, PNL-2, and COX-2 antibodies.

Molecular markers	Oral	Cutaneous
S-100	100% (29/29)	100% (56/56)
melan-A	93% (27/29)	100% (56/56)
PNL-2	100% (12/12)	100% (8/8)
COX-2		
Score 0	7% (2/29)	12% (7/56)
Score 1–5	59% (17/29)	45% (25/56)
Score 6–12	34% (10/29)	43% (24/56)

The immunoreactivity for S-100, melan-A, and PNL-2 was observed as brown cytoplasmic staining. All samples were positive for S-100 protein and PNL-2, and 75 of 85 were positive for melan-A. Of the 10 cases that were negative for melan-A the diagnosis was confirmed by positive staining for PNL-2 (**Table 4**). Overall, a high COX-2 expression revealed a statistically significant correlation with Ki-67 ($p = 0.03$), however when oral and skin melanomas were analysed independently only the skin tumours presented statistical significance ($p = 0.01$) (representative images of Ki-67, melan-A, PNL-2 and COX-2 staining are shown in **Figure 1**).

We found that COX-2 was located in the cellular membrane, cytoplasm, and nuclear membrane in a diffuse and homogeneous pattern. In the primary tumour the labelling intensity of COX-2 was comparable to that of neoplastic intravascular emboli, however, the extension of COX-2 staining in neoplastic intravascular emboli was always diffuse. These results confirmed that COX-2 was expressed, at some degree, in 90% of all tumours examined (**Figures 1E,F**) and that 34 of 85 of these tumours had a combined distribution and intensity score of 6–12. Of the 29 oral melanomas, 10 showed a COX-2 score of 6–12, whereas 24 of 56 of cutaneous melanomas scored 6–12 (**Table 4**).

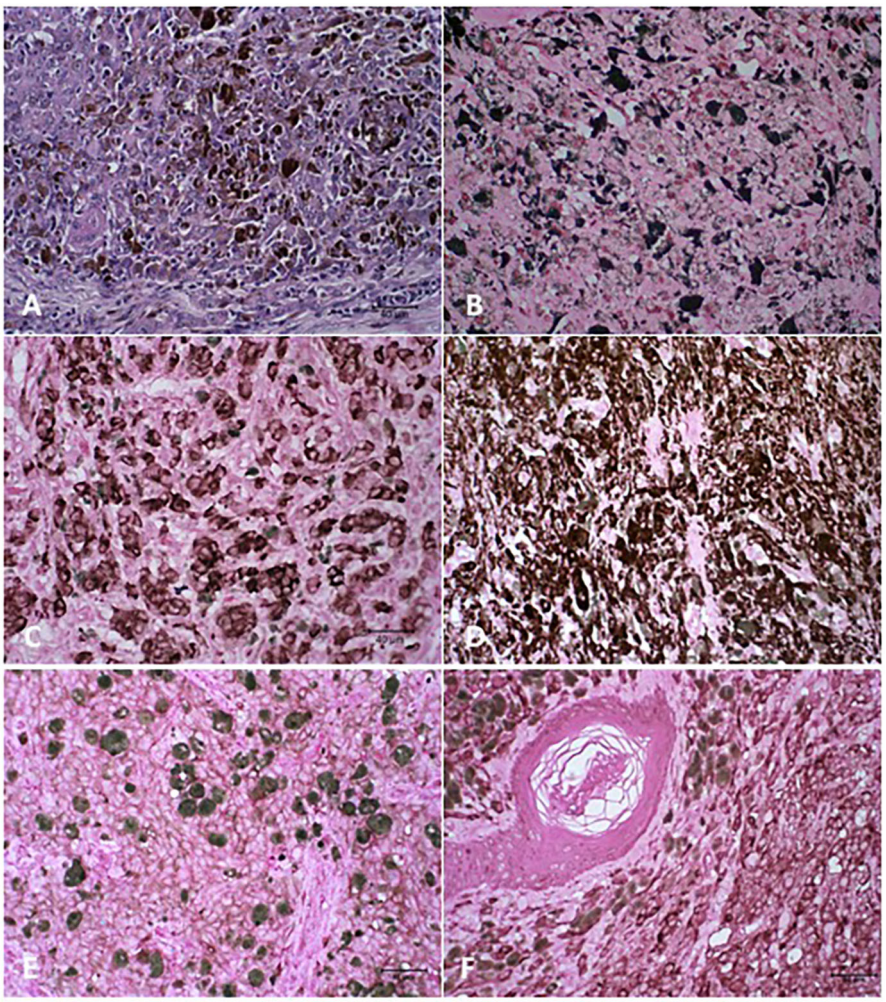
The high COX-2 expression revealed a statistically significant correlation with both oral and cutaneous tumours with Ki-67 ($r = 0.45$; $p = 0.03$), MI ($r = 0.49$; $p < 0.001$), lymphatic invasion ($r = 0.41$; $p = 0.04$) and the histological type of tumour (epithelioid and mixed) ($r = 0.51$; $p = 0.003$). In the cutaneous tumours the presence of skin ulceration ($r = 0.49$; $p = 0.02$) was also statistically correlated with high COX-2 levels. Thus, these results suggest that high COX-2 protein levels could be associated clinicopathological aggressiveness parameters. To correlate this data to COX-2 gene expression we isolated RNA from the FFPE samples and determined COX-2 gene expression by qRT-PCR. The samples were divided according to the previous IHC results into low and high COX-2 protein levels. The gene expression revealed a statistically significant inverse correlation with the COX-2 protein expression ($r = 0.64$; $p = 0.0008$) (**Figure 1G**).

Construction of an Inducible COX-2 miRNA Expression Vector

Artificial miRNAs were engineered to have 100% homology to the target COX-2 sequence and result in cleavage. To test the candidate miRNAs for suppression of COX-2 expression, three canine COX-2miRNA vectors were transiently transfected by lipofection into a canine bladder cancer cell line (K9TCC) that highly expresses COX-2. The vector contains a CMV promoter-driven puromycin resistance gene and a green fluorescent protein (GFP) gene to select and identify transfected cells. EmGFP/COX-2miRNA and EmGFP/miRNeg expressing cells were sorted 48 h post-transfection by fluorescence-activated cell sorting (FACS). COX-2 down-regulation was assessed by qRT-PCR using the comparative CT ($2\Delta\Delta CT$) method. The pcDNAemGFPmiRCOX2 vectors are Gateway® compatible (**Figure 2A**(i)). After removal of emGFP, canine COX-2miRNAs and miRNeg expression cassettes were transferred to the pTRIPZdest destination vector (a kind gift from Dr Peter Hohenstein, Roslin Institute) (**Figure 2A**(ii)). This is an inducible system activated by DOX. COX-2 down-regulation was assessed by qRT-PCR in K9TCC inducible clones grown over a week in DOX (2 μg/ml) (Data not shown).

Generation and Characterisation of Melanoma Cell Lines Stably Expressing an Inducible COX-2 miRNA Vector

The basal gene expression of COX-2 in each cell line was determined by qRT-PCR. COX-2 expression was highest in CMGD2, whereas the lowest level COX-2 expression was detected in CML10 cells (**Figure 2B**). The CMGD2 and TLM1 melanoma cell lines were stably transfected with the pTRIPZdestmiRCOX2-346 vector and control cells with the pTRIPZdestmiRNeg plasmid. Puromycin was applied 48 h post-transfection to select for stable expression of the plasmid. CMGD2 and TLM1 puromycin-resistant colonies were pooled 10–14 days following transfection, expanded and frozen for subsequent experiments. To test the inducible system, CMGD2 and TLM1 puromycin-resistant pools expressing the pTRIPZdestmiRCOX2-346 (miRNACOX2-346) vector or miRNeg control vector were exposed to 1 μg/ml of DOX. The pTRIPZdestmiRCOX2-346 vector contains a tetracycline-dependent promoter which in the presence of DOX drives the expression of the miRNA and the expression of a TurboREP reporter, allowing assessment of promoter activity. Here



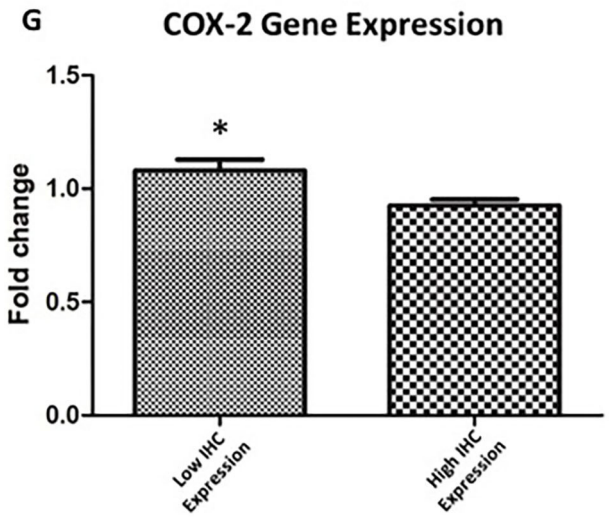


FIGURE 1 | COX-2 expression in oral and skin melanoma in the dog. **(A)** Oral, mucosal melanoma, no pigmentation. H&E staining. **(B)** Skin. Ki-67 staining in the nucleus of neoplastic cells. DAB staining of chromogen. Giemsa counterstain. **(C)** Oral. Melan-A staining in the cytoplasm of neoplastic cells. DAB staining of

(Continued)

FIGURE 1 | chromogen. Giemsa counterstain. **(D)** Skin. Melanoma Antigen (PNL-2) staining in the cytoplasm of neoplastic cells. DAB staining of chromogen. Giemsa counterstain. **(E)** Oral. COX-2 staining, low expression (score 4) in neoplastic cells. DAB staining of chromogen. Giemsa counterstain. **(F)** Skin. COX-2 staining, high expression (score 12) in neoplastic cells. DAB staining to chromogen. Giemsa counterstain. All images are objective 40x. Scale bar represents 40 μM. **(G)** Relative COX-2 mRNA expression comparing IHQ samples with low and high COX-2 immunostaining ($p = 0.02$*).

TurboRFP expression was assessed by fluorescence microscopy. We confirmed that COX-2 gene expression was decreased in CMGD2 cells expressing miRNACOX2-346 +DOX and TLM1 cells expressing miRNACOX2-346 +DOX compared to cells expressing the miR negative control (**Figure 2C**(i) and (ii), respectively).

Effects of COX-2 Knockdown on Cell Proliferation

To determine if COX-2 has an effect on cell viability of melanoma cell lines we used the CMGD2 and TML1 cell lines stably expressing the miRNACOX2-346 or the miR negative control. Here we induced knock-down of COX-2 by treating cells with 1 μg/ml DOX (+) or water (–) as a vehicle control. Cell viability was assayed 72 h after treatment. Here we showed that cell viability is COX-2 dependent in both CMGD2 (**Figure 3A**(i)) and TML1 cell lines (**Figure 3A**(ii)). Clonogenic survival analysis, as a measure of cell reproductive death after treatment, shows that only cells expressing the miRNACOX2-346 vector and treated with DOX have a reduction in colony-forming ability (**Figure 3B**). Taken together, these results indicate that COX-2 may be an important driver of melanoma cell survival.

Invasiveness and Migration Are COX-2 Dependent

The invasive capacity of CMGD2 and TLM1 cells stably expressing miRNACOX2-346 was determined using a Boyden chamber assay. CMGD2 miRNACOX2-346 and TLM1 miRNACOX2-346 cells treated with 1 μg/ml DOX (+) showed a significant reduction in invasiveness compared to both the vehicle control and the miRNeg control (**Figures 4A,B**). Similarly, we assessed migration potential using a standard scratch assay and showed that induced silencing of COX-2 reduced the ability of both TML1 (**Figure 5**) and CMGD2 (data not shown) to close the wound. These results indicate that COX-2 enhances the migration and invasion capabilities of canine melanoma cells.

DISCUSSION

The COX-2 pathway is the evolutionarily conserved master switch that activates the inflammatory response. Under normal conditions, acute inflammation is a short-term, self-limited process activated by an inflammatory stimulus. However, tumours covet a level of "smouldering" inflammation within their microenvironment and as such over-expression of COX-2 is a prominent feature of several human cancers, and has been associated with tissue re-modelling, angiogenesis, cancer cell survival, metastasis, and immune evasion (8). In human melanoma COX-2 expression has been linked to malignant behaviour. In this study we examined the relationship between COX-2 expression and canine melanoma. Here we present evidence that COX-2 is overexpressed in both oral and cutaneous melanomas and that this is related to the degree of pigmentation, MI, Ki-67 proliferation index and the expression of validated melanoma markers including S-100 and melan-A. We also developed a novel inducible COX-2 miRNA vector to effectively knock-down COX-2 expression in a panel of canine melanoma cell lines, and showed that a number of aggressive phenotypes are COX-2 dependent.

Our data is consistent with the notion that canine melanoma is an effective model for comparative oncology. Spontaneous melanocytic neoplasms arise in many domestic animals including dogs, cats, horses and pigs. However, malignant melanoma is more common in the domestic dog than other species and a majority of cases arise in either the oral cavity or the skin (4). Canine oral melanomas are highly aggressive and frequently metastasise, principally to local lymph nodes and lungs, whereas cutaneous melanomas are most often benign. In contrast to dogs, cutaneous melanomas are more common in humans, and melanoma of the oral cavity is extremely rare but exceedingly aggressive, metastatic and associated with poor patient outcomes (27). Because of their rareness, accounting for <1% of all melanomas in humans, there is currently a lack of knowledge regarding pathogenesis including etiological factors, risk factors and molecular drivers (28). We propose that there is a significant opportunity to utilise the dog model to improve molecular and mechanistic understanding of melanoma progression.

Previously, overexpression of COX-2 has been correlated with the development and progression of human cutaneous melanoma (29, 30) and has been proposed as a prognostic marker. In oral melanoma, COX-2 is highly expressed but negative in oral nevi indicating that COX-2 is a valuable marker to distinguish melanocytic lesions of the oral cavity (31), and elevated COX-2 expression is correlated with poor prognosis (32). Similarly, in canine melanoma COX-2 levels are related to Ki-67 proliferation index and survival rate indicating that COX-2 is related to the acquisition of malignancy (7, 16, 33, 34). These results are consistent with our own findings that are presented here; where 89% of all tumours (76 of 85) express some degree of COX-2, with 34% of oral melanomas (10 of 29) and 42% of cutaneous tumours (24 of 56) expressing the highest level of combined distribution and intensity. We also show that COX-2 protein level correlates with Ki-67 proliferation rate, MI and the expression of established melanoma markers.

A mouse model of ovarian cancer has shown a COX-2 gene dosage effect in promoting tumour development, and

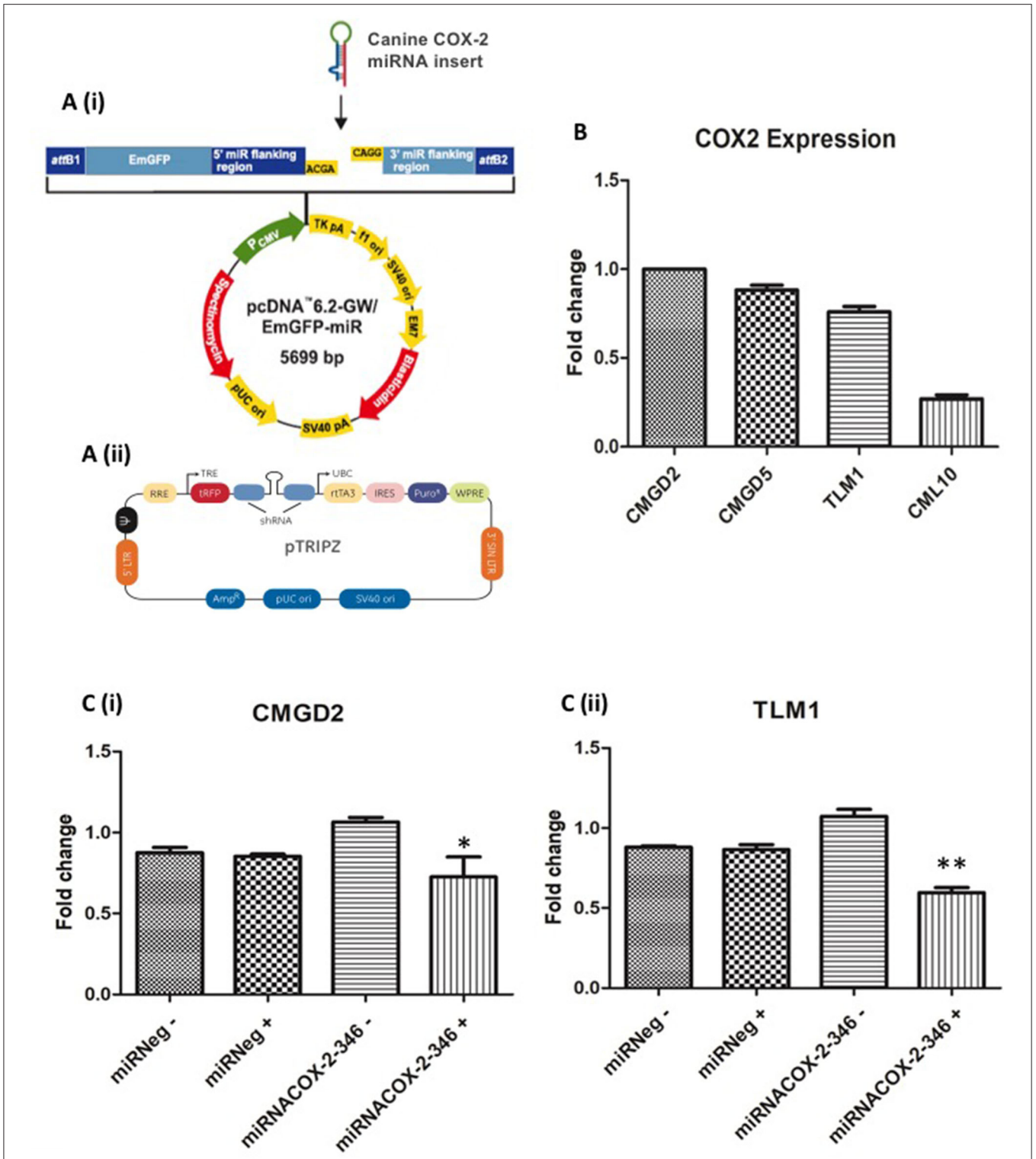


FIGURE 2 | Construction of an inducible COX-2 miRNA expression vector in canine melanoma cell lines. **(A)** Canine double-stranded COX-2 miRNAs were generated and cloned into (i) pcDNA^{TM}6.2-GW/EmGFP-miR vector (Invitrogen) and transferred to (ii) pTRIPZdest destination vector. **(B)** Basal expression level of COX-2 in CMGD2, CMGD5, TLM1, and CML10 canine melanoma cell lines. **(C)** Validation of inducible COX-2 mi-RNA expression: (i) CMGD2 cells and (ii) TLM1 cells were transfected with 2.5 μg of the vector or negative control after 24 h cells were treated with 1 μg/ml DOX (+) or vehicle control (–) and COX-2 expression was determined by qRT-PCR. Data shown as fold change relative to miRneg (+) expression ($p = 0.03$*, $p = 0.002$**).

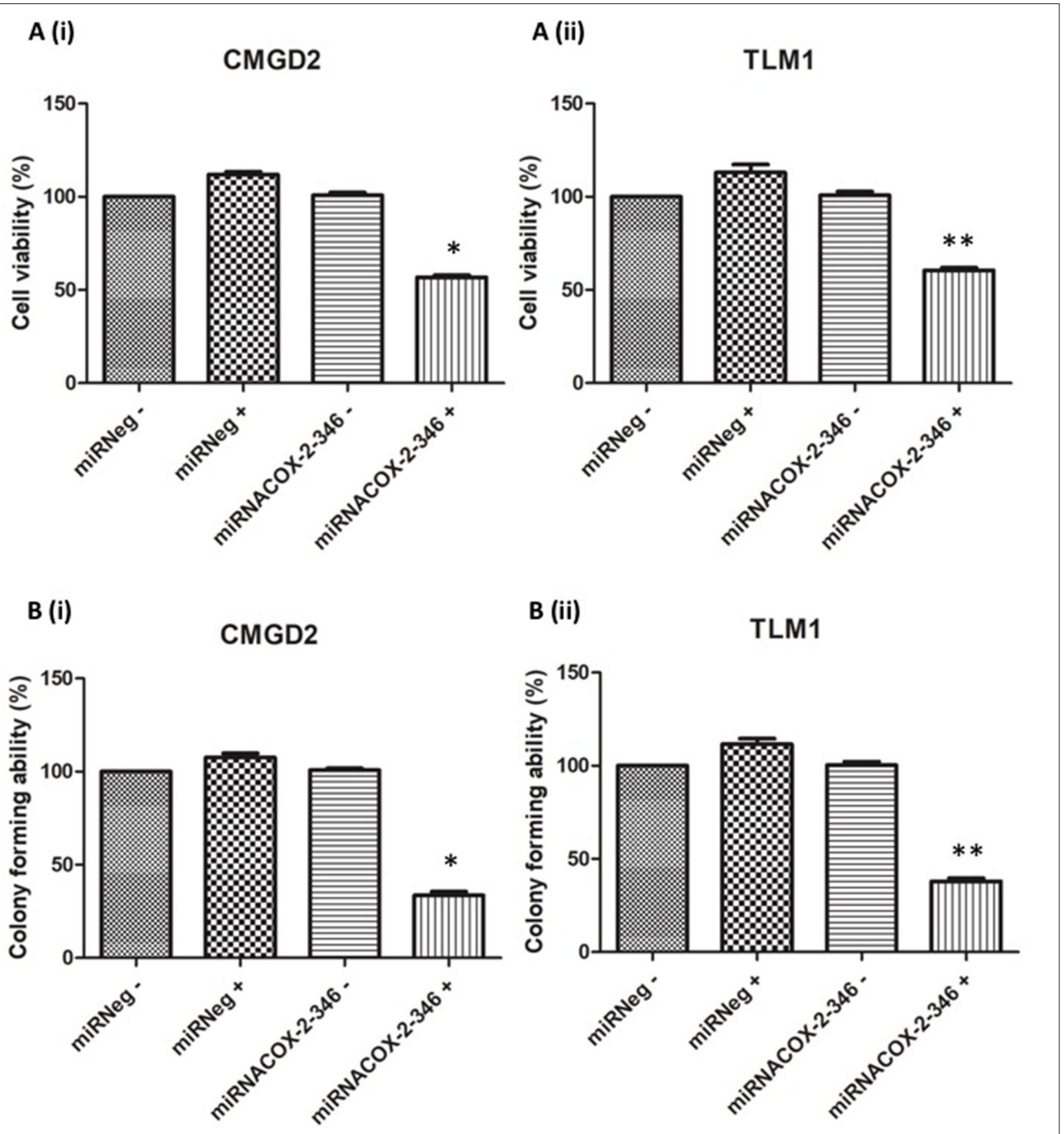


FIGURE 3 | Melanoma cell viability and colony-forming ability is COX-2 dependent. **(A)** Cell viability of CMGD2 (i) and TLM1 (ii) was determined after transfection with either miRNACOX2-346 or the miRneg negative control with (+) or without (–) DOX. Cell viability was assayed 48 h after DOX treatment ($p = 0.001$*, $p = 0.003$**). **(B)** Colony forming ability of CMGD2 (i) and TLM1 (ii) was determined after transfection with either miRNACOX2-346 or the negative control with (+) or without (–) DOX ($p = 0.0001$*, $p = 0.01$**).

interestingly they showed that COX-2 may be important for tumour initiation whereas progression and malignancy is driven by COX-1 (35). This is consistent with previous work from our lab showing that COX-2 is overexpressed in the canine osteosarcoma stem cells and that inhibition of COX-2 may be sufficient to inhibit tumour initiation (10). At what times,

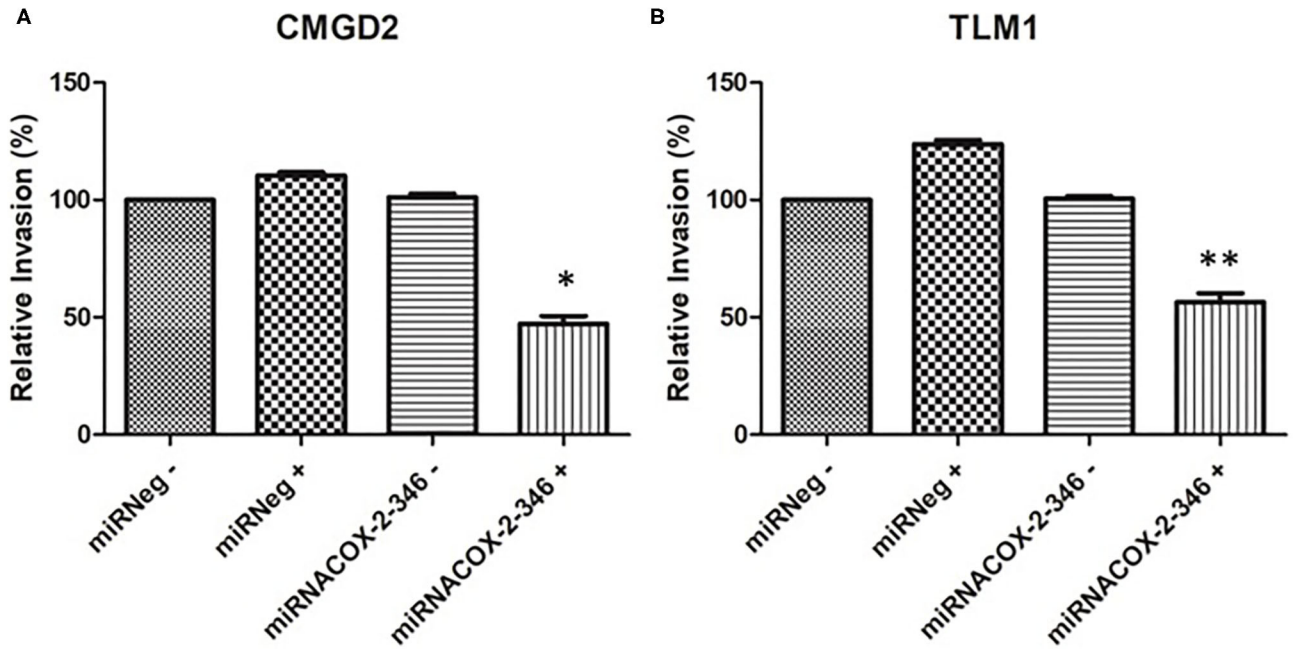


FIGURE 4 | COX-2 regulates cell invasion. **(A)** CMGD2 and **(B)** TLM1 cells were transfected with either miRNACOX2-346 or the miRneg negative control with (+) or without (–) DOX. Invasion potential was determined 48 h after seeding into a transwell chamber assay (QCM™ collagen-based cell invasion assay kit) ($p = 0.0004$*, $p = 0.0002$**).

and in which sub-set of cells, COX-2 expression is required to promote tumourigenesis is unclear. A disadvantage of working with the canine model is a lack of the molecular tools to elucidate the varied roles of COX-2 in tumourigenesis. In this study we describe the creation of two canine melanoma cell lines in which COX-2 expression can be reversibly down-regulated by a doxycycline-inducible miRNA that targets the COX-2 transcript. The vector used has a red fluorescent reporter to allow investigators to assess the feasibility of the inducible system for the cell line specific applications. The ability to reversibly knock-down COX-2 gene expression allows for reversible manipulation of COX-2 expression at any time during tumour progression, in contrast to conventional COX-2 gene disruption strategies which permanently eliminate global COX-2 expression at all times. Here we utilised this system to show that, in the context of canine melanoma, COX-2 is required for cellular proliferation, migration and invasion. Previous studies, consistent with our data, have shown that, by immunohistochemical analysis of COX-2, there is a significant association between COX-2 levels and the proliferation status of cells within a tumour cell population (5, 7, 29, 31). Several studies have also revealed the inhibition of COX-2 by NSAIDs and COXIBs leads to a dose-dependent reduction in cell proliferation in a range of cancer cell lines (15, 36–38). Using CMGD2 and TLM1 cells we have shown that when COX-2 expression is knocked-down there is a significant decrease in cell proliferation and colony forming efficiency compared to the negative control. Although COX-2 has been observed to promote cell proliferation by regulating the activation of downstream oncogenic pathways (39), the underlying molecular mechanism of COX-2 driving cellular proliferation remains to be elucidated.

Melanoma is an aggressive and highly metastatic disease (28). The metastatic process involves the migration of a cell with tumour-initiating potential from the tumour microenvironment followed by the subsequent invasion and colonisation of a secondary site within the body. Here we showed that both the invasive capacity and migration potential of melanoma cells is dependent on COX-2. The epithelial-to-mesenchymal transition (EMT) is a key process involved in acquisition of metastatic potential in number of different types of cancer, including melanoma. During EMT of cancer cells, epithelial cells lose the apical-basal polarity and detach from surrounding cells and transition to invasive mesenchymal cells, which ultimately leads to the active entry of cancer cells into circulation (40). Previous studies have linked COX-2 to the EMT process: COX-2 overexpression has been associated with upregulation of mesenchymal markers and downregulation of epithelial markers (41); and in breast cancer, COX-2 induced production of PGE_2 inhibited Smad signalling thereby enhancing EMT progression and metastasis (42). Moreover, treatment with various COXIBs led to the reversion of EMT in several cancer cell lines (43, 44). EMT is a key mediator of cancer stem cell plasticity, where by non-cancer stem cells can dedifferentiate and acquire stem cell characteristics, and it is these cells that are responsible for

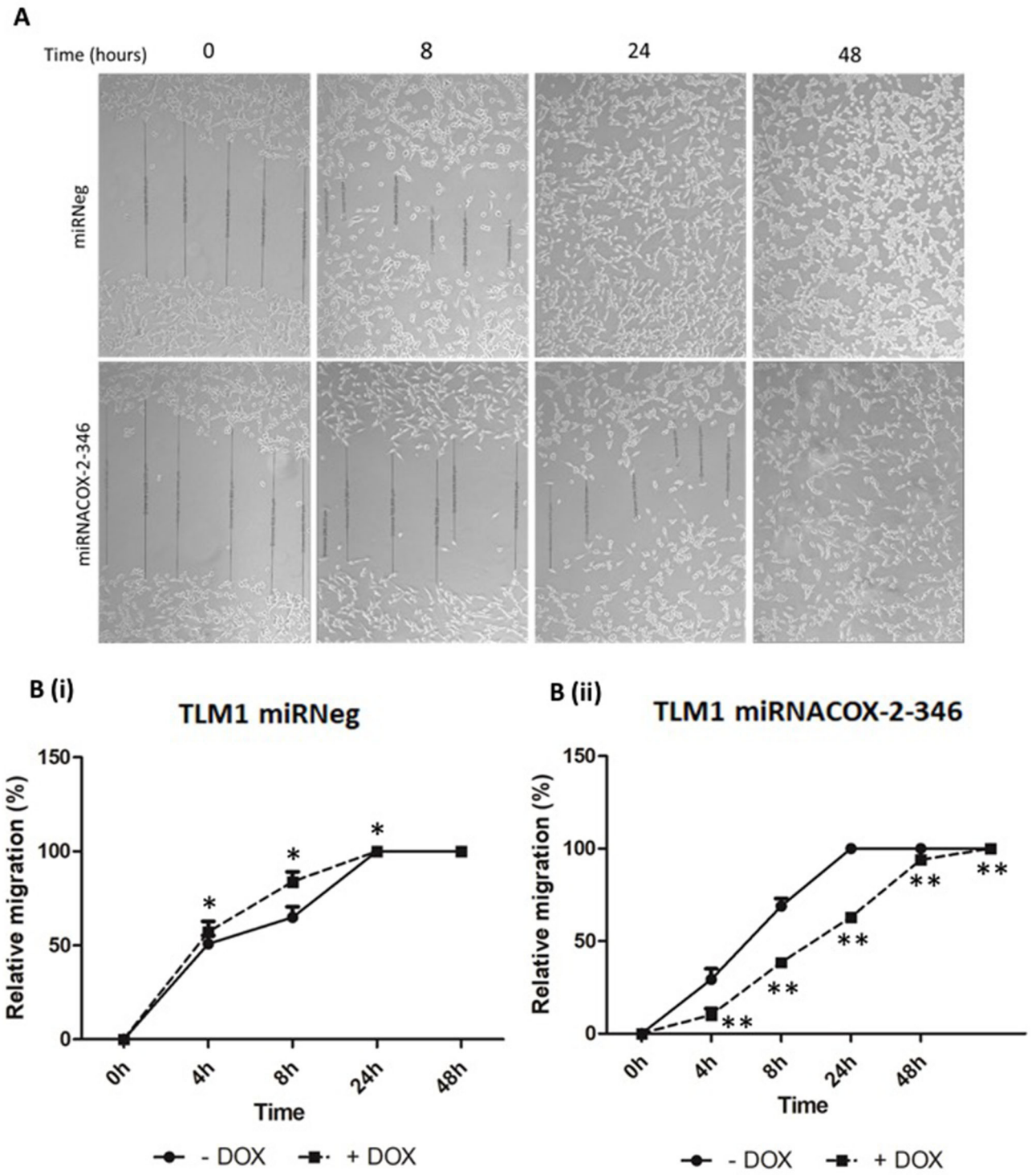


FIGURE 5 | Migration of canine melanoma cells is COX-2 dependent. **(A)** Representative image of a scratch assay of TML1 cells transfected with either miRNACOX2-346 or the miRneg negative control, treated with 1 μg/ml DOX. After 24 h incubation a scratch was made and the gap distance was measured at regular intervals until the gap closed. **(B)** Quantification of this data (i) miRneg negative control and (ii) miRNACOX2-346 ($p < 0.05$*, $p < 0.0001$**).

metastatic dissemination. Given that COX-2 has been shown to be overexpressed in cancer stem cells it is interesting to speculate that COX-2 may maintain the cancer stem cell population by driving EMT. The presence of cancer stem cells in different melanoma cell lines and tumours has previously been reported for humans but not for dogs (45–48). To date an evaluation of the

role of COX-2 in melanoma stem cell biology has not yet been undertaken. By extension, future research should address the mechanistic role that COX-2/PGE_2 axis plays in melanoma biology and determining if pharmacological inhibition of COX-2 could prevent cancer stem cell survival, EMT and metastasis.

CONCLUSIONS

Our study provides empirical evidence that COX-2 protein expression is negatively correlated with a worse prognosis in canine patients with melanoma. Extensive clinical studies should be performed to determine the prognostic significance of COX-2 as a marker of canine melanoma. COX-2 is associated with almost all stages of tumour development including tumour initiation, immunosuppression, EMT and metastasis, which all contribute to therapeutic resistance. This is the first study to have developed and characterised a canine-specific DOX-inducible COX-2 miRNA vector. This is a valuable molecular tool for further understanding the mechanisms through which COX-2 effects melanoma progression. Here we have shown that cellular proliferation, invasion and migration are COX-2 dependent in an *in vitro* canine melanoma model. Further studies are required to establish COX-2 as a bona fide therapeutic target in the treatment of canine melanoma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee in Animal Experimentation (COMISSÃO DE ÉTICA NO USO DE ANIMAIS/UNIVERSIDADE FEDERAL DE MINAS GERAIS). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

LP wrote the manuscript with support from TS. TS and EF conducted and verified histological classification of patient samples. TS conducted all *in vitro* experiments. AD designed COX-2 miRNA expression vectors. LP and TS performed data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.633170/full#supplementary-material

Supplementary Figure 1 | Panel of negative and positive controls of immunohistochemistry. **(A)** Cutaneous melanoma, negative control, Ki-67 marker. Giemsa counterstain **(B)** Cutaneous melanoma, positive internal control. Ki-67 staining in the nucleus of epidermal cells. DAB staining of chromogen. Giemsa counterstain. **(C)** Cutaneous melanoma, negative control. Melan-A marker. Giemsa counterstain. **(D)** Cutaneous melanoma, positive internal control. Melan-A staining in the cytoplasm of melanocytes cells of the epidermis. DAB staining of chromogen. Giemsa counterstain. **(E)** Cutaneous melanoma. Melanoma Antigen (PNL-2) marker. Giemsa counterstain. **(F)** Cutaneous melanoma. Melanoma Antigen (PNL-2) staining in the cytoplasm of melanocytes cells of the epidermis. DAB staining of chromogen. Giemsa counterstain **(G)** Mucosal Melanoma, negative control. COX-2 marker. Giemsa counterstain. **(H)** Cutaneous melanoma, positive internal control. COX-2 staining in inflammatory cells. DAB staining to chromogen. Giemsa counterstain. All images are objective 40x. Scale bar represents 40 μM.

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T Cell Immune Profiles of Blood and Tumor in Dogs Diagnosed With Malignant Melanoma

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Investigation of canine T cell immunophenotypes in canine melanomas as prognostic biomarkers for disease progression or predictive biomarkers for targeted immunotherapeutics remains in preliminary stages. We aimed to examine T cell phenotypes and function in peripheral blood mononuclear cells (PBMC) and baseline tumor samples by flow cytometry, and to compare patient ($n = 11$–20) T cell phenotypes with healthy controls dogs ($n = 10$–20). CD3, CD4, CD8, CD25, FoxP3, Ki67, granzyme B, and interferon-γ (IFN-γ) were used to classify T cell subsets in resting and mitogen stimulated PBMCs. In a separate patient cohort ($n = 11$), T cells were classified using CD3, CD4, CD8, FoxP3, and granzyme B in paired PBMC and single cell suspensions of tumor samples. Analysis of flow cytometric data of individual T cell phenotypes in PBMC revealed specific T cell phenotypes including FoxP3+ and CD25+FoxP3- populations that distinguished patients from healthy controls. Frequencies of IFN-γ+ cells after ConA stimulation identified two different patient phenotypic responses, including a normal/exaggerated IFN-γ response and a lower response suggesting dysfunction. Principle component analysis of selected T cell immunophenotypes also distinguished patients and controls for T cell phenotype and revealed a clustering of patients based on metastasis detected at diagnosis. Findings supported the overall hypothesis that canine melanoma patients display a T cell immunophenotype profile that is unique from healthy pet dogs and will guide future studies designed with larger patient cohorts necessary to further characterize prognostic T cell immunophenotypes.

Keywords: melanoma, canine, dog, immune profile, one health, comparative oncology

INTRODUCTION

Immunotherapy has shown great promise in the treatment of melanoma in humans, including those with an advanced stage of disease, but a majority of patients still fail to respond long term to these therapies and a high rate of toxicity is associated with their use (1–4). Elucidation of the most critical immunologic components of the immune environment that impact response to

such therapeutics will be essential for increasing response rates and duration of responses (5). The canine cancer model affords a unique opportunity to characterize immunologic phenotypes and biomarkers that may be predictive of responses to specific immunotherapeutics, particularly for malignant melanoma. Spontaneously arising melanoma in the dog has been shown to be a viable model system for human melanoma, with canine oral melanoma and human mucosal melanoma being the most studied (6–10). Oral melanoma is the most commonly occurring malignant tumor in the oral cavity of dogs (11). The majority of these canine tumors are malignant in nature with a high metastatic rate, although a more benign variant has been reported (12). Despite standard chemotherapy, radiotherapy and clinical trials with traditional cancer vaccines (13–16), the majority of dogs with oral melanoma die of their disease as was the case in human medicine prior to the advent of more targeted immunotherapies, although most patients in human medicine still are not cured despite these advances (4, 17).

Knowledge of dog immunology and tumor immunology in particular, has been limited by the availability of reagents for canine immune markers and validated flow cytometry panels (18). Reagents for identification of canine lymphocyte subsets have evolved from veterinary investigations focused on characterizing and typing of canine and feline lymphomas (19–24) over the past 30 years. However, additional canine-specific or cross-reactive reagents for critical immune markers are now emerging rapidly (25–32). These new reagents and tools have encouraged the development of diagnostic and experimental tools (33–37). This will facilitate examination of the canine immune response to melanoma and immunologic interventions for multiple cancers including melanoma. In turn, these reagents support and encourage the use of this model to investigate novel immunologic interventions that will impact naturally occurring cancers in pet dogs as well as human cancer.

It is well-understood that host T cell immunity plays a critical role in control and progression of cancer in humans (38–40). The emergence of cancer immunotherapeutics that target cancer-related T cell dysregulation such as the currently approved immune checkpoint inhibitors based on PD1, PD-L1, and CTLA-4 have encouraged the investigation of additional checkpoint pathways as immunotherapeutic targets. Naturally occurring canine oral melanoma may provide a very useful model for testing of emerging or very novel therapeutics (41, 42). Furthermore, these studies may provide viable alternative therapeutics for certain intractable canine cancers such as oral melanoma. However, investigations of canine T cell subsets and their role as biomarkers for canine melanoma progression or response to immunotherapeutics, remain in early stages. The aims of this preliminary study were to examine T cell phenotypes and function in peripheral blood mononuclear cells (PBMC) and tumor tissue in untreated cases of melanoma by use of flow cytometry panels, and to compare T cell phenotypes of canine melanoma patients with healthy control dogs. The study was designed to test the overall hypothesis that canine melanoma patients demonstrate a T cell immunophenotype that is different and unique from healthy pet dogs. Our studies revealed specific T cell phenotypes that do distinguish canine melanoma patients from healthy dogs.

MATERIALS AND METHODS

Canine Melanoma Patient and Healthy Control Cohorts

Dogs presenting to the University of California Davis (UC Davis) William R. Pritchard Veterinary Medical Teaching Hospital (VMTH) with a histological diagnosis of a malignant melanoma and gross disease were recruited to the study. Dogs were required to have no prior treatment of their melanoma. Blood and tissue were collected with informed owner consent under approval from the UC Davis Institutional Animal Care and Use Committee (IACUC) and Clinical Trials Review Board (protocols 22172 and 22002). Healthy client owned dogs served as controls and were recruited through clients and staff of the UC Davis VMTH and blood was drawn with informed consent and IACUC approval (22172). Demographic and tumor specific information for patient and control dogs is presented in **Supplementary Table 1**.

Isolation of Canine Peripheral Blood Mononuclear Cells (PBMC) From Whole Blood

Whole blood was obtained from canine melanoma patients and healthy dogs using vacutainer tubes containing EDTA anticoagulant. Blood was processed as described previously (25). Briefly, PBMC were isolated by density centrifugation using Histopaque 1077 (Sigma-Aldrich, Saint Louis, MO, USA). PBMC pellets were further processed by treatment with RBC lysis buffer (Biolegend, San Diego, CA, USA). Isolated PBMC were subsequently tested as fresh PBMC by flow cytometric analysis, or cryopreserved in media containing 45% heat inactivated fetal bovine serum (FBS), 45% heat inactivated dog serum (Equitech-bio, Kerrville, TX, USA), and 10% DMSO. Cryopreserved PBMC were used for all studies with the exception of a single study comparing tumor associated cells and PBMC from a select patient cohort where fresh tumor associated cells and fresh PBMC from patients (and controls) were stained and tested. Fresh PBMC or thawed cryopreserved PBMC were rested/cultured in culture media overnight prior to staining the next day for flow cytometry analysis as described previously (33).

Preparation of Tumor Samples for Flow Cytometry Analysis

Canine melanoma tumor biopsy samples were cut into small pieces in Dulbecco's phosphate-buffered saline (DPBS) (Gibco, Carlsbad, CA, USA) and digested in DPBS containing 1 mg/ml collagenase IV, 0.5% bovine serum albumin, with or without 0.1 mg/ml DNase for 2 h on a shaker at 37°C. Tumor associated cells were washed with DPBS, filtered by a 70 μm filter, and tumor cells were stained on the same day.

TABLE 1 | Antibodies used in flowcytometry panels.

Target/species	Conjugates	Clone #	Vendor
CD3/Human	FITC	CD3-12	Bio-Rad
CD4/Dog	Pacific Blue	YKIX302.9	Bio-Rad
CD4/Dog	PE-Cy7	YKIX302.9	Bio-Rad
CD8a/Dog	Alexa Fluor 700	YCATE55.9	Bio-Rad
CD8a/Dog	PerCP-eFluor 710	YCATE55.9	Thermo Fisher
CD25/Human	PE	ACT1	Dako
CD25/Dog	PE	P4A10	Thermo Fisher
Foxp3/Human	Alexa Fluor 700	FJK-16s	Thermo Fisher
Foxp3/Human	eFluor 660	FJK-16s	Thermo Fisher
Granzyme B/Human	PE-Texas Red	GB11	Thermo Fisher
Interferon-γ /Bovine	PE	CC302	Bio-Rad
Ki67/Human	PE-Cy7	20Raj1	Thermo Fisher

Stimulation of PBMC With Concanavalin A (ConA)

PBMC were treated with ConA (Sigma-Aldrich) (5 μg/ml) and brefeldin A (Sigma-Aldrich) (1 μg/ml) in cell culture media for 16 h at 37°C before assay for expression of interferon (IFN-γ) by intracellular cytokine staining (ICS) the next day. Unstimulated control PBMC for each sample (patient and control) were incubated in media with brefeldin A and without ConA for 16 h at 37°C.

Flow Cytometry

Staining protocols were previously described (25, 33). Briefly, when possible a minimum of 1 million cells were stained for PBMC and 2 million cells were stained for tumor associated cells. For all experiments, cells were stained for viability with a fixable viability dye (LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, or LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, Thermo Fisher Scientific, Waltham, MA, USA). Cells were stained with cell surface antibodies in DPBS with 3% heat inactivated fetal bovine serum (FBS). Permeabilization, fixation and intracellular staining of cells were performed using eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific). Cell surface staining, permeabilization, fixation, and intracellular staining were performed for 20 min at 4°C.

Antibodies staining for cell surface markers were directed against CD4, CD8α, and CD25. Antibodies staining for intracellular markers were directed against CD3, FoxP3, Ki67, granzyme B, and IFN-γ. One panel included a fixable viability dye (Aqua or Near-IR) and antibodies for CD3, CD4, CD8, CD25, FoxP3, and Ki67. A second panel contained the same markers and in addition, antibodies for detection of granzyme B and IFN-γ. These two panels were utilized for PBMC. Tumor associated cells and corresponding PBMC were tested with a fixable viability dye and antibodies for CD3, CD4, CD8, FoxP3, and granzyme B. All antibodies are described for species, clone number, fluorochrome, and vendor in **Table 1**. Of note, PBMC from four melanoma patients were tested only with the anti-human CD25 antibody.

Stained PBMC were washed in buffers and under conditions as previously described (33), suspended in 1% paraformaldehyde (Affymetrix, Thermo Fisher Scientific), and stored at 4°C for subsequent flow cytometer acquisition. A BD LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) was utilized for most flow acquisitions with a BD Fortessa flow cytometer (BD Biosciences) serving as an alternative when the BD LSRII was unavailable for use. For PBMC, minimum acquisition events were 63,000. For tumor associated cells, minimum acquisition events were 26,000. Typically, 100,000 events were acquired. Acquired flow cytometric data utilized FlowJo v10.6.1 (BD Biosciences) software.

Statistics

Data was collected in a commercially available spreadsheet software program (Microsoft Excel, Microsoft Corporation, Redmond, WA, USA) and descriptive statistics were calculated. Initial exploration of the data was done using a principal component analysis (PCA). PCA was performed using R version 4.1.0 (R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/). PCA plots were generated using R package ggplot2_3.3.3 (43). PERMANOVA was performed using the default parameters with the adonis function and beta-dispersion was calculated using the permutest function with the betadisper function in the vegan_2.5-7 package (vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan). Data was checked for normality using a Shapiro-Wilks test. Non-parametric analysis was performed as most data sets did not meet the criteria of normality. To determine differences between patients and controls, a pair-wise analysis was conducted using the Mann Whitney *U*-test. To investigate differences in flow cytometry parameters between matched tumor and PBMC, a Wilcoxon matched-pairs signed rank test was used. Comparisons of frequencies between three or more subsets were performed using a Kruskal-Wallis test with the Dunn's *post-hoc* test for multiple comparisons. To test for differences in the demographic data between continuous variables between patients and controls, a Mann Whitney *U*-test was performed while a Fischer's exact test was done to look for differences in proportions of categorical data between patients and controls. Statistical tests and graphing utilized GraphPad Prism software (GraphPad Prism version 9.2, GraphPad Software, San Diego, CA USA) for flow cytometry data and Stata (Stata/IC version 14.2., StataCorp, College Station, TX, USA) for demographic and clinical tumor related data. A p-value < 0.05 was considered statistically significant.

RESULTS

Cohort Description

A total of 31 dog patients diagnosed by histopathology with a malignant melanoma and 23 healthy control dogs were enrolled. Healthy dogs were followed for at least 1 year after sample collection to ensure that they did not develop a melanoma. The median age of patients at sample collection was 10.2 years (range 5.9–15.1 years) while the median age of control dogs at time of

sample collection was 8.5 years (range 4–15.5 years). These were statistically different at $p = 0.03$. Amongst the patients there was 1 intact female dog, 14 female spayed dogs, 15 castrated male dogs and one intact male, while for the control group there was 11 female spayed dogs and 12 castrated male dogs. The proportions of dogs in each category were not different at $p = 1.0$. In the patient group there were 10 mixed breed dogs, four Labrador retrievers, and a range of other breeds (**Supplementary Table 1**). In the control group there were seven mixed breed dogs, three rat terriers, three golden retrievers, and one or two of other breeds as listed in **Supplementary Table 1**. Weights were available for 31 of the cases and 19 of the control dogs. The median weight in the patient group was 24.7 kg (range 4.9–50 kgs) while the median weight in the control group was 14.9 kg (range 5.2–47 kg). These were not statistically different at $p = 0.24$.

Twenty-six patients were diagnosed with an oral melanoma, two had dermal melanomas, and one each of a digital and anal gland melanoma and in one dog the primary could not be identified with a diagnosis made by biopsy of an enlarged submandibular lymph node. Twenty-nine of the cases had primary tumor measurements available. The median largest diameter tumor was 2.8 cm (range 0.5–7 cm). Mitotic index was available for 22 cases. The median number of mitotic figures in 10 high-power fields was 6.5 (range 0–40). Ten cases had metastasis at the time of diagnosis.

Development of Staining Panels for Flow Cytometric Analysis of Canine T Cells in Blood

Flow cytometry panels were developed for phenotyping canine T cells in cryopreserved PBMC isolated from blood to explore changes in regulatory and activated T cell populations in canine melanoma patients and healthy dogs. Representative gating strategies are shown for characterizing frequencies of T cell (CD3+) subsets including CD4+, CD8+, and CD4-CD8- cells (**Figure 1A**) and frequencies of regulatory and activated populations within each T cell subset (**Figure 1B**). The CD4-CD8- T cell subset was included in analyses for different T cell markers as a population of interest based on a recent report (44) and was consistently observed in all patient and control blood samples. Based on these gating strategies different T cell subsets were interrogated for FoxP3, CD25, Ki67, and granzyme B to distinguish regulatory, activated, and putative quiescent subsets. Regulatory T cell (Treg) populations were identified as FoxP3+ (including both CD25- and CD25+ cells) or CD25+FoxP3+. Activated subsets were based on expression of CD25 in the absence of FoxP3 expression (CD25+FoxP3-) and quiescent populations as CD25-FoxP3-. All T cell subsets and derivative populations were also analyzed for Ki67 expression as a marker for proliferation (45). CD8+ T cells were assessed for granzyme B expression which can be considered both an activation and exhaustion marker for this subset (46–50) (**Figure 1B**). A subsequent panel included IFN-γ staining using ICS in addition to regulatory and activation markers to explore IFN-γ expression after ConA stimulation as a marker for T cell function and T cell programming (**Figure 1C**). A preliminary panel included an anti-human CD25 monoclonal antibody (clone ACT1) reported to be cross-reactive for canine T cells (51–53). However, a subsequent analysis comparing CD25 staining by this anti-human CD25 to an anti-canine CD25 monoclonal antibody (clone P4A10) (54) revealed that the anti-human antibody failed to detect all CD25+ CD4+ T cells as shown in **Supplementary Figure 1**, although some level of cross-reactivity was observed. A representative staining pattern by the anti-human CD25 antibody **Supplementary Figure 1A** revealed that detection of the CD25+FoxP3- population was particularly less efficient compared to the anti-canine antibody (**Supplementary Figure 1B**). Overall staining of CD4+ T cells by the anti-human CD25 antibody resulted in significantly lower frequencies compared to staining by the anti-canine antibody ($p < 0.0001$) (**Supplementary Figures 1C,D**). Use of this anti-human CD25 in the initial patients resulted in loss of data for this marker in a small subset of patients ($n = 4$) (**Supplementary Table 2**). **Supplementary Tables 1, 2** describe patient and control cohorts and which markers were tested for each cohort.

Principal Component Analysis (PCA) of Canine Melanoma Patients Reveals a Distinctive T Cell Phenotype in Blood Compared to Healthy Dogs

Principal component analysis (PCA) was performed to determine whether canine melanoma patients and healthy controls expressed different T cell immunophenotypes. PCA including 17 immunophenotypes identified as specific T cell subset frequencies (**Supplementary Table 3**) determined by flow cytometry and 36 animals including 20 healthy controls and 16 melanoma patients which are described in **Supplementary Tables 1, 2**, revealed a significant difference between healthy controls and canine melanoma patients (PERMANOVA, $p = 0.002$) (**Figure 2A**). These results suggested that melanoma patients display an overall T cell phenotype in blood that is distinguishable from that of healthy dogs. Furthermore, PCA of 23 immunophenotypes or T cell subset frequencies (**Supplementary Table 3**) observed after ConA stimulation was conducted for a subset of melanoma patients ($n = 11$) and healthy dogs ($n = 10$) (**Supplementary Tables 1, 2**), and also revealed a significant difference between healthy controls and melanoma patients (PERMANOVA, $p = 0.046$) (**Figure 2B**). These findings supported that canine melanoma patients demonstrate a T cell phenotype that is unique and different when compared to healthy dogs.

PCA was also performed on samples from melanoma patients ($n = 16$) with 17 immunophenotypes (same immunophenotypes and patients as described for **Figure 2A**) to determine if clinical parameters were associated with immunophenotypes in melanoma patients. No significant associations were found between oral vs. non-oral tumor, tumor size, mitotic index or metastasis at time of diagnosis, with immunophenotype (PERMANOVA, $p > 0.05$) (**Figure 2C**). However, PCA of melanoma patients ($n = 11$) (same patients described for

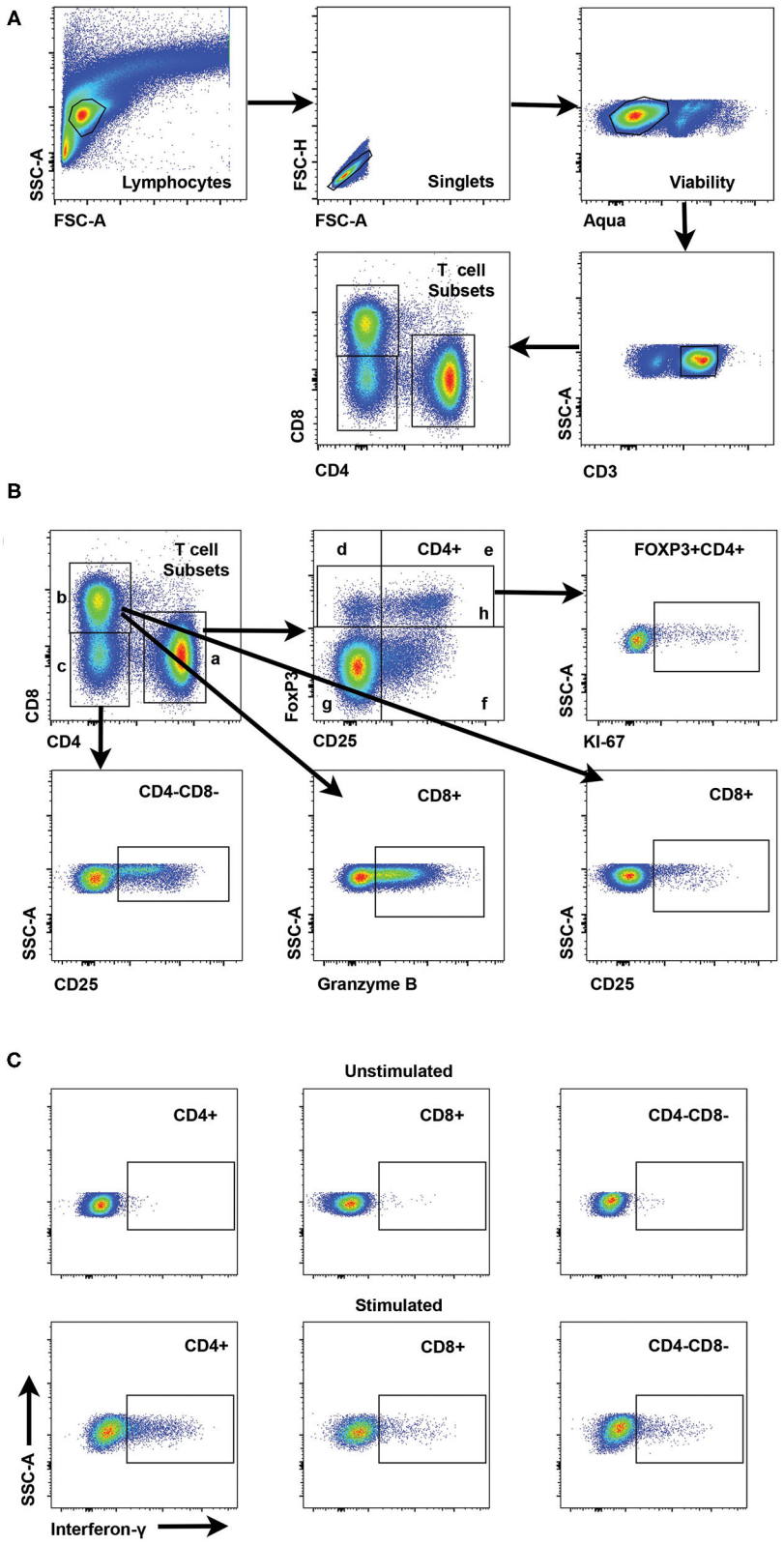

FIGURE 1 | Gating strategies for interrogation of canine T cell subsets by flow cytometry. **(A)** Representative scatter plots reveal the gating strategy for detection of CD4+, CD8+, and CD4-CD8- T cell subsets starting with establishment of a lymphocyte gate, followed by gating on singlets, exclusion of dead cells by a viability

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FIGURE 1 | stain and gating on CD3+ cells as the parental gate for T cell subsets. **(B)** Scatter plots showing interrogation of CD4+ T cells are representative for gating of specific populations for all T cell subsets. Regulatory CD4+ T cells include FoxP3+ (h) and CD25+FoxP3+ (e). CD25+FoxP3- cells (f) represent an activated T cell subset and CD25-FoxP3- cells (g) are designated as a quiescent T cell subset. Interrogation of FoxP3+ cells for Ki67 reveals the gating strategy for Ki67+ cells for all subsets. Gating strategies for CD25 and Granzyme B are shown for both CD4-CD8- and CD8+ T cells. **(C)** Representative scatter plots show interferon-γ staining and gating for CD4+, CD8+ and CD4-CD8- T cell subsets with or without stimulation with Con-A.

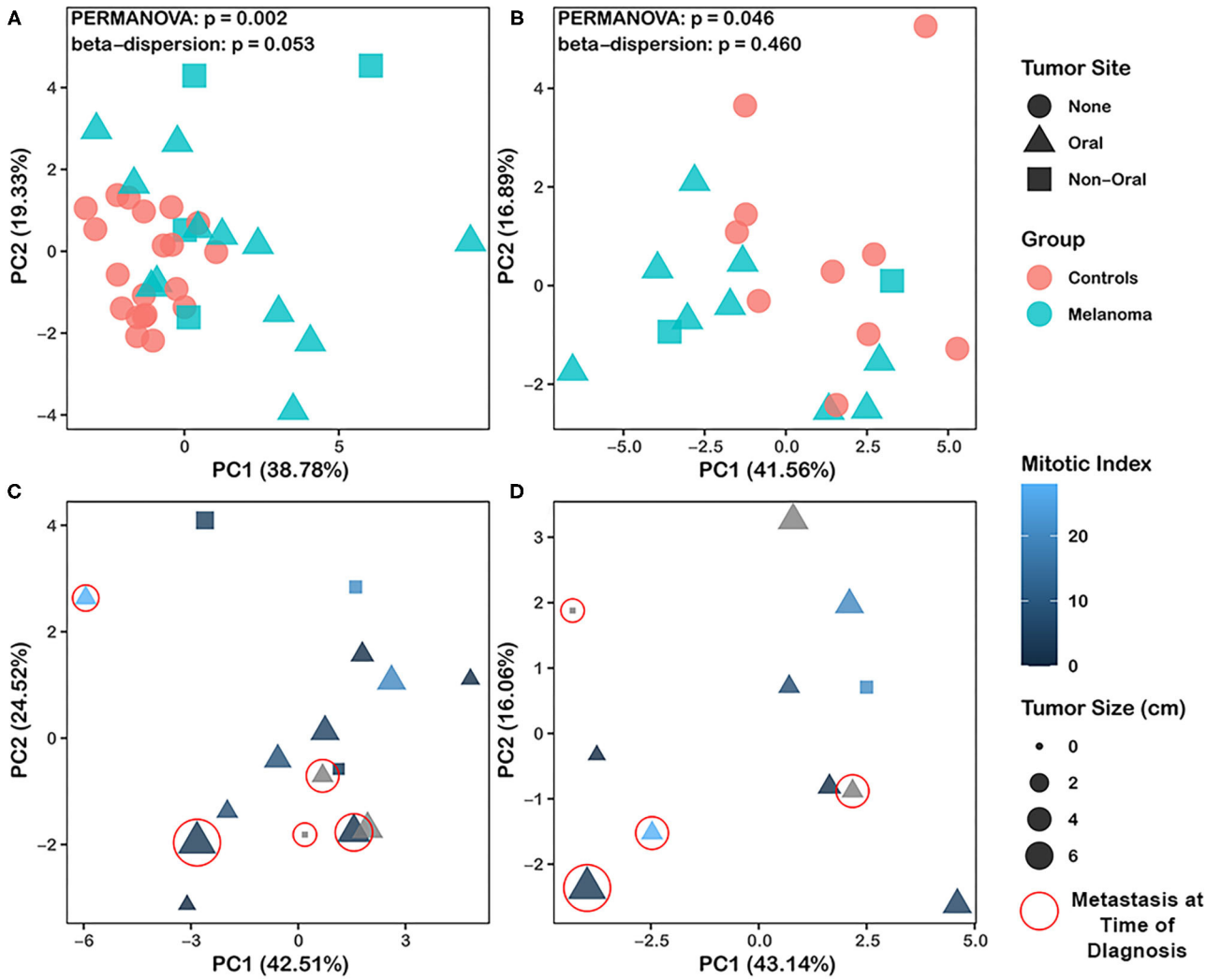

FIGURE 2 | Principal component analysis (PCA) of immunophenotype in melanoma and healthy control patients. **(A)** PCA including 17 immunophenotypes and 36 animals (20 healthy controls, 16 melanoma patients) is shown. **(B)** PCA including 23 background subtracted immunophenotypes after stimulation with ConA and 21 animals (10 healthy controls, 11 melanoma patients) is shown. **(A,B)** Patients are discriminated for melanoma site. Orange circles represent healthy control animals; teal-colored dots denote melanoma patients, which are further categorized into oral melanoma (triangle) and non-oral melanoma (square). **(C)** PCA including 17 immunophenotypes and 16 melanoma patients. **(D)** PCA including 22 background subtracted immunophenotypes after stimulation with ConA and 11 melanoma patients. **(C,D)** Patients are discriminated for melanoma site, tumor mitotic index, tumor size, and metastasis. Triangle points represent patients with oral melanoma; square points represent patients with non-oral melanoma. Points are colored based on mitotic index, from dark to bright blue. Patients with missing mitotic index are denoted by gray color. Size of data points correspond to size of the tumor. Patients with metastasized melanoma are circled in red. PCA was performed using R version 4.1.0. *P*-values < 0.05 are considered significant.

Figure 2B) with 22 immunophenotypes post ConA stimulation (**Supplementary Table 3**) showed clustering of animals with metastasis at time of diagnosis ($n = 4$) compared to animals without metastasis ($n = 7$) (PERMANOVA, $p = 0.032$) (**Figure 2D**). Similar to analysis shown in **Figure 2C**, no significant associations of tumor site, tumor size, or mitotic index with immunophenotype were observed for this PCA (PERMANOVA, $p > 0.05$) (**Figure 2D**).

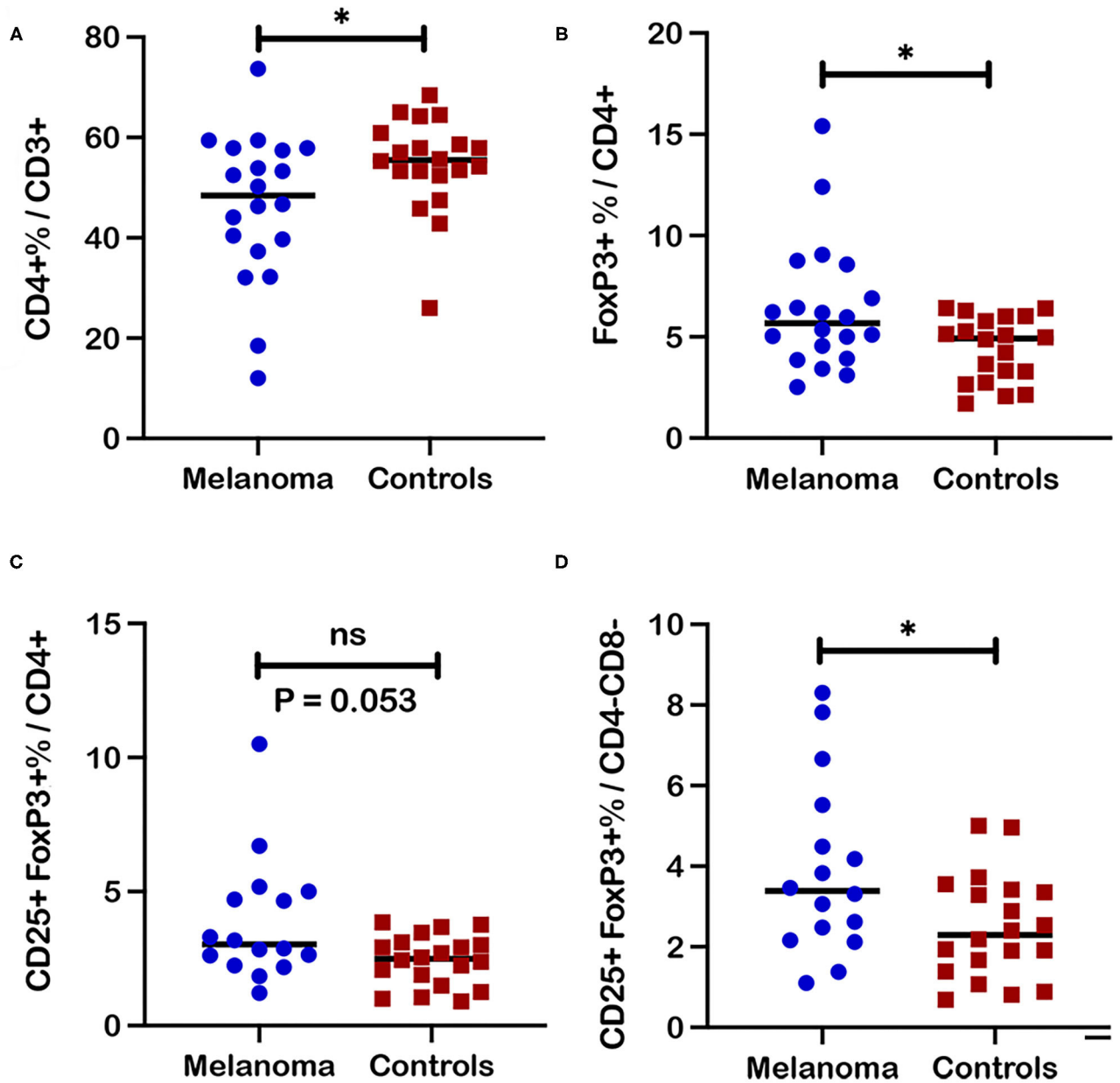

FIGURE 3 | Frequencies of regulatory T cell subsets in blood from canine melanoma patients and healthy controls. **(A)** A comparison of median frequencies for CD4+ cells within the T cell population for patients and controls is shown. **(B)** Median frequencies of FoxP3+ and **(C)** CD25+FoxP3+ cells within the CD4+ T cell population are also compared between patients and healthy controls. **(D)** Median frequencies of CD25+FoxP3+ cells within the CD4-CD8- T cell population are compared between patients and healthy controls. Pair-wise analysis between patients and controls was conducted with the Mann Whitney test using GraphPad Prism software and two-tailed analysis. "ns" denotes not significant. A single asterisk (*) denotes a *P*-value < 0.05. *P*-values < 0.05 are considered significant.

Canine Melanoma Patients Demonstrated Increased Frequencies for Selected Regulatory T Cell Subsets in Blood

Further assessment of a canine melanoma patient T cell immunophenotype involved comparison analysis of individual T cell subset frequencies between patients and healthy control dogs. Analysis of canine melanoma patients ($n = 20$; P1-20) revealed decreased frequencies of CD4+ T cells compared to healthy controls ($n = 20$, $p = 0.039$) (**Figure 3A**); however, no significant differences for frequencies of CD8+ and CD4-CD8- T cell subsets in blood were detected between patients and controls ($n = 20$) (**Supplementary Figures 2A,B**). For examination of Treg subsets, T cell subsets were analyzed with FoxP3 as a single marker (FoxP3+) or for co-expression of CD25 and

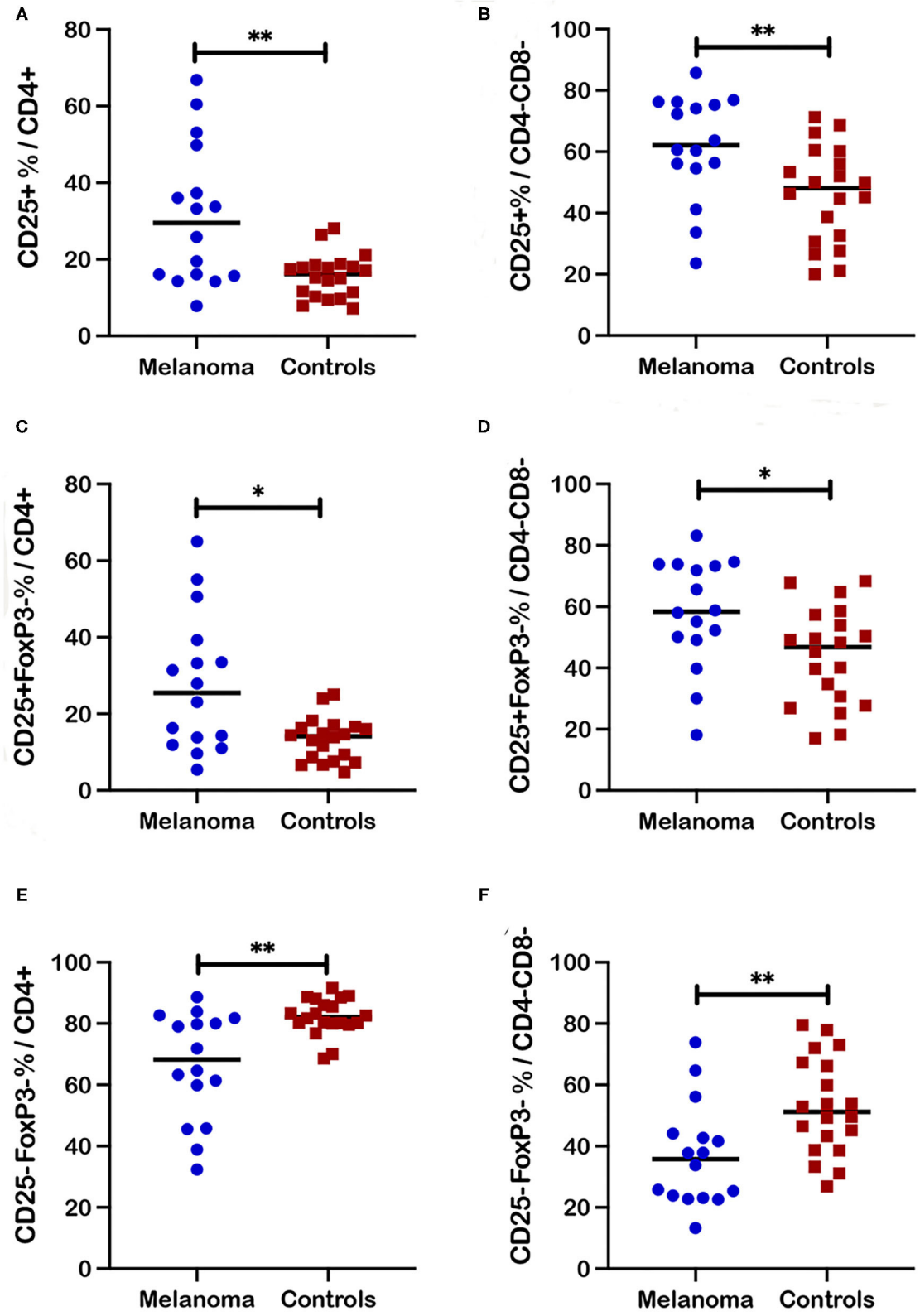

FIGURE 4 | Frequencies of activated and quiescent T cell subsets in blood from canine melanoma patients and healthy controls. Median frequencies of CD25+ cells within the CD4+ **(A)** and CD4-CD8- **(B)** T cell subsets are compared between patients and healthy controls. Frequencies of CD25+FoxP3- cells within the CD4+

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FIGURE 4 | (C) and CD4-CD8- **(D)** T cell subsets are compared between patients and healthy controls. Median frequencies of CD25-FoxP3- cells within the CD4+ **(E)** and the CD4-CD8- **(F)** T cell subsets are compared between patients and healthy controls. Pair-wise analysis between patients and controls was conducted with the Mann Whitney test using GraphPad Prism software and two-tailed analysis. A single asterisk (*) denotes a *P*-value < 0.05 and two asterisks (**) denote a *P*-value < 0.01. *P*-values < 0.05 are considered significant.

FoxP3 (CD25+FoxP3+). An increased frequency of FoxP3+ cells within the CD4+ subset was detected for patients compared to controls ($n = 20$, $p = 0.038$) (**Figure 3B**). Assessment of the regulatory CD25+FoxP3+ population also showed a trend for an increased frequency in the CD4+ subset ($p = 0.053$) (**Figure 3C**) and an increased frequency for the CD4-CD8- subset ($p = 0.036$) (**Figure 3D**) for patients ($n = 16$, P5-20) compared to controls ($n = 20$). Significant differences between patients and controls for regulatory T cell subsets were modest and were not detected for FoxP3+ populations within the CD4-CD8- and CD8+ T cell subsets (**Supplementary Figures 2C,D**). Furthermore, analysis of the ratio of CD8+ frequency to FoxP3+ frequency within the CD4+ subset revealed no significant difference between patients and controls ($n = 20$) (**Supplementary Figure 2E**).

Canine Melanoma Patients Demonstrated Increased Frequencies for Selected T Cell Subsets Based on CD25 Expression in Blood

CD25 as the alpha chain of the trimeric IL-2 receptor is considered a critical marker for T cell activation that is associated not only with regulatory but other activated T cell subsets. Significantly increased frequencies of CD25+ cells that include both FoxP3+ and FoxP3- populations, were observed for CD4+ ($p = 0.01$) and CD4-CD8- ($p = 0.0038$) T cell subsets in blood from canine melanoma patients ($n = 16$) compared to healthy controls ($n = 20$) (**Figures 4A,B**). Similarly, frequencies of CD25+FoxP3- cells were also significantly increased for the CD4+ ($p = 0.017$) and CD4-CD8- ($p = 0.012$) T cell subsets (**Figures 4C,D**). In contrast, frequencies of a putative quiescent phenotype CD25-FoxP3- were significantly decreased in patients compared to controls for both the CD4+ ($p = 0.002$) and CD4-CD8- ($p = 0.0035$) T cells (**Figures 4E,F**).

Expression of the proliferation marker Ki67 was also analyzed for multiple T cell subsets in blood from canine melanoma patients ($n = 16$) and healthy controls ($n = 10$). Ki67+ frequencies for both CD4+ and CD4-CD8- T cells within the CD3+ population were not significantly different between patients and controls (**Supplementary Figures 3A,B**). Frequencies of Ki67+ cells within regulatory (FoxP3+ and CD25+FoxP3+) CD4+ subsets (**Supplementary Figures 3C,D**) and CD4-CD8- T cell subsets (**Supplementary Figures 3E,F**) were also not significantly different. Absence of statistical significance between patients and controls was also observed for activated (CD25+FoxP3-) CD4+ and CD4-CD8- T cell subsets (**Supplementary Figures 4A,B**) and quiescent (CD25-FoxP3-) CD4+ and CD4-CD8- T cell subsets (**Supplementary Figures 4C,D**). Lastly, in contrast to CD4+ and CD4-CD8- T cell subsets, significant differences in frequencies for any activation/proliferation phenotypes including granzyme B+, CD25, Ki67, or CD25+FoxP3-, were not observed for the CD8+ T cell subset in blood for patients compared to controls (**Supplementary Figures 5A–D**). Frequencies in the CD25-FoxP3- quiescent population within the CD8+ T cell subset were also not significantly different between patients and controls (**Supplementary Figure 5E**).

Canine Melanoma Patients Exhibited Two Unique Phenotypes for Interferon-γ T Cell Responses to ConA Stimulation

IFN-γ responses to ConA stimulation were measured as frequencies of IFN-γ+ cells within each T cell subset after stimulation of PBMC. IFN-γ expression after stimulation was utilized as a functional assessment of circulating T cells in canine melanoma patients ($n = 11$) and as another marker for differentiating the T cell immunophenotype of canine melanoma patients from healthy controls ($n = 10$). Although median values for IFN-γ+ frequencies were higher for CD4+, CD4-CD8-, and CD8+ T cell subsets (**Figures 5A–C**) in patients compared to controls, statistical differences were not observed between patients and controls, although a trend toward significance ($p = 0.051$) was observed for CD8+ T cells. Median values for IFN-γ+ cell frequencies were also higher for CD25+FoxP3- (activated phenotype) and CD25-FoxP3- (quiescent phenotype) populations within the CD4+T cell subset (**Figures 5D,E**) in patients compared to controls, but differences were not statistically different. Interestingly five patients (P10, P11, P12, P14, P15) demonstrated lower IFN-γ+ cell frequencies compared to other patients, for at least one T cell subset including CD4+, CD4-CD8-, CD25+FoxP3-CD4+, and CD25-FoxP3-CD4+ populations. For each subset, three of these five patients formed a cluster showing frequencies that were significantly lower ($p = 0.012$ for all four subsets) compared to other patients. Patients P10 and P11 demonstrated lower frequencies for all four subsets, with P14 showing lower frequencies for CD4+ and CD25+FoxP3-CD4+ populations, P12 for the CD4-CD8- population, and P15 for the CD25-FoxP3-CD4+ population. One patient (P10) was diagnosed with melanoma with the primary site unknown and the remaining four patients were characterized by oral tumors (**Supplementary Table 1**). The only other commonality for these patients was evidence of metastasis at time of diagnosis for three of the five patients (P10-12; **Supplementary Table 1**). These data revealed two unique phenotypes for T cell IFN-γ response to polyclonal stimulation by ConA by patient T cells that characterized patients as normal/high vs. low responders. IFN-γ+ cell frequencies for CD25+FoxP3- and CD25-FoxP3- subpopulations of other T cell subsets are not described due to extremely low events

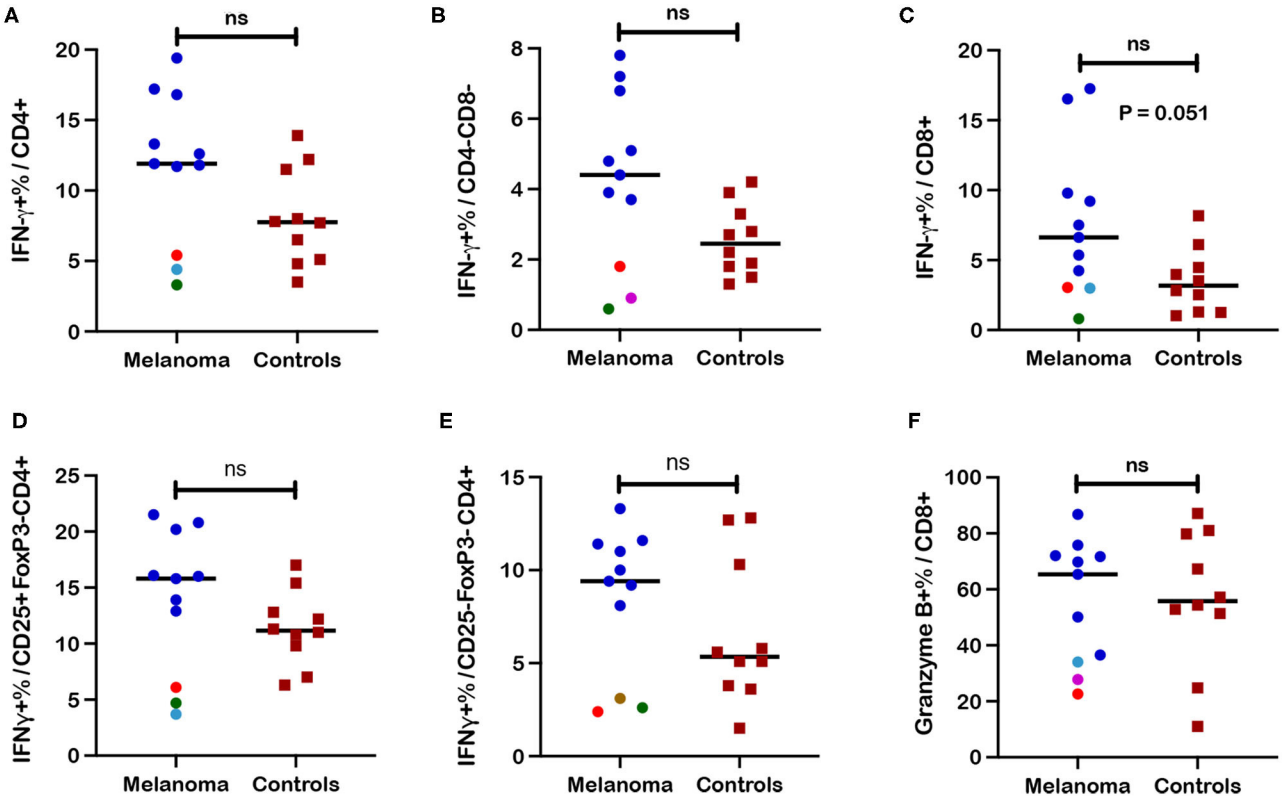

FIGURE 5 | Frequencies of interferon-γ+ (IFN-γ) and granzyme-B+ T cell subsets after ConA stimulation in blood from canine melanoma patients and healthy controls. Median frequencies of IFN-γ+ cells after ConA stimulation were determined in **(A)** CD4+, **(B)** CD4-CD8-, and **(C)** CD8+ T cells by intracellular cytokine staining (ICS) and flow cytometry and compared between patients and healthy controls. Median frequencies of INF-γ+ cells after ConA stimulation are also shown for **(D)** CD25+FoxP3- (activated) and **(E)** CD25-FoxP3- (quiescent) CD4+ T cells and compared between patients and controls. **(F)** Induction of granzyme B after ConA stimulation is represented by median frequencies of granzyme B+ cells within the CD8+ T cell subset and compared between patients and controls. Colored dots represent outlier patients with green indicating subject P10, red indicating subject P11, purple indicating subject P12, turquoise indicating subject P14, and brown representing P15. Values described for IFN-γ+ frequencies reflect values for stimulated cells after subtraction of values for unstimulated cells. Values described for granzyme B+ frequencies do not reflect subtraction of values for unstimulated cells. Pair-wise analysis between patients and controls was conducted with the Mann Whitney test using GraphPad Prism software and two-tailed analysis. "ns" denotes not significant.

detected by flow cytometric analysis. Finally, analysis of granzyme B expression, an activation marker for CD8+ T cells after ConA stimulation, did not reveal statistical differences between frequencies of granzyme B+ CD8+ T cells after ConA stimulation between patients and healthy controls (**Figure 5F**). Three of the five low responder patients also demonstrated lower frequencies of either IFN-γ+ (**Figure 5C**) or granzyme B+ cells (**Figure 5F**) within the CD8+ T cell subset after ConA stimulation, although clustering of these patients from other patients was not as striking.

Canine Melanoma Patients Demonstrated Increased Frequencies of Multiple T Cell Subsets Based on CD25 Expression in Blood After ConA Stimulation

Frequencies of CD25 expression in different T cell subsets after ConA stimulation (**Figure 6**) revealed patterns very similar to those observed for unstimulated T cells as shown in **Figure 4** with the exception that significant differences between patients and controls were also revealed for the CD8+ T cell subset after stimulation. Significantly higher frequencies of CD25+ cells within the CD4+ ($p = 0.0003$), CD4-CD8- ($p = 0.0045$), and CD8+ ($p = 0.011$) T cell subsets were observed after ConA stimulation in patients compared to healthy controls (**Figures 6A–C**). Significantly higher frequencies were also observed for the activated CD25+FoxP3- population within the CD4+ ($p = 0.0003$), CD4-CD8- ($p = 0.017$), and CD8+ ($p = 0.013$) T cell subsets after ConA stimulation for patients compared to controls (**Figures 6D–F**). Also similar to the results for unstimulated cells, significantly lower frequencies for the quiescent CD25-FoxP3- population within the CD4+ ($p = 0.0002$), CD4-CD8- ($p = 0.008$), and CD8+ ($p = 0.01$) T cell subsets after ConA stimulation were observed for patients compared to controls (**Figures 6G–I**). In contrast, no significant differences between patients and controls were observed for frequencies of regulatory FoxP3+ and CD25+FoxP3+ cells within the CD4+ (**Supplementary Figures 6A,B**) or the

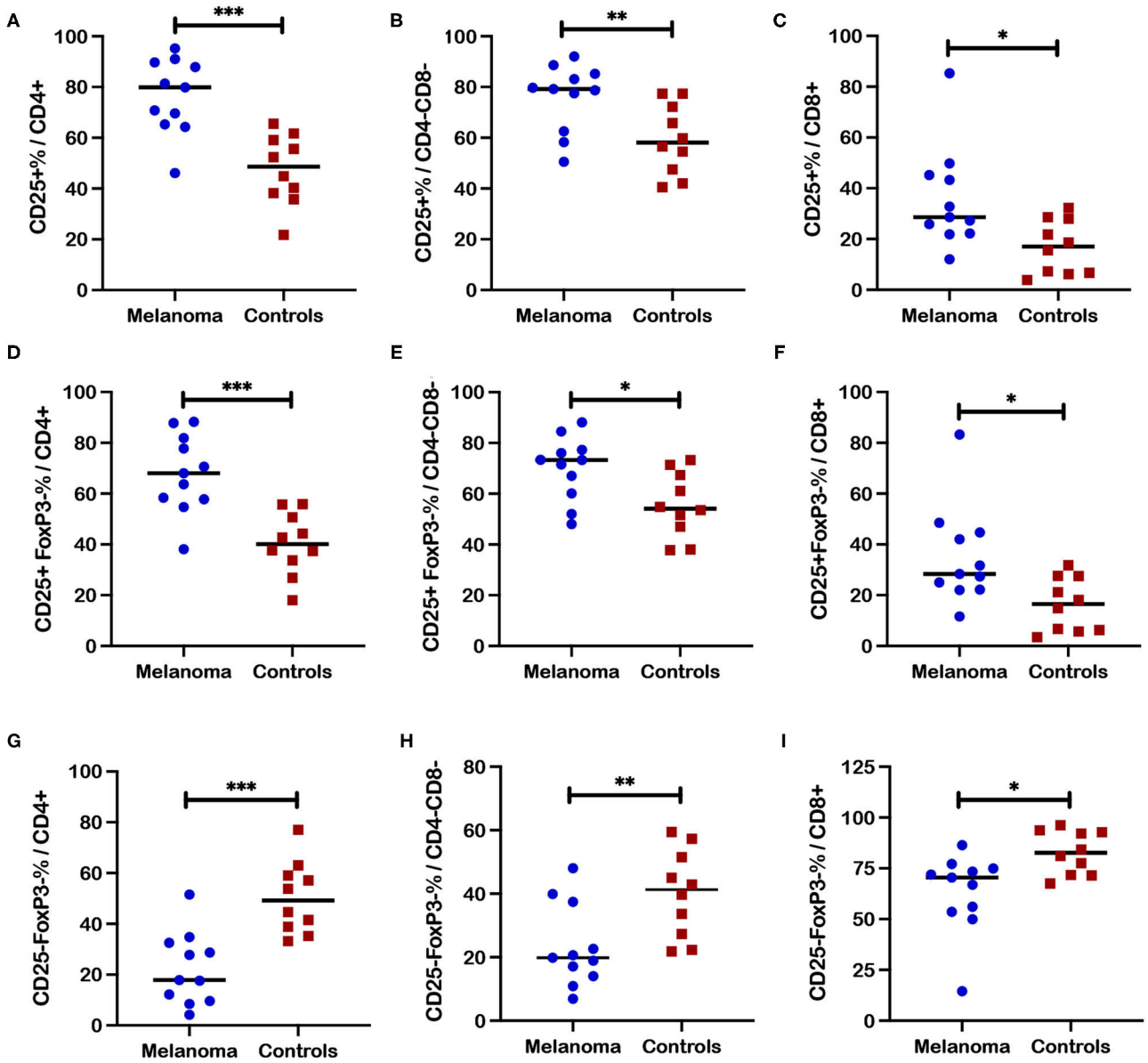

FIGURE 6 | Frequencies of CD25+ T cell subsets after ConA stimulation in blood from canine melanoma patients and healthy controls. Median frequencies of CD25+ cells after ConA stimulation were determined in **(A)** CD4+, **(B)** CD4-CD8-, and **(C)** CD8+ T cells and compared between patients and healthy controls. Median cell frequencies after ConA stimulation are next shown for the activated phenotype CD25+FoxP3- within **(D)** CD4+, **(E)** CD4-CD8-, and **(F)** CD8+ T cells and compared between patients and controls. Median cell frequencies after ConA stimulation are shown for T cell phenotype CD25-FoxP3- within **(G)** CD4+, **(H)** CD4-CD8-, and **(I)** CD8+ T cell populations and compared between patients and controls. Values described for CD25+ frequencies do not reflect subtraction of values for unstimulated cells. Pair-wise analysis between patients and controls was conducted with the Mann Whitney test using GraphPad Prism software and two-tailed analysis. A single asterisk (*) denotes a *P*-value < 0.05; two asterisks (**) denote a *P*-value < 0.01; and three asterisks (***) denote a *P*-value < 0.001. *P*-values < 0.05 are considered significant.

CD4-CD8- (**Supplementary Figures 6C,D**) T cell subsets post ConA stimulation. Event numbers were too low for analysis of regulatory cell populations with CD8+ T cell subset.

Proliferation responses based on Ki67+ cell frequencies are not reported for T cell subsets as no induction of Ki67 expression was observed for either patients or controls after ConA stimulation. The absence of such responses was likely due to the need for an extended time frame in culture for restoration of proliferative function for cryopreserved lymphocytes (55, 56), as well as an extended time frame of ConA stimulation for optimal induction of Ki67: both conditions were not accommodated by our experimental protocol.

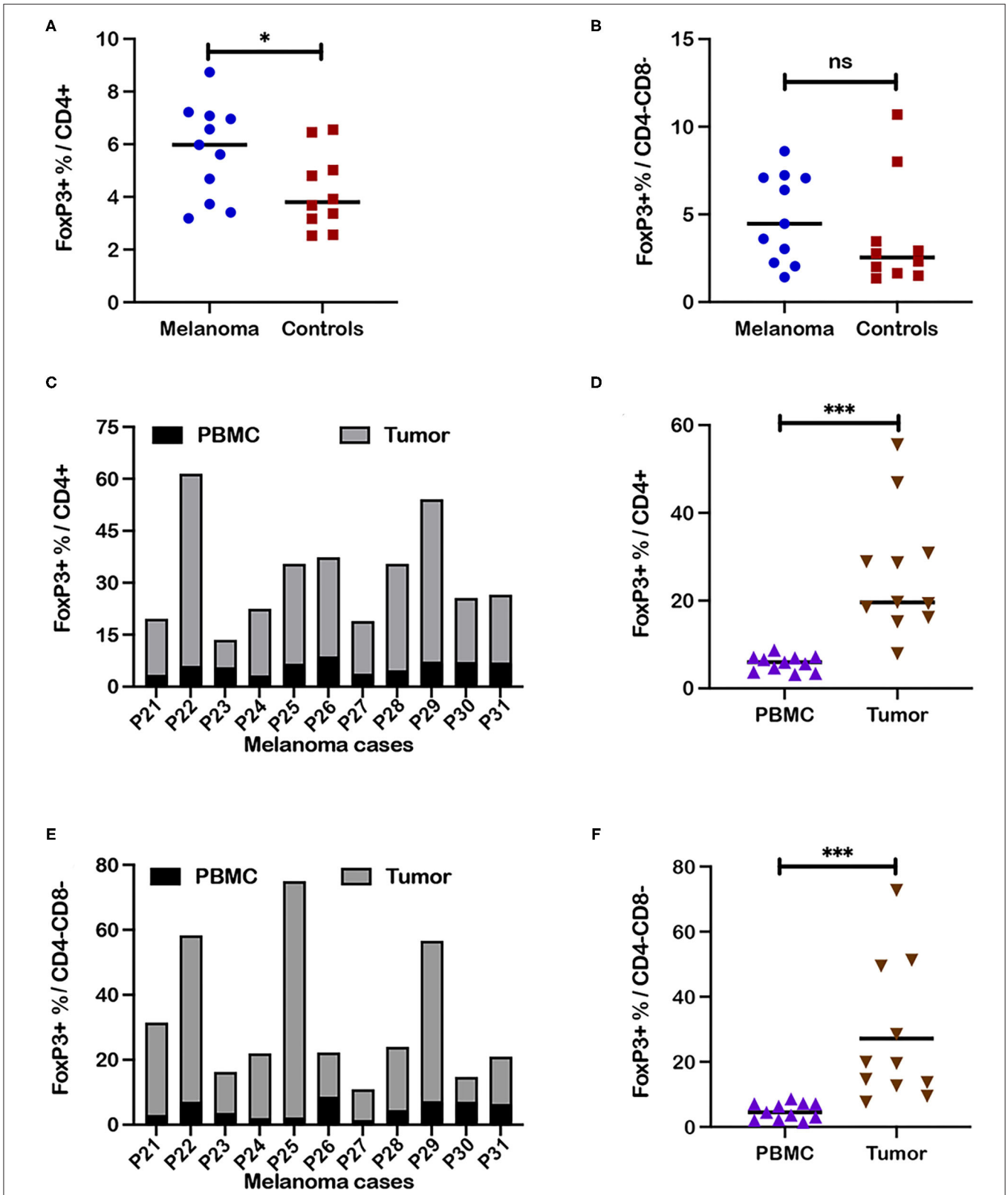

FIGURE 7 | Comparison of frequencies of regulatory T cell subsets in tumor associated cells and blood in canine melanoma patients. PBMC isolated from blood and tumor associated cells from a separate cohort of 11 canine melanoma patients were assessed for T cell phenotypes by flow cytometry. **(A)** Median frequencies of

(Continued)

FIGURE 7 | FoxP3+ within CD4+ and **(B)** CD4-CD8- T cell populations in blood were compared between patients of this patient cohort and healthy controls. **(C)** Each stacked bar reflects frequencies of FoxP3+ cells within the CD4+ T cell subset in blood vs. tumor associated cells for a single patient. A comparison of frequencies assessed for blood and tumor associated cells is shown for all patients. **(D)** Median frequencies of FoxP3+ cells within the CD4+ T cell subset are compared between PBMC and tumor as a grouped analysis for the same patients. **(E)** A stacked bar analysis of frequencies of FoxP3+ cells within the CD4-CD8- T cell subset in blood vs. tumor associated cells from each patient is shown for all patients. **(F)** Median frequencies of FoxP3+ cells within the CD4-CD8- T cell subset are compared between PBMC and tumor as a grouped analysis for the same patients. Pair-wise analysis between frequencies for patients and controls was conducted with the Mann Whitney test and between PBMC and tumor associated cells using the Wilcoxon matched-pairs signed rank test, using GraphPad Prism software and a two-tailed analysis. "ns" denotes not significant. A single asterisk (*) denotes a *P*-value < 0.05; three asterisks (***) denote a *P*-value < 0.001. *P*-values < 0.05 are considered significant.

Significantly Higher Frequencies of FoxP3+ T Cells Were Detected in Tumor Associated Cells Compared to Blood

Fresh PBMC and tumor associated cells were isolated from a separate cohort of canine melanoma patients (P21-31; **Supplementary Tables 1, 2**) and tested by an abbreviated flow cytometry panel that included interrogation for CD3+ T cell subsets (CD4+, CD4-CD8-, and CD8+), FoxP3 and granzyme B (**Supplementary Figure 7**). FoxP3+ frequencies within the CD4+ T cell subset in fresh PBMC for this patient cohort ($n = 11$) were higher compared to fresh PBMC isolated from healthy controls ($n = 10$; C14-23) ($p = 0.043$) (**Figure 7A**; **Supplementary Figure 7B**), although the difference was modest as shown for other melanoma patients (**Figure 3B**). Although median frequencies for FoxP3+ cells within the CD4-CD8- T cell subset were higher for patients compared to controls, a significant difference was not detected (**Figure 7B**). A comparison of frequencies of FoxP3+ cells within the CD4+ T cell subset for tumor associated cells and blood (PBMC) for each patient revealed a significantly higher frequency of FoxP3+ cells in tumor vs. blood for each patient (**Figures 7C,D**) ($p = 0.001$). A similar result was noted for comparison of FoxP3+ cell frequencies within the CD4-CD8- T cell subset with higher frequencies detected in tumor associated cells compared to blood for each patient (**Figures 7E,F**) ($p = 0.001$). Comparison of frequencies of CD4+, CD4-CD8-, and CD8+ T cell subsets between PBMC and tumor associated cells did not reveal significant differences (**Supplementary Figures 8A–F**). Significant differences in frequencies of granzyme B+ cells within the CD8+ T cell subset were also not detected between tumor associated cells and PBMC for each patient (**Supplementary Figures 8G,H**). Importantly intratumoral frequencies of FoxP3+ cells within the CD4+ and CD4-CD8- subsets were strikingly higher compared to frequencies in PBMC.

Frequencies of Regulatory, Activation, and Proliferation Markers Differed Between Specific T Cell Subsets in Blood for Healthy Controls

Data generated from these studies of canine melanoma patients afforded an opportunity to analyze different canine T cell subsets and subpopulations for specific T cell markers based on healthy control dogs. An interesting CD4-CD8- CD3+ T cell subset was reported and characterized previously to express regulatory markers including FoxP3 and CD25 (44). Our results for 20 healthy control dogs also show FoxP3 expression by CD4-CD8- T cells although frequencies of FoxP3+ cells for this subset are lower compared to CD4+ cells with a difference trending for significance ($p = 0.052$) (**Figure 8A**). Regardless frequencies of FoxP3+ cells for CD4-CD8- T cells (median = 3.23%) were still comparable to CD4+ cells (median = 4.93%) and significantly higher than those assessed for CD8+ T cells (median = 0.54%) ($p < 0.0001$).

Analysis of healthy controls ($n = 10$) revealed higher Ki67+ cell frequencies within the FoxP3+CD4+ T cells compared to frequencies within CD25+FoxP3- ($p = 0.001$) and CD25-FoxP3- ($p = 0.002$) subpopulations of CD4+ T cells and also compared to Ki67+ cell frequency with the total CD4+ T cell subset ($p = 0.018$). Ki67+ cell frequencies were also higher in the CD25-FoxP3+CD4+ population compared to CD25+FoxP3-CD4+ ($p = 0.0001$) and CD25-FoxP3-CD4+ ($p = 0.002$) populations and also compared to Ki67+ cell frequency within the total CD4+ T cell subset ($p = 0.024$). Ki67+ cell frequencies were not significantly different between regulatory populations FoxP3+CD4+ and CD25-FoxP3+ CD4+ T cells, or between different FoxP3-CD4+ T cell populations (CD25+FoxP3- and CD25-FoxP3-) or compared to Ki67+ cell frequency within the total CD4+ T cell subset (**Figure 8B**). Similar results were shown for the CD4-CD8- subset where Ki67+ cell frequencies detected within the FoxP3+CD4-CD8- T cell subset were higher compared to Ki67+ cell frequencies for CD25+FoxP3- ($p < 0.0001$) and CD25-FoxP3- ($p = 0.0097$) populations of the CD4-CD8- T subset, and also compared to Ki67+ cell frequency with the total CD4-CD8- T cell subset ($p = 0.015$). Ki67+ cell frequencies were also higher in CD25-FoxP3+CD4-CD8- population compared to CD25+FoxP3- ($p < 0.0001$) and CD25-FoxP3- ($p = 0.0022$) populations of CD4-CD8- T cells, and also compared to Ki67+ cell frequency with the total CD4-CD8- T cell subset ($p = 0.0035$). Ki67+ cell frequencies were not significantly different between FoxP3+ and CD25-FoxP3+ populations within CD4-CD8- subset, or between FoxP3-CD4-CD8- T cell populations (CD25+FoxP3- and CD25-FoxP3-) or between FoxP3-CD4-CD8- T cell subsets and the total CD4-CD8- T cell subset (**Figure 8C**). These results overall show higher Ki67+ cell frequencies within FoxP3+ populations of both CD4+ and CD4-CD8- T cell subsets. Assessment of similar populations within the CD8+ T cell subset was not performed due to extremely low numbers of events detected by flow cytometric analysis for this T cell subset.

Frequencies of CD25+ cell populations within each T cell subset were analyzed and revealed a significantly higher

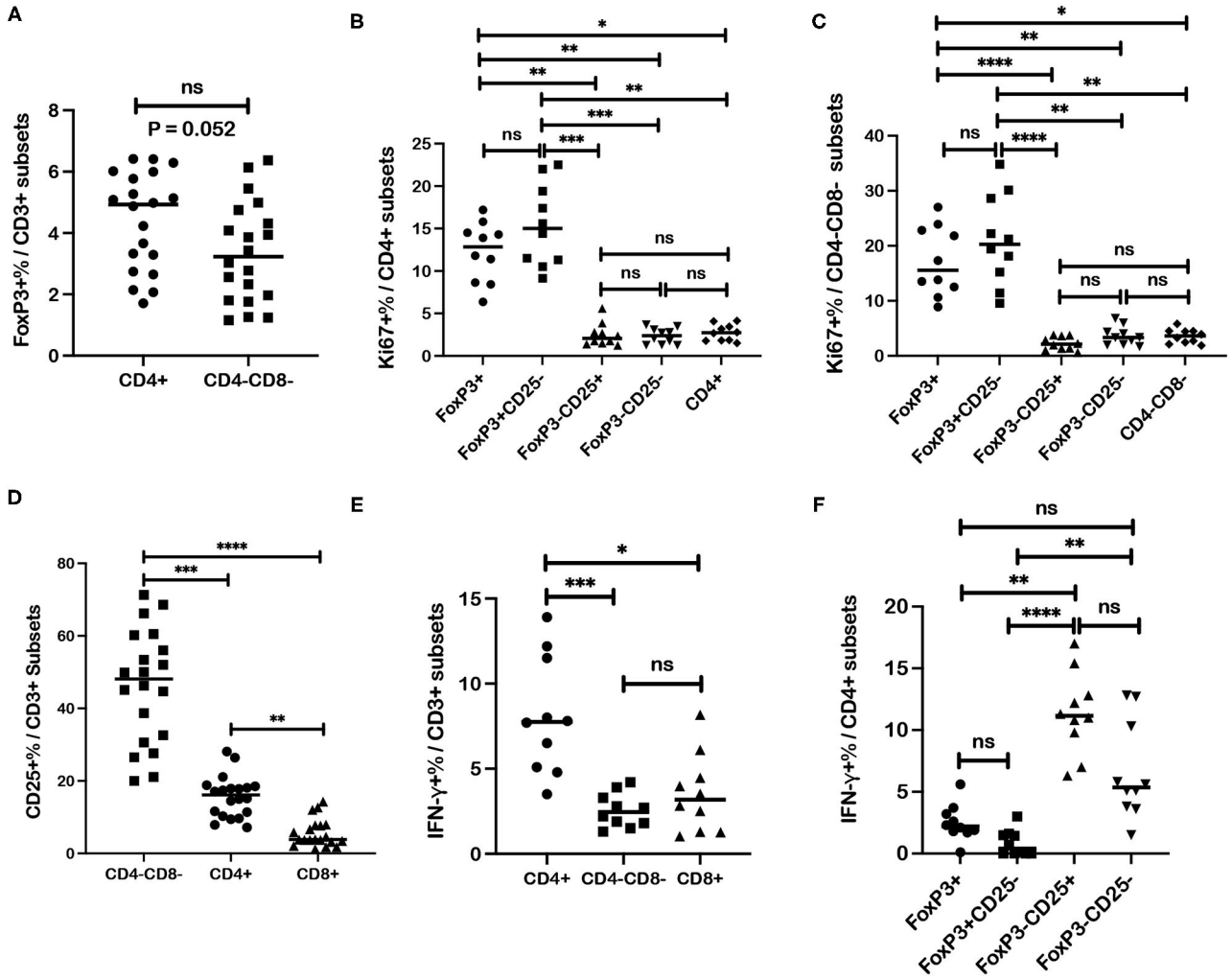

FIGURE 8 | Associations between regulatory and activation markers for specific T cell subsets in healthy dog PBMC. **(A)** Median frequencies of FoxP3+ cells are compared between CD4+ and CD4-CD8- T cell subsets. **(B)** Median frequencies of Ki67+ cells within different subsets of CD4+ T cells including regulatory (FoxP3+; CD25-FoxP3+), activated (CD25+FoxP3-) and quiescent (CD25-FoxP3-) populations, are compared. **(C)** Median frequencies of Ki67+ cells within different subsets of CD4-CD8- T cells including regulatory (FoxP3+), activated (CD25+FoxP3-) and quiescent (CD25-FoxP3-) populations, are compared. **(D)** CD4+, CD4-CD8-, and CD8+ T cells subsets are compared for median frequencies of CD25+ cells. **(E)** Median frequencies of interferon-γ (IFN-γ)+ cells after ConA stimulation were compared between CD4+, CD4-CD8-, and CD8+ T cells subsets. **(F)** Median frequencies of IFN-γ+ cells after ConA stimulation were compared between different CD4+ T cell subsets including regulatory (FoxP3+; CD25-FoxP3+), activated (CD25+FoxP3-) and quiescent (CD25-FoxP3-) populations. Values described for IFN-γ+ frequencies reflect values for stimulated cells after subtraction of values for unstimulated cells. Comparisons of frequencies between three more subsets were performed with a Kruskal-Wallis test with Dunn's *post-hoc* test for multiple comparisons. Pair-wise analysis between frequencies of two subsets **(A)** was conducted with the Mann Whitney test and two-tailed analysis. All analyses used GraphPad Prism software. "ns" denotes not significant. A single asterisk (*) denotes a *P*-value < 0.05; two asterisks (**) denote a *P*-value < 0.01; three asterisks (***) denote a *P*-value < 0.001 and four asterisks (****) denote a *P*-value < 0.0001. *P*-values < 0.05 are considered significant.

frequency in the CD4-CD8- T cell subset compared to CD4+ ($p = 0.0006$) and CD8+ ($p < 0.0001$) T cells (**Figure 8D**). As expected CD25+ cell frequencies for CD4+ cells were also higher compared to the CD8+ subset ($p = 0.004$). Frequencies of activated CD25+FoxP3- cells were also higher for the CD4-CD8- subset compared to CD4+ cells ($p = 0.0004$) and CD8+ cells ($p < 0.0001$) (**Supplementary Figure 9**). Also as expected, frequencies of CD25+FoxP3- cells were higher in CD4+ cells compared to CD8+ cells ($p = 0.009$). These results reveal the highest frequency of activated CD25+ cells within the CD4-CD8- T cell subset followed by the CD4+ subset.

IFN-γ+ cell frequencies after ConA stimulation were higher for CD4+ T cells compared to CD4-CD8- T cells ($p = 0.0009$) and CD8+ T cells ($p = 0.012$) (**Figure 8E**). Differences in IFN-γ+ cell frequencies were not significant between CD4-CD8- and CD8+ T cells. Analysis of IFN-γ+ cell frequencies within FoxP3+ and FoxP3- CD4+ subsets after ConA stimulation was also performed. Significantly

higher IFN-γ+ cell frequencies were detected in CD25+FoxP3-CD4+ T cells compared to regulatory FoxP3+CD4+ cells ($p = 0.004$) and CD25-FoxP3+CD4+ cells ($p < 0.0001$) (**Figure 8F**). IFN-γ+ cell frequencies were also higher in the CD25-FoxP3-CD4+ T cells compared to CD25-FoxP3+CD4+ cells ($p = 0.002$). Comparisons of IFN-γ+ cell frequencies between FoxP3+CD4+ and CD25-FoxP3+CD4+ subsets, FoxP3+CD4+ and CD25-FoxP3-CD4+ subsets, and CD25+FoxP3-CD4+ and CD25-FoxP3-CD4+ subsets revealed no significant differences (**Figure 8F**). Overall, these data show higher IFN-γ+ cell frequencies for the CD4+ T cell subset and particularly for the CD25+FoxP3-CD4+ subset with low IFN-γ+ cell frequencies found in FoxP3+ CD4 subsets. Also of note, IFN-γ+ cell frequencies detected with the CD4-CD8- subset were comparable to those detected in the CD8+ subset and distinguishes one property shared by the CD4-CD8- and CD8+ subsets.

DISCUSSION

T cell immunophenotyping studies described herein were predicated on the hypothesis that canine melanoma patients exhibit a T cell immune profile that is different from healthy control pet dogs. A secondary hypothesis states that frequencies of circulating T cell subsets may not match intratumoral frequencies and that blood vs. tumor T cell immunophenotypes will differ. Findings by PCA indeed determined that canine melanoma patients display a T cell immunophenotype that is unique from healthy pet dogs. These findings were further supported by analysis of frequencies of individual T cell immunotypes in patients and healthy controls. Key findings revealed increased frequencies of circulating regulatory FoxP3+ T cell subsets and activated CD25+FoxP3- T cell subsets in canine patients compared to healthy controls. Furthermore, T cell function measured by IFN-γ responses to ConA stimulation in PBMC distinguished canine melanoma patients into two groups with a larger group demonstrating a higher frequency of IFN-γ+ cells indicating a competent and possibly inflated functional response. A second smaller group of patients showed a considerably weaker response suggestive of a dysregulated response. Another key finding was the dichotomy determined between frequencies of FoxP3+ CD4+ and FoxP3+ CD4-CD8- cells in blood compared to tumor where frequencies of FoxP3+ cells were significantly higher in tissue than detected in circulation. Lastly, the CD4-CD8- T cell population frequently displayed an immunophenotype similar to CD4+ T cells in blood and tumor with the exceptions of amplified CD25 expression and an IFN-γ response that was more similar to CD8+ T cells. These findings although not surprising, collectively characterized a T cell immunophenotype that clearly distinguished canine melanoma patients from healthy controls and suggested immune pathways for further investigation.

Previous reports have described increased frequencies of circulating and intratumoral Tregs defined either as FoxP3+CD4+ or CD25+FoxP3+CD4+ T cell subsets for canine melanomas by either flow cytometry analysis (57–61) or immunohistochemistry (IHC) (60–63). Our findings also revealed significantly increased frequencies for either FoxP3+ or CD25+FoxP3+ populations in either CD4+ or CD4-CD8- T cell subsets in PBMC from patients compared to controls, although differences were fairly small and CD4+ T cell frequencies were actually lower in patients. A finding of greater interest was the significantly higher frequency of FoxP3+ cells within both the CD4+ and CD4-CD8- T cell subsets in tumor associated cells compared to corresponding patient blood samples from a small case cohort, confirming the importance of examining the local tumor environment. Although few in number, other reports have described similar observations of increased frequencies of FoxP3+ cells detected within either tumor or draining lymph node for canine melanoma patients assessed by flow cytometry (57, 60) or by IHC Treg (61). Although intratumoral Tregs have been reported to confer immunosuppression by multiple mechanisms including inhibition of anti-tumor effector responses and to promote disease progression for a wide range of human cancers (64, 65) there are conflicting reports as to whether FoxP3+ tumor-infiltrating lymphocytes (TILs) confer a negative prognosis for human cutaneous melanoma (64). Two reports describing Tregs within TILs for canine melanoma concluded that higher frequencies of intratumoral Treg were a negative prognostic factor (60, 63) whereas a more recent report (61) did not find intratumoral Treg frequency to associate with more aggressive disease. Our findings on a small canine melanoma cohort did not find significant differences between frequencies of other T cell subsets (CD4+, CD8+, and CD4-CD8-) or frequencies of granzyme B+ CD8+ T cells in blood vs. tumor. Furthermore, the canine cohort was insufficient in case number to determine an association of intratumoral Treg frequency and disease outcome. Future studies with larger patient cohorts and flow cytometry panels with additional markers along with corresponding IHC analysis will be necessary to determine the relevance of the large Treg frequencies in tumor associated cells as observed in this study and to identify intratumoral T cell phenotypes as correlates for prognosis and potential immunotherapeutics.

CD25 proved to be a major marker of interest in characterizing T cell immunophenotypes in canine melanoma patients. CD25 expression has classically been defined as a Treg marker when co-expressed with FoxP3+ in CD4+ T cells (66, 67). In contrast to reports for frequencies of healthy human CD25+ CD4+ T cell (68, 69), our studies revealed a higher frequency of this subset within both healthy controls and patients which proved to include both FoxP3- as well as FoxP3+ cell populations. Other reports describing CD25+ cell frequencies within the CD4+ T cell subset in healthy dogs also revealed similar or higher frequencies compared to our current studies (44, 70, 71). Our results also revealed increased frequencies of the CD25+ cells for both CD4+ and CD4-CD8- T cell subsets in canine melanoma patients compared to healthy dog controls. A more interesting finding related to the significant frequencies of CD25+FoxP3- cell population within both CD4+ and CD4-CD8- T cell subsets for healthy controls and patients. Furthermore, frequencies of the CD25+FoxP3- cell population in both T cell subsets were significantly higher in patients.

Reports specifically describing CD25+FoxP3- cells within either CD4+ and CD4-CD8- T cell subsets for different species are few. However, CD25 as the alpha chain of the trimeric IL-2 receptor is considered a critical marker for T cell activation for not only regulatory, but also recently activated effector and antigen-experienced or resting memory T cells in mice and humans (69, 72–76). Moreover, frequencies of both CD25+ and CD25+FoxP3- populations were increased for all T cell subsets including CD8+ T cells after stimulation by ConA, again with patients again showing significantly higher frequencies compared to healthy controls. Conversely no differences in frequencies of FoxP3+ or CD25+FoxP3+ populations for either CD4+ or CD4-CD8- T cell subsets after stimulation by ConA were observed between patients and controls. These findings would suggest that the CD25+FoxP3- population within each T cell subset, particularly the CD4+ and CD4-CD8- subsets, represented an activated population primed for expansion upon polyclonal stimulation with a heightened response shown by patients.

Given that higher frequencies of the activated CD25+FoxP3- population were higher in patients, lower frequencies of the putative quiescent CD25-FoxP3- population were detected within both CD4+ and CD4-CD8- subsets for patients compared to healthy controls in unstimulated and ConA-stimulated cells. Stimulated CD8+ T cells also revealed lower frequencies for the CD25-FoxP3- population in patients compared to controls. Based on the absence of expression by FoxP3 (considered to be a regulatory and activation marker) and absence of activation marker CD25, this population was tentatively considered a "quiescent" T cell population. However, both CD25+FoxP3- and CD25-FoxP3- populations are most likely heterogenous populations and the CD25-FoxP3- population will include both naive and memory populations. Therefore, these populations will require further interrogation by memory and other activation markers for an accurate identification and determination of specific sub-populations that may be accountable for differences between canine melanoma patients and healthy controls. It is important to note that proliferation marker Ki67 did not clearly distinguish any T cell subsets or sub-populations for differences in frequencies between patients and controls. Similarly, granzyme B as an activation and exhaustion marker for CD8+ T cells, did not detect differences in frequencies of granzyme B+ CD8+ cells between patients and healthy controls for either unstimulated or stimulated cells. However, patients demonstrated a large variation in values for frequencies of granzyme B+ cells compared to controls and revealed selected outlier patients showing very high frequencies. Further examination of granzyme B as a CD8+ T cell phenotype will be warranted in larger patient cohorts.

T cell dysfunction or T cell exhaustion is a well-recognized outcome of chronic antigenic stimulation and cancer and may result in a progressive loss of T cell functions including proliferation, cytokine release and cytolytic activity. This is a consequence of the actions of multiple inhibitory receptors including PD1 and CTLA-4 that are induced by chronic antigenic stimulation or inhibitory receptor ligands expressed by tumor cells (39). IFN-γ is a type 2 pleiotropic interferon that displays antiproliferative, anti-angiogenic and pro-apoptotic activity against tumor cells through multiple complex mechanisms (39, 77). IFN-γ is produced predominantly by activated CD4+ T cells (Th1), CD8+ T cells, γδ T cells, and natural killer (NK) cells and may also demonstrate suppressive effects on anti-tumor immune responses by induction of multiple immunoregulatory factors on tumor cells including indoleamine-2,3-dioxygenase (IDO) and PDL1. To assay for T cell dysfunction in a small cohort of canine melanoma patients, induction of IFN-γ expression in response to mitogen (ConA) stimulation was measured in PBMC by ICS in both patients and healthy controls. Increased IFN-γ+ cell frequencies within T cell subsets after ConA stimulation were observed for patients compared to healthy controls for all subsets. However, differences were not statistically significant although a trend for a significant difference between patients and controls was noted for CD8+ T cells. Of note, patients revealed two different phenotypic responses with five out of 11 patients showing IFN-γ+ cell frequencies much lower than those of other patients for at least one or more CD4+ or CD4-CD8- T cell subsets. These findings suggested that IFN-γ release as a T cell function was spared in a moderate proportion of patients (6/11) whereas this specific function was affected in a subset of patients. Our findings therefore slightly differed from results of the one other report describing IFN-γ release after mitogen stimulation in canine melanoma patients which revealed significantly lower frequencies of IFN-γ+ cell frequencies for patients compared to controls (58). Conditions in this previous report utilized PMA and ionomycin for stimulation and therefore direct comparisons of the two studies are not possible. The patient cohort in our study is too small to determine the relevance of this dysfunctional response to disease progression or as a prognostic marker. In summary, assay of additional T cell functions including induction of release of other cytokines and perhaps using different stimulation protocols, should be examined in larger patient cohorts in future studies to further characterize T cell functions as biomarkers in canine melanoma patients.

Characterization of regulatory (FoxP3+), activation (CD25+FoxP3-), proliferation (Ki67+), and functional (IFN-γ+) markers for different canine T cell subsets was not a goal of these studies. However, analysis of healthy dog controls within these studies allowed this type of analysis for canine T cells. The CD4-CD8- T cell subset in dogs was described previously as an activated subset that demonstrates significantly higher frequencies of CD25+ cells (44) compared to CD4+ and CD8+ T cells. This finding was also observed in our studies with a large range of values for frequencies of CD25+ cells within the CD4-CD8- T cell subset, but with a median frequency well above that of CD4+ T cells. Likewise, higher frequencies of CD25+FoxP3- cells were detected for the CD4-CD8- T cell subset when compared to CD4+ and CD8+ subsets. No significant difference between frequencies of FoxP3+ cells within the CD4-CD8- and CD4+ subsets was detected by our results or by Rabiger et al. Moreover, CD4+ and CD4-CD8- T cell subsets demonstrated similar

patterns for increased Ki67+ cell frequencies within FoxP3+ populations compared to FoxP3- populations including the CD25+FoxP3- population. Higher frequencies of Ki67+ cells within circulating Tregs compared to FoxP3-CD4+ T cells have also been reported in humans along with the observation that Treg populations are highly proliferative (78, 79). Furthermore, another report identified Ki67+ cell frequencies within the Treg as a negative prognostic factor (80) for human ovarian cancer. However, a comparison of IFN-γ+ cell frequencies between T cell subsets after ConA stimulation revealed frequencies for the CD4-CD8- subset were comparable to CD8+ T cells and significantly lower than those for CD4+ T cells. This finding revealed a property that distinguished the CD4-CD8- T cells from CD4+ T cells. With the exception of IFN-γ expression, our studies suggest that canine CD4-CD8- T cells share similar phenotypes with CD4+ T cells although the expression of CD25, typically a property of CD4+ T cells, was significantly higher for this subset. It is important to note that the CD3 monoclonal antibody used in these studies detects the epsilon domain of CD3 (CD3ε) and therefore detects both TCR-αβ+ and TCR-γδ+ T cell subsets. Furthermore, the CD3ε domain has been reported to be expressed on a small subset of human natural killer (NK) cells (81–83), whereas canine NK cells have been characterized as TCR-αβ+ CD3+CD5-low cells (84–87). Accordingly, the CD4-CD8- CD3+ population detected in our studies may include multiple populations including both TCR-αβ+ and TCR-γδ+ T cells, NK cells and possibly NKT cells. Gene expression analysis of the canine T cells subsets will be necessary to further distinguish CD4-CD8- T cell from the conventional CD4+ and CD8+ subsets. Lastly, analysis of IFN-γ+ frequencies after stimulation within CD4+ T cell subsets revealed frequencies within the CD25+FoxP3- population, that were significantly higher compared to FoxP3+ populations and also higher than CD25-FoxP3- CD4+ cells. These findings were not unexpected based on other reports describing restricted IFN-γ expression by Tregs in other species and instead Tregs typically secrete regulatory immunomodulators including IL-10 and Transforming growth factor b (TGF-b) (88–90). However, these results further defined CD25+FoxP3- T cells as an activated population unique from canine Treg, and also as a T cell population of interest for further investigation in canine cancer by both gene expression analysis and an expanded examination for additional T cell markers.

Selection of T cell markers utilized in panels for these studies was based on availability of canine cross-reactive antibodies available and also reported in the literature at the time the project was initiated. Based on reports in humans and dogs, markers for regulatory T cells (FoxP3 and CD25), proliferation/activation, (Ki67), (granzyme B), and functionality and T cell programming (IFN-γ) were feasible for a single panel and provided a diverse set of parameters for a preliminary assessment of T cell phenotyping of canine melanoma patients and controls. Limitations of the selected markers are acknowledged and additional T cell markers currently available would include eomes (34), PD-1 (25, 91, 92), T-bet and GATA-3 (44), memory markers CD62L and CD45RA (33) and additional other markers including cytokines including such as TNF-α.

The tumor and patient information compared to T cell phenotypes was examined by PCA which provided an additional strategy for addressing the basic question of whether canine melanoma patients display a T cell phenotye that is different from healthy dogs. However, PCA precludes cases where a single component of data is absent (example: mitotic index) and related to immunophenotype, exclusion of a marker for which only a limited number of samples were assayed (Ki67 missing for 50% of controls). Additionally, the canine melanoma case cohort overall was small and low in numbers for less progressive melanoma phenotypes which hindered analysis for correlations of immunophenotypes with survival or disease progression. An analysis of progression or survival was also not possible in this survey study as some of the subjects had no treatment and those that did undergo treatment had a variety of therapies. Accordingly, analysis of individual T cell phenotypes also did not reveal significant differences for different clinical parameters. Despite these limitations PCA determined an association between immunophenotype and melanoma patients with metastasis, which should be explored in further studies.

Importantly PCA determined that melanoma patients displayed a unique T cell phenotype by analysis of both unstimulated and stimulated T cell populations when compared to healthy control and provided further support of our overall hypothesis. Furthermore, findings from analysis of individual T cell immunophenotypes determined by flow cytometry revealed specific phenotypes defined by both regulatory and activation markers that distinguished canine melanoma patients and healthy controls. These results represent as survey investigation of canine melanoma patients with an array of T cell markers that has not previously been reported when tested in combination. As such, these findings will direct future assessment of a larger canine melanoma patient cohort with flow cytometry panels that accommodate additional T cell markers to further define specific T cell subsets as biomarkers for tumor stage, disease progression and response to specific immunotherapeutics.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by UC Davis IACUC and Clinical Trials Review Board. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MK, ES, RR, and AM contributed to conception and design of the study. ES, MK, HC, HK, SW, and RC contributed to flow cytometry panel development. ES, NC, and MK performed the statistical analysis. ES and MK wrote the first draft of the manuscript. HC and NC wrote sections of the manuscript. All

authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.772932/full#supplementary-material

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Molecular and Immunohistochemical Expression of LTA4H and FXR1 in Canine Oral Melanoma

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Oral melanoma is a common canine tumor whose prognosis is considered ominous, but poorly predicted by histology alone. In the present study the gene and protein expression of Leukotriene A4 hydrolase (LTA4H) and Fragile-X-mental retardation-related protein1 (FXR1), both reported as related to metastatic potential in different tumors, were investigated in canine oral melanoma. The main aim of the study was to confirm and quantify the presence of LTA4H and FXR1 genes and protein in oral melanomas. A secondary aim was to investigate their association with histologic prognostic criteria (mitotic count, Ki-67 index). Formalin-fixed-paraffin-embedded canine oral melanomas (36) were collected and histopathological evaluation carried out. Immunolabelling for LTA4H and FXR1 and Ki-67 were performed. RT-PCR evaluated LTA4H and FXR1 gene expressions. Histologically, most tumors were epithelioid cell melanomas (19/36) and were amelanotic, mildly or moderately pigmented (5, 12 and 13/36 respectively), only 6 were highly pigmented. Mitotic count ranged 1-106, Ki-67 index ranged 4.5–52.3. Thirty-two (32/32) melanomas immunolabelled for LTA4H and 33/34 for FXR1. RT-PCR values ranged 0.76–5.11 ΔCt for LTA4H and 0.22–6.24 ΔCt for FXR1. Molecular and immunohistochemical expression of both LTA4H and FXR1 did not statically correlate with mitotic count or Ki-67 index. The present study demonstrates LTA4H and FXR1 gene and protein in canine oral melanoma, however their expression is apparently unrelated to histopathologic prognostic criteria. Although LTA4H and FXR1 seem unrelated to tumor behavior, their extensive expression in the present cohort of cases suggest that they may play a role in canine oral melanoma oncogenesis.

Keywords: dog, oral melanoma, LTA4H, FXR1, immunohistochemistry, prognostic markers

INTRODUCTION

Melanoma is a common neoplasia in dogs, that can be a diagnostic and prognostic challenge. Melanoma represents about 3% of all canine tumors and 7% of all canine malignant tumors. It originates in the oral cavity (62%), the skin (27%), the digit (6%) and, less commonly in the eye (5%) (1, 2). Melanoma is the most common malignant tumor of the oral cavity in dogs (3).

Oral melanomas are traditionally considered malignant, rapidly growing, invasive tumors that often recur after surgical resection and frequently metastasize, via lymphatic or blood vessels, to

regional lymph nodes, lungs and viscera (2, 4). In dogs, the reported average survival time after diagnosis of oral melanoma spans 5–7 months (1, 5, 6). However, a subset of oral melanocytic tumors with a more favorable clinical course and prolonged survival have been reported, mainly in dogs with histologically well-differentiated melanocytic neoplasms (6–10). Several studies evaluated histological and immunohistochemical prognostic markers and possible threshold values to define morphological standards (6–8), but obtained different or even conflicting results. Currently, a parameter to effectively predict the likely progression of the disease in individual dogs is still not available.

Recent studies both in human and veterinary medicine have focused on finding genetic markers that may have a good predictive value, with specific emphasis on the potential development of metastatic disease (11–14). The studies of Onken and Malho investigated genetic biomarkers of uveal melanoma in humans and dogs, respectively (11–13). They highlighted the existence of different molecular classes of expected survival based on the differential expression of a set of genes. Among them, an increased expression in Leukotriene A4 hydrolase (LTA4H) and Fragile X mental retardation-related protein 1 (FXR1) genes were related to metastasizing behavior in both human and canine uveal melanoma.

LTA4H is a cytosolic hydrolytic enzyme, which catalyzes the conversion of leukotriene A4 into leukotriene B4 inside the arachidonic acid cascade. LTA4H expression is widely distributed in several different tissues (15). Leukotrienes are mediators of inflammation and chronic tissue inflammation has been linked to increased risk for the development of cancer (16). Aberrant arachidonic acid metabolism is suspected to have a role in carcinogenesis due to the imbalance shifted toward the pro-carcinogenic lipoxygenase pathways (5-, 8- and 12-LO) instead that anti-carcinogenic (15-LO) (16, 17). *LTA4H* has been previously described to be over-expressed in different types of tumors in humans, mice, rats, dogs and cats (18–28). *LTA4H* was also specifically demonstrated to be overexpressed in human, canine and feline ocular melanomas (10, 12, 28).

FXR1 is a cytoplasmic RNA binding protein, highly conserved among vertebrates and expressed in different tissues. FXR1 belongs to a family of RNA binding proteins consisting of the Fragile X Mental Retardation Protein (FMRP), responsible for the human fragile X mental retardation syndrome, and the Fragile X Mental Retardation Syndrome-Related Protein 2 (FXR2) (29, 30). FXR1 acts in the inflammatory process controlling the expression of tumor necrosis factor-α (TNF-α) at a post transcriptional level (31, 32) and has a role in muscular cells development (33). Moreover, its role has been investigated in different tumors such as squamous cell carcinoma (34), pulmonary carcinomas (35, 36), colorectal cancer (37), prostate cancer (38) and Wilms tumor (39). FXR1 is supposed to affect DNA stability with two pathways (40), either using miRNA pathway to regulate target mRNA expression (41) or playing a role in post-transcriptional regulation directly interacting with mRNA and affecting its stability (33). In lung tumorigenesis, for example, FXR1-dependent regulation of mRNA may specifically regulate ERK (extracellular-signal-regulated kinases) signaling pathway (36). Moreover, FXR1 recruits transcription factor STAT1 or STAT3 to gene promoters and, through the regulation of transcription, mediates cell proliferation in human cancers harboring TP53 homozygous deletion (42).

Our study aimed to investigate the expression of LTA4H and FXR1 in canine oral melanoma, at both genetic and protein level. A secondary aim of the study was to identify possible correlations between the expression of LTA4H/FXR1 and statistically proven histologic prognostic parameters, i.e., mitotic count and Ki-67 index.

MATERIALS AND METHODS

Cases and Histopathology

Oral melanomas samples ($n = 36$) were collected and routinely formalin-fixed and paraffin-embedded (FFPE) (43) from the diagnostic service of histopathology at the University of Milan and the private San Marco Laboratory in Padova (Italy) during the period 2011–2017.

Four micrometer-thick sections of each sample were stained with hematoxylin and eosin (H&E) and evaluated on light microscope by two board certified pathologists (LN, CG). Mitotic count was calculated as the number of mitoses on 10 consecutive high power fields, starting in the area (0.237 mm^2) with the highest mitotic activity (7).

Pigmentation of melanomas was semi-quantitatively evaluated on the whole section as: "negative/–" when no pigment was detectable, "mild/+" when 1 to 10% of neoplastic cells contain pigment, "moderate/++" 10 to 50% of cells were pigmented, and "high/+ + +" when >50% of cells were pigmented. In samples with no detectable pigment, the diagnosis of melanoma was confirmed through anti-PNL2 immunohistochemistry (see following section) (5, 44).

Additionally to the FFPEs, out of the 36, fresh samples were collected in double (two melanoma plus the adjacent not affected tissue) and stored in RNA later at −80°C for the subsequent molecular genetic analyses.

Immunohistochemical Detection of LTA4H, FXR1, Ki-67, and PNL2

Serial paraffin sections were cut 4 μm thick and mounted on poly-lysine coated slides (Menzel-Gläser, Braunschweig, Germany). Immunohistochemical staining with the standard avidin-biotin-peroxidase complex (ABC) method was performed.

Briefly, sections were deparaffinized in xylene and rehydrated through a descending series of ethanol concentrations. Incubation with H_2O_2 10% in Tris buffer saline (TBS; pH 7.4) for 2 h at 55°C in a laboratory stove was used to bleach heavily pigmented sections and to block endogenous peroxidase activity (45). In the set up phase of the protocol, selected samples of pigmented oral melanomas were treated with and without bleaching to assess if immunoreactivity was maintained.

Antigen retrieval was then performed by heating the slides in citrate buffer solution (pH 6.5) in a water bath at 95°C for 30 min (for LTA4H and FXR1) (46), in citrate buffer solution in pressure cooker for 18 min (for Ki-67) (47) and in microwave oven in EDTA buffer solution (pH 8.5) for 10 min at 500 W

(for PNL2) (44), followed by cooling down in buffer at room temperature (RT) for 30 min. Sections were therefore incubated for 20 min at RT with normal horse (LTA4H, Ki-67, PNL2) or goat (FXR1) serum (dilution 1:70) to block any non-specific protein binding and therefore incubated with primary antibodies at 4°C overnight in a humidified chamber:

- mouse monoclonal leukotriene A4 hydrolase/LTA4H antibody (1E9 clone) (NBP1-47829; Novus Biologicals, Littleton, Co, USA), 1:100 dilution;
- rabbit polyclonal anti-FXR1 antibody (ab50841; Abcam, Cambdrige, UK), 1:100 dilution;
- mouse monoclonal anti-human Ki-67, clone MIB-1 (GA626; Dako, Glostrup, Denmark;), 1:600 dilution;
- mouse monoclonal anti Melanoma-Associated Antigen, clone PNL2 (MON3307; Monosan, Uden, Netherlands), 1:50 dilution.

Sections were then rinsed in TBS three times for three minutes each and incubated with the secondary anti-mouse (LTA4H; Ki-67; PNL2) or anti-rabbit (FXR1) biotinylated antibody (1:200, 30 min RT) (Vector Laboratories, Burlingame, CA, USA, BA 1000 and BA 2000, respectively) followed by rinsing in TBS and therefore incubation with the ABC reagent (30 minutes RT) (PK-6100; Vector Laboratories, Burlingame, USA). After rinsing in TBS, the 3-amino-9-ethylcarbazole (AEC) chromogen (SK-4200; Vector Laboratories, Burlingame, CA, USA) was applied for 15 min and, after rinsing in tap water, slides were counterstained with Mayer's haematoxylin (SS C030X; Diapath srl, Martinengo, Italy) for 2 min. Slides were therefore dried and mounted in aqueous mounting agent (108562; Aquatex, Merck, Darmstadt, Germany).

Canine skeletal myocytes adjacent to the neoplasm, and neutrophils were adopted as internal positive controls for FXR1 and LTA4H immunolabelling, respectively (46, 48). Epithelium of intestinal crypts in a section of normal canine intestine was adopted as positive control for Ki-67, a section of partially pigmented canine melanoma was adopted as positive control for PNL2. Negative controls were carried out by replacing the primary antibodies with mouse or rabbit IgG (Santa Cruz; Dallas, TX, sc-2025 and sc2027, respectively).

Immunolabelled sections were evaluated at optic microscope. LTA4H and FXR1 labeled sections were semi-quantitatively scored, considering the percentage of positive cells (<10, 11–30, 31–50, 51–70, >70%), cellular localization of the signal (nuclear/cytoplasmic), intensity of staining (mild, moderate, intense). The immunoreactive score adapted from Remmele and Stegner (IRS score) (49) was calculated combining the intensity and percentage of positivity as follows: IRS score (0–12) = percentage of positive cells (no positive cells = 0, 1–10% positive cells= 1, 11–50% of positive cells= 2, 51–70% of positive cells= 3, >70% of positive cells = 4) * intensity of staining (no positive cells = 0, +/mild = 1, 2+/moderate = 2, 3+/marked = 3). For statistical purposes, IRS scores were further categorized into three cumulative classes of expression, as follows: class 1 (mild expression) corresponded to IRS scores 0–3, class 2 (intermediate expression) to IRS scores 4–8 and class 3 (marked expression) to IRS scores 9–12.

Ki-67 index was calculated as the mean number of positively labeled neoplastic cell nuclei in 5 fields at 400× (7). The number of positive nuclei was counted using a digital automatic counter (QCapture Pro 6.0). PNL2 positivity was evaluated as presence or absence of the signal.

Relative Expression of *LTA4H* and *FXR1*

Total RNA was extracted from eight sections 10 μm-thick cut from each FFPE tissue block using the RecoverAll Total Nucleic Acid Isolation Kit (AM1975; Thermofisher Scientific; Massachusetts, USA) and from frozen fresh samples using RNeasy Minikit (74104; Qiagen; Hilden, Germany). RNA was eluted in a final volume of 60 μl of water after an on-column DNase treatment (Qiagen; Hilden, Germany), quantified using NanoDrop ND-100 Spectrophotometer (Thermofisher Scientific; Massachusetts, USA) and immediately stored at −80°C until molecular analyses.

Two hundred nanograms of RNA were retro-transcribed to cDNA using the QuantiTect Reverse Transcription kit (205311; Qiagen; Hilden, Germany) following the manufacturer's protocol. An additional reaction without retrotranscriptase enzyme was performed to verify the complete DNA removal. cDNAs were stored at −80°C until subsequent use.

Amplification condition, the dynamic range and the efficiency of each qPCR reaction were assessed on cDNA produced from a fresh tissues. Furthermore, the integrity of the RNA extracted from FFPE samples was verified through the amplification of a fragment of *B2M* gene from all cDNA.

The relative expression of *LTA4H* and *FXR1* genes for each FFPE was calculated after a ΔCt measure using the *B2M* and *ACTB* genes as references. The synthesized cDNA samples were amplified in duplicate using the iQ5 Real-Time PCR instrument (Bio-Rad, California, USA) and Universal SYBR® Green Supermix (1708880; Bio-Rad, California, USA) as fluorescent molecules. Primers sequences were described by Malho and coauthors (11) and the final concentration of forward and reverse primers was 250 nM for *FXR1*, *B2M* and *ACTB* and 400 nM for *LTA4H* genes, respectively (**Supplementary Table 2**). The thermal profile was 98°C for 30 s, 40 cycles of 98°C for 15 s, 50–58°C for 15 s, 72°C for 15 s and a melting profile was included after the last amplification cycle in order to exclude the presence of aspecific amplifications. Cycle threshold (Ct) values of each target were determined for each sample to estimate ΔCt measure.

Statistical Analysis

Molecular and immunohistochemical levels of LTA4H and FXR1 were compared between tumors with low (<19.5) and high Ki-67 index (≥19.5) and tumors with low (<4) and high (≥4) mitotic count, according to the prognostic thresholds proposed by Bergin and colleagues (7). Adopted statistical tests were the Mann Whitney U test and the Kruskal-Wallis test (p <

0.05). Correlation between immunohistochemical IRS scores and molecular ΔCt was assessed by the Spearman Rho test ($p < 0.05$).

RESULTS

Case Selection and Histology

Thirty-six specimens of oral melanomas from 32 dogs were included in the study (two samples were recurrence and one was a necropsy sample of cases already included in the study, and one dog had two distinct masses in different sites of the oral cavity). Dogs belonged to different breeds and ranged 5–18 years of age (mean 11.8, median 12). Fifteen animals were male, fifteen were female, and for two animals sex was not reported. When the specific site of the tumor was provided, oral melanomas were located in the gum ($n = 10$, 27.8%), labial mucosa ($n = 5$, 13.9%), palate ($n = 2$, 5.6%) and tonsillary region ($n = 1$, 2.8%).

Histologically, melanomas exhibited different morphological types (epithelioid, spindle or mixed) and various degree of pigmentation. Nineteen tumors (52.8%) were classified as epithelioid, nine (25%) as spindle and eight (22.2%) as mixed. Pigmentation was evaluated as absent in 5 tumors (13.9%), mild/+ in 12 (33.3%), moderate/++ in 13 (36.1%) and high/+++ in 6 (16.7%). Mitotic count ranged 1–106 (mean 23.8, median 15). Five tumors had a mitotic count <4, whereas 31 had mitotic count ≥4 (**Supplementary Table 1**).

Immunohistochemical Expression of LTA4H, FXR1 and Ki-67

Of the originally selected 36 cases, 2 cases were excluded from the immunohistochemical results because they were poorly reactive, and 2 cases were excluded from the anti-LTA4H staining because no further material was available in the paraffin block. Immunoreactivity was of equal intensity in the preliminary subset of samples treated with and without bleaching, confirming the maintenance of immunoreactivity after the bleaching protocol (**Supplementary Table 1**).

Thirty-two melanomas were LTA4H-positive (32/32) (**Figures 1A,B**), with a percentage of positive neoplastic cells of 31–50% in 2 cases, 51–70% in five and above 70% in 25 cases. The intensity of staining was from mild to intense. The localization of the positivity was cytoplasmic ($n = 19$), nuclear ($n = 3$), or both nuclear and cytoplasmic ($n = 10$). IRS score varied from 3 to 12. 18/32 cases (56.3%) were classified as IRS score class 2, 14/32 cases (43.8%) as class 3.

FXR1 immunolabelling stained positive in 33/34 tested oral melanomas (**Figure 1C**). The percentage of stained cells was <10% in 6 cases, between 11 and 30% in 5 cases, between 51 and 70% in 9 and >70% in 8 cases. Fourteen cases were intensely stained, 13 moderately and 6 mildly. The immunostaining was always cytoplasmic. IRS score varied from 0 to 12. 6/34 cases (17.6%) were classified as IRS score class 1, 13/34 cases (38.2%) as class 2 and 11/34 cases (32.4%) as class 3.

Ki-67 index was assessed in 28/34 cases, due to insufficient paraffin block material. The average number of positive neoplastic nuclei ranged 4.5–52.3, with a mean of 16.9 and a median of 13.7. Ki-67 index was beneath 19.5 in 19 cases and above 19.5 in 9 cases.

The comparison of groups with low and high Ki-67 index (<19.5 and ≥19.5) with the levels of immunohistochemical scores of LTA4H and FXR1 revealed no statistically significant differences ($p = 0.212$ and 0.138, respectively). Groups with low and high mitotic count (<4 and ≥4) had no significantly different levels of immunohistochemical FXR1 ($p = 0.153$) but had significantly different IRS scores of LTA4H ($p = 0.017$). The intracellular localization of LTA4H immunohistochemical signal (nuclear, cytoplasmic, or nuclear and cytoplasmic) showed no statistical correlation with LTA4H IRS score ($p = 0.532$).

LTA4H and *FXR1* Gene Expression

Thirty-six FFPE cases of canine oral melanoma were analyzed with qPCR to quantify the expression of the target genes *LTA4H* and *FXR1* (**Supplementary Table 1**).

B2M and *ACTB* were preliminary tested as housekeeping genes in a subset of 10 samples, to assess if the expression trend was stable. Despite the presence of a difference in the threshold cycles (Ct) of *B2M* and *ACTB* (paired *t*-test: $t9 = 8.48$; $p < 0.0001$), the relation between the two genes expression was linear ($p < 0.0001$), thus indicating that the ratio of expression between *B2M* and *ACTB* was constant. The Ct of these two housekeeping genes were related by the equation:

$$Ct\ B2M = 7.3 + .82^{*} Ct\ ACTB\ (R2 = 0.92).$$

Therefore, *B2M* was chosen as housekeeping gene to complete the analyses of the samples in order to quantify the relative expression of the investigated target genes.

Among the analyzed samples, RNA extraction suitable for cDNA synthesis and qPCR assays was obtained in twenty-three out of thirty-six cases and in these samples *LTA4H* and *FXR1* were successfully amplified. Gene expression results, reported as ΔCt, comparing the Ct of the target gene with the Ct of the housekeeping gene, were ranged 0.76–5.11 for *LTA4H* and 0.22–6.24 for *FXR1*. In three samples the amplification of *FXR1* gene was not quantified due to the Ct value being out of the dynamic range of the reaction.

Molecular expression of both *LTA4H* and *FXR1* genes did not differ among groups with low and high Ki-67 index (<19.5 and ≥19.5) (*LTA4H* $p = 0.502$, *FXR1* $p = 0.635$), nor among groups with low and high mitotic count (<4 and ≥4) (*LTA4H* $p = 0.501$, *FXR1* $p = 0.140$). *LTA4H* molecular and immunohistochemical values were correlated ($p = 0.014$, rho = −0.539), but *FXR1* was not ($p = 0.122$, rho = 0.337) (**Figure 2**).

DISCUSSION

In the present study, a caseload of canine oral melanomas was investigated for the expression of two targets, i.e., LTA4H and FXR1, at both gene and protein level.

Anamnestic and histological data of dogs included in the study were mostly consistent with previous literature reports: dogs were equally distributed among sexes and variably distributed among breeds, with a preponderance of mixed breed. Adult-old aged dogs were most represented (mean 11.8 years of age) (7, 50) and tumors mainly affected the gum (2). Consistently with

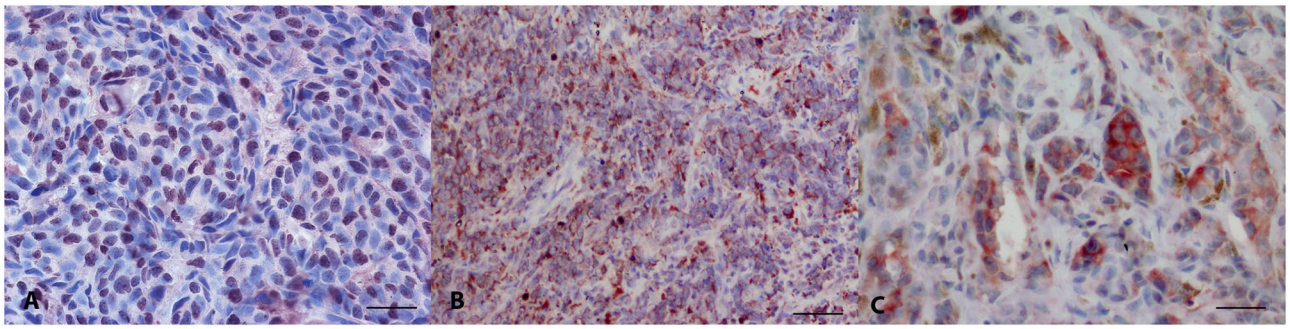

FIGURE 1 | Canine oral melanoma, immunohistochemistry. **(A)** Anti-LTA4H staining, diffuse nuclear signal. AEC chromogen, bar 50 μm. **(B)** Anti-LTA4H staining, diffuse cytoplasmic signal. AEC chromogen, bar 100 μm. **(C)** Anti-FXR1 multifocal intense signal. AEC chromogen, bar 50 μm.

literature (1–5, 8), neoplastic cell histo-morphology was highly variable, with epithelioid cell type predominantly observed, and in the vast majority of cases <50% of neoplastic cells were pigmented Although a "high" degree of pigmentation (at least 50% of pigmented cells) has been correlated with longer survival times in canine oral melanomas, the prognostic significance of the degree of tumor pigmentation is still debated, most likely due to the subjectivity in the quantification of the pigment (7, 8).

The main aim of the present study was to investigate the occurrence of LTA4H and FXR1 genes and proteins in canine oral melanoma. *LTA4H* and *FXR1* have been previously found to be over-expressed in both human and canine uveal melanomas (11, 12, 14) and *LTA4H* has also been reported to be upregulated in feline ocular melanomas (28). Additionally, the immunohistochemical expression of FXR1 protein has been demonstrated in a small caseload of canine uveal and oral melanocytic tumors (46).

In the present cohort of cases, LTA4H and FXR1 were detected at both gene and protein levels. The qPCR relative expression of *LTA4H* and *FXR1* genes exhibited marked differences among tested cases of oral melanoma, conversely immunohistochemical positivity showed negligible expression differences. Comparing immunohistochemical results, expressed as IRS score, with qPCR results, expressed as ΔCt, a correlation among protein and gene expression was significant only for LTA4H. This discrepancy between immunohistochemical and qPCR results can have different explanations. First, immunohistochemistry, although useful in providing information concerning the localization of antigens, is not a quantitative technique, as opposite to RT-PCR. Second, protein tissue expression does not instantly reflect possible alterations during the synthesis process, which can take place from transcription to post-translational modifications, i.e., protein expression does not necessarily mirror gene expression. Third, while the use of FFPE tissues allows to enroll archive specimens, formalin ficxation procedure can induce degradation of the tested molecules. However, in the present caseload, DNA quality was assessed through the amplification of house-keeping genes and only samples in which the RNA extraction was considered suitable for cDNA synthesis and qPCR assays were further included in the study. Finally, molecular analyses may be influenced by residual constitutive expression of genes in normal adjacent tissues or extracellular matrix.

The secondary aim of the present study was to evaluate the possible association LTA4H and FXR1 genes and proteins with established histologic prognostic criteria, i.e., mitotic count and Ki-67 index (7, 42), in order to test if LTA4H and FXR1 presence and level of expression could be related to the biological behavior of the tumor.

Data on the expression of the investigated targets were compared with mitotic count, and no difference between melanomas with mitotic count < or > 4/10 HPF (8) was observed for FXR1 neither at gene nor at protein levels, while a significantly difference was observed for LTA4H protein but not gene expression No statistically significant association was observed between LTA4H and FXR1 when their gene and protein expressions were compared with Ki-67 index (adopting a value of 19.5 as a discriminating threshold) (7).

Overall, the results of this study indicate that LTA4H and FXR1 are extensively expressed in canine oral melanomas and suggest that they can play a role in tumor oncogenesis process. However, their gene and protein expressions resulted poorly related to histological prognostic criteria suggesting that they are not associated to canine oral melanoma biological behavior, conversely to what has been demonstrated in canine and human uveal melanomas.

It should be noted that, in previous studies, the overexpression of *LTA4H* and *FXR1* genes has been specifically associated with increased metastatic risk of uveal melanoma, while in the present study LTA4H and FXR1 were compared with histopathologic prognostic criteria, but not specifically with the presence of metastases. In this regard, it is worth considering that canine uveal and oral melanocytic tumors are characterized by different biological behavior. Indeed, while uveal melanomas are generally slowly progressive and slowly metastasizing tumors, oral melanomas are aggressive tumors with rapid progression and frequently already metastasized at the time of diagnosis (2, 4, 51, 52). Therefore, the more homogeneous, rapidly aggressive, behavior of canine oral melanomas enrolled in the study

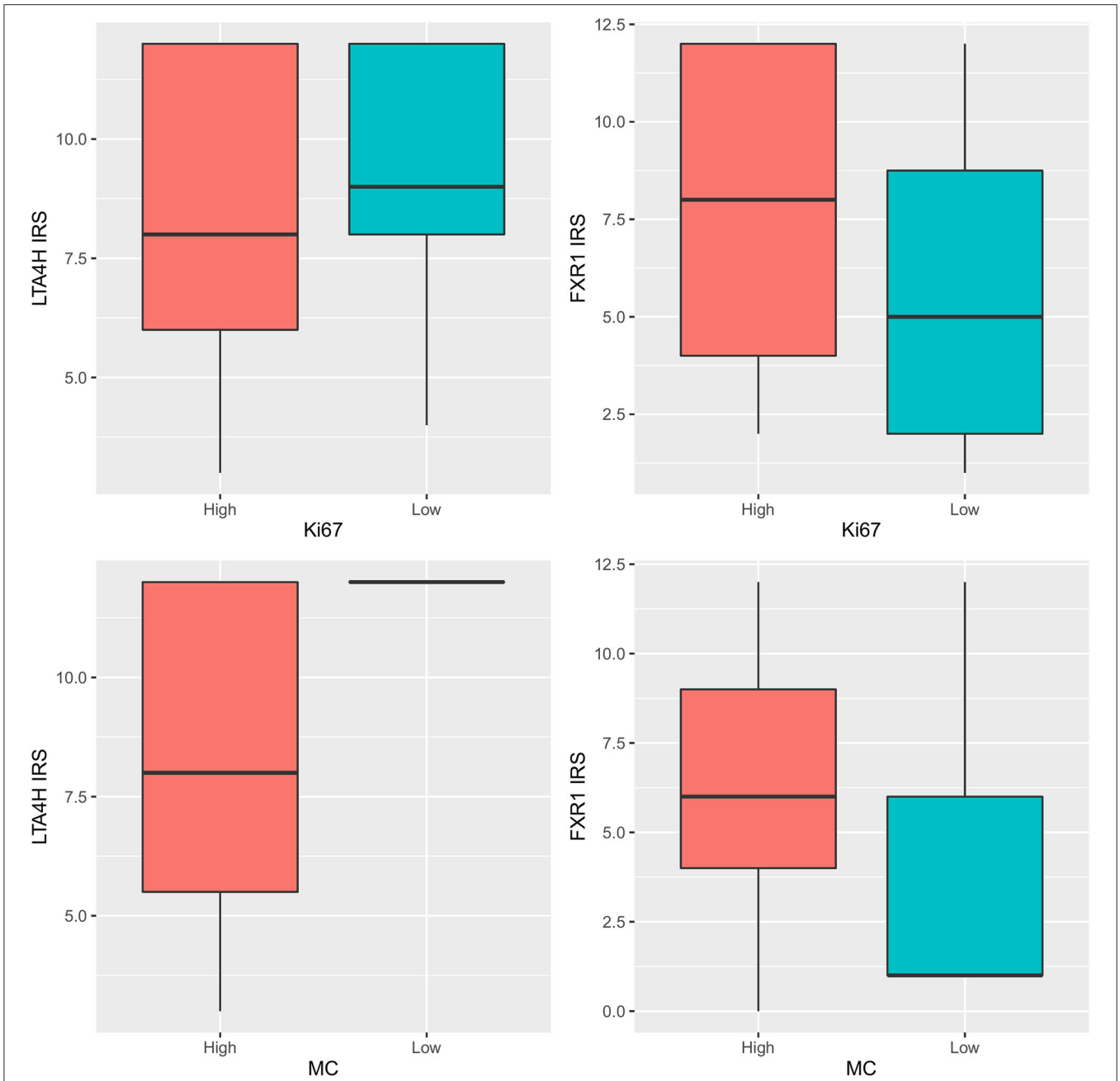

FIGURE 2 | LTA4H and FXR1 IRS scores: comparison among groups with low and high Ki-67 index (upper row) (upper left $p = 0.212$, upper right $p = 0.138$) and among groups with low and high mitotic count (MC) (lower row) (lower left $p = 0.017$, lower right $p = 0.153$).

may affect the lack of discriminating value of the markers that we investigated.

In conclusion, in the present study the expression of LTA4H and FXR1 has been verified in canine oral melanoma at both gene and protein levels, and even though we failed to identify a significant correlation with known histopathologic criteria of prognosis, further investigations on a larger cohort of cases and possibly including follow up data are necessary to define the role of FXR1 and LTA4H in the pathogenesis of canine oral melanoma.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this study did not include experimental animals, it was performed on histological specimens. All animal owners signed an informed consensus for clinical procedures and data recording and using for studies.

AUTHOR CONTRIBUTIONS

LN and CG performed the histology and immunohistochemistry. LN, CG, and CB wrote the first draft of the manuscript. MR and DS contributed to case selection. CB, FG, VS, and ML performed the molecular analyses. GF performed the statistical analysis. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.767887/full#supplementary-material

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Canine Melanoma Immunology and Immunotherapy: Relevance of Translational Research

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In veterinary oncology, canine melanoma is still a fatal disease for which innovative and long-lasting curative treatments are urgently required. Considering the similarities between canine and human melanoma and the clinical revolution that immunotherapy has instigated in the treatment of human melanoma patients, special attention must be paid to advancements in tumor immunology research in the veterinary field. Herein, we aim to discuss the most relevant knowledge on the immune landscape of canine melanoma and the most promising immunotherapeutic approaches under investigation. Particular attention will be dedicated to anti-cancer vaccination, and, especially, to the encouraging clinical results that we have obtained with DNA vaccines directed against chondroitin sulfate proteoglycan 4 (CSPG4), which is an appealing tumor-associated antigen with a key oncogenic role in both canine and human melanoma. In parallel with advances in therapeutic options, progress in the identification of easily accessible biomarkers to improve the diagnosis and the prognosis of melanoma should be sought, with circulating small extracellular vesicles emerging as strategically relevant players. Translational advances in melanoma management, whether achieved in the human or veterinary fields, may drive improvements with mutual clinical benefits for both human and canine patients; this is where the strength of comparative oncology lies.

Keywords: canine melanoma, immunotherapy, vaccination, CSPG4, comparative oncology

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INTRODUCTION

The interplay between the immune system and cancer has been widely investigated for over a century and has provided the groundwork for the emerging field of research known as immuno-oncology. In this setting, immunotherapy aims to exploit the immune system to change a patient's fate toward cancer eradication and has seen outstanding positive results.

Just like humans, dogs naturally develop a multitude of diseases, including cancer, with six million of new diagnosis each year in the USA alone (1). Developing over a long-time period in an intact immune system, complex interactions between a tumor and the immune system can occur in canine patients, as they do in humans, making cancer cells susceptible to the selective pressure of spontaneous immunity. The similarities between cancer in dogs and humans, and the recent success of immunotherapy in human oncology, have increased enthusiasm for applying it to the treatment of canine cancer. However, advancements in canine medicine are still running behind

human clinics, and efforts to develop immunotherapies for dogs are still limited (2). A deeper investigation of the canine immune system and its response to evolving cancers would speed up the development and successful application of immunotherapy in tumor-bearing dogs.

Some of the most relevant comparative aspects of melanoma immunology and immunotherapy will be discussed in this perspective.

Finally, despite several in-human studies have already highlighted the role of small extracellular vesicles (SEVs), including exosomes (EX), in disease progression and their potential as biomarkers in liquid biopsies for diagnosis and therapy, this is a relatively new topic that requires more work before reaching clinical applicability. At the same time, the research in veterinary medicine is still at an early stage, although the potential of SEVs also in this setting is clear (3). As a step forward in this direction, a mention to preliminary examples of the potential of SEVs as promising non-invasive diagnostic and prognostic tools in the melanoma precision veterinary medicine has been added, with potential implications also for the human medicine.

CANINE ORAL MALIGNANT MELANOMA

Malignant melanoma is among the most common cancers in dogs (4, 5). The most diffuse and fatal subtype is oral malignant melanoma (OMM), which accounts for 30–40% of all canine oral malignancies (6, 7) and 20.3 cases per 100,000 dogs per year (4, 5). OMM is characterized by local invasiveness and high metastatic propensity (6, 8, 9). Up to 74% of OMM rapidly develop distant metastasis, which are the leading cause of death. The survival time of OMM-affected dogs is very short being of about 200 days after diagnosis (8, 10, 11). Surgery is the first-line treatment of choice for the local control of the tumor and correct surgical excision plays a fundamental role in the outcome of the disease (7). It can be flanked by radiotherapy and/or chemotherapy (12). However, metastatic lesions are generally resistant to chemotherapy (4).

Thanks to the release of the canine genome (13), the annotation and deposition of genomic, transcriptomic, and proteomic data derived from canine neoplastic lesions has improved the characterization of the molecular foundations of canine cancers. Although genetic alterations in OMM have not yet been fully described, the mutation profiles of OMM resemble UV-independent molecular etiology, which are typical of human non-UV-induced cutaneous, mucosal, and uveal melanomas (4, 14–16). The same MAPK and PI3K/AKT/mTOR pathways have been found to be activated in OMM and human melanomas, highlighting the overlap between OMM and specific human melanoma subtypes molecular signature. These acquisitions may have an influence on the clinical management of canine OMM, and the first demonstration is the targeted combination treatments with specific human MAPK and PI3K/AKT/mTOR pathway inhibitors that have recently been tested *in-vitro* in the canine setting (17). Of note, one of the most frequently investigated human-melanoma-associated antigens, chondroitin sulfate proteoglycan (CSPG)4, is expressed by most OMM and appears to play a relevant role in clinical outcome, as discussed below (8, 10, 18–20).

Beyond the molecular background, considering the ever more relevant role of immunotherapy in human melanoma management, an increased attention should be dedicated to the immunological aspects of OMM. While the "immune-phenotyping" of human melanoma has been extensively characterized (21), less data are available for OMM. This could slow the development and the translation of effective immunotherapies to veterinary care. Deeper investigations into canine melanoma immunology could guide the application of immunotherapies that have already been approved for use in humans and the possibility of developing novel strategies that could see long-lasting applications in both clinical settings.

THE IMMUNE LANDSCAPE OF OMM

The OMM immune microenvironment is still widely unexplored. Only recently the interplay between OMM and the immune cells in the tumor microenvironment (TME) has gained attention (22), while several studies have already documented the immunogenicity of human melanoma (23), with a dynamic crosstalk between cells within the TME. Tumor-infiltrating lymphocytes (TILs) are the histopathological reflection of the host's immune response against cancer cells, with the $CD3^+$, $CD4^+$ and $CD8^+$ TILs having a favorable prognostic role in overall survival in the human setting (24).

In a recent study conducted on canine samples, the majority of OMM biopsies were found to be "briskly" infiltrated by different T-lymphocyte subsets (25). A positive correlation between a high TIL level, a high percentage of $CD8^+$ T-lymphocytes infiltrating the tumor and better patient survival was observed, which implies the importance of TIL characterization for predicting tumor aggressiveness and prognosis in melanoma-bearing dogs as well (25).

B cells may also play a relevant part in TIL composition. A recent retrospective study conducted on tissue samples collected from canine melanocytic tumors, including the OMM subtype, revealed that there existed a correlation between higher $CD20^+$ cell infiltration and the risk of metastasis and tumor relapse (22). Worse survival was observed for dogs whose tumor was more highly infiltrated by $CD20^+$ cells, which finally indicates the negative role played by B-lymphocytes in canine melanomas (22). As far as human melanoma is concerned, contradictory roles have

Abbreviations: ADCC, Antibody-dependent cellular cytotoxicity; B7-H3, B7 homolog 3 protein; CD40L, CD40-Ligand; CDC, Complement-dependent cytotoxicity; CSPG4, Chondroitin sulfate proteoglycan 4; CTLA-4, Cytotoxic T-Lymphocyte Antigen 4; DLA, Dog Leukocyte Antigen; EVs, Extracellular vesicles; EX, Exosomes; FDA, Food and drug administration; ICIs, Immune checkpoint inhibitors; Ig, Immunoglobulin; LAG3, Lymphocyte-activation gene 3; LMR, Lymphocyte-to-monocyte ratio; mAbs, Monoclonal antibodies; MDSC, Myeloid derived suppressor cells; NLR, Neutrophil-to-lymphocyte ratio; OMM, Oral Malignant Melanoma; PD-1, Programmed death-1; PD-L1, Programmed death-ligand 1; SEVs, Small Extracellular Vesicles; TAM, Tumor-associated macrophages; TIGIT, T cell immunoglobulin and ITIM domain; TIM3, T cell immunoglobulin and mucin-domain containing-3; TIL, Tumor infiltrating lymphocytes; TME, Tumor microenvironment; Tregs, Regulatory T cells.

been observed for tumor-infiltrating B cells, with both positive and negative correlations with patient clinical outcome being suggested (26–30). Tumor localization and the markers used to detect B cells may be relevant aspects to take into consideration in explaining the discrepancies. A deeper investigation into the role of melanoma-infiltrating B cells in canine and human patients may help in formulating new hypotheses that can be mutually informative in both veterinary and human clinics.

It is also well known that the activation of immunosuppressive cell subpopulations, such as regulatory T cells (Tregs), in human melanoma constitutes an immune-escape mechanism that facilitates tumor growth and progression (31). Starting from this point, a number of studies in veterinary medicine have focused on the characterization of Tregs in canine melanomas (22, 32, 33). An increase in Tregs has been linked to a higher hazard of death in dogs, confirming the connection between Tregs infiltration and worse prognosis (22).

Finally, alterations in peripheral blood leukocytes that mirror systemic inflammation triggered by cancer have already been characterized in human melanoma patients (34). The neutrophil-to-lymphocyte ratio (NLR) and the lymphocyte-to-monocyte ratio (LMR) are prognostic indicators of the evolution of the disease in humans (35, 36), being low LMR counts linked to poorer prognosis in several types of cancers, including metastatic melanoma (37). As such, this aspect is also being investigated in both hematological and solid canine tumors, and LMR is now widely accepted as a prognostic indicator of patients' outcome (38–41). As well, pre-treatment high LMR has been reported to be of significant prognostic value in melanoma-bearing dogs that received anti-PD1 treatment (42), establishing this parameter as a possible indicator of response to immunotherapy. However, neither prognostic nor predictive significance has yet been found for NLR and LMR in a small cohort of canine patients which received anti-CSPG4 immunotherapy (see below), nor any correlations to histological/immunohistochemical parameters of melanoma well-known prognostic factors (43).

Overall, the interest in the contribution of the immune system in shaping the TME in canine patients is growing. The identification of common features in the immunology of human and canine melanoma could help to accelerate the acquisition of novel information that could be exploited to design more effective therapeutic interventions for both settings. Nonetheless, the development of new investigation tools specific for dogs are required to reach the level of knowledge that has been obtained in human oncology.

NOVEL IMMUNOTHERAPEUTIC TARGETS FOR OMM TREATMENT

Many advancements have been made in immunotherapeutic management of melanoma, especially with the introduction of immune checkpoint inhibitors (ICIs). Regardless of the still-high percentage of patients who do not respond to such therapies (44), ICIs are a true breakthrough in human melanoma treatment, strikingly improving the prognosis of responder patients. Hence, veterinary medicine is now shifting attention to the use of ICIs as a potentially effective systemic treatment also for tumor-bearing dogs.

The expression of Cytotoxic T lymphocyte associated protein 4 (CTLA-4), Programmed death-1 (PD-1) and of PD-1 ligand-1 (PD-L1) on canine immune cells and/or cancer cells has already been investigated and reported (45, 46). Chimeric rat-dog anti-PD-1 and anti-PD-L1 (45) and "caninized" anti-CTLA-4 (46) and anti-PD-1 (42, 47) monoclonal antibodies (mAbs) have been developed. Anti-PD-1 mAbs tested in canine cancer patients, including OMM cases, exerted a significant effect on the inhibition of the PD-1/PD-L1 axis in pilot clinical studies and exhibited remarkable anti-tumor activity, resulting in the increased survival of treated dogs, compared to conventionally treated controls (45, 47).

However, no specific ICI therapy has yet been commercially approved for the treatment of dogs. Nevertheless, in line with human findings (48, 49), veterinary medicine is going toward the characterization of other poorly explored immune checkpoint targets to increase the immunotherapeutic armamentarium. The "next generation immune checkpoints" include B7 homolog 3 protein (B7-H3), lymphocyte activation gene-3 (LAG- 3), T cell immunoglobulin and mucin-domain containing-3 (TIM-3), T cell immunoglobulin and ITIM domain (TIGIT), and CD200. These molecules exert a co-inhibitory function, and strictly act by co-operating with CTLA-4 and PD-1/PD-L1 axes to modulate the anti-cancer immune response (48, 49).

As already demonstrated in humans, TIGIT is upregulated on NK cells of dogs with naturally occurring metastatic osteosarcoma after IL-15 treatment, suggesting a successful possible combinatorial approach for treating metastatic tumors in dogs (50). CD200 blocking in high grade glioma-bearing dogs by means of synthetic peptide ligands, has revealed an increased therapeutic efficacy when combined to an autologous tumor cell lysate vaccine (51). Equally, agonistic mAbs targeting co-stimulatory molecules belonging to the tumor necrosis factor receptor superfamily, such as CD27, OX40, and CD40, have shown impressive anti-tumor effects in pre-clinical and clinical studies (52–54). Interestingly, promising results have been obtained in melanoma-bearing dogs treated with an adenovirus encoding the CD40 ligand (CD40L) (55).

Therefore, targeting different checkpoint molecules and/or finding novel combinatorial strategies in patients which do not respond to PD-1/PD-L1 blockade is essential, and could represent a promising approach to achieve a greater therapeutic effect in a variety of both human and canine tumors.

B7-H3 CHECKPOINT MOLECULE AS A TARGET FOR OMM

Among the novel immune checkpoints, B7-H3 has recently emerged as an interesting target (56–59). B7-H3 is a type I transmembrane protein member of the B7-superfamily (60). The human B7-H3 gene codes for four immunoglobulin (Ig)-like domains; two pairs of IgV-IgC. The transcribed RNA can be alternatively spliced to generate two proteins, 4IgB7-H3 (B7-H3b) and 2IgB7-H3 (B7-H3) (61). B7-H3 has conserved its

amino acid sequence throughout evolution (61, 62) and both the 4Ig- and 2Ig-B7-H3 isoforms are expressed in other species besides humans, including dogs (61, 63). Furthermore, there is 94% amino acid homology between the dog and human B7-H3 sequences (64, 65).

B7-H3 has been suggested to play both a co-stimulatory and inhibitory role in human tumor immunity, depending on the context (62, 66–70). In parallel, the B7-H3 over-expression on tumor cells and its role in promoting tumorigenesis through non-immunologic mechanisms is becoming evident and clinically relevant. The efficacy of the first anti-B7-H3 therapeutic mAb (Enoblituzumab) against B7-H3 expressing tumors, including melanoma, is under examination, alone or in combination with other ICIs, in Phase I-II human clinical trials (71) (https://clinicaltrials.gov/ct2/show/NCT02475213).

B7-H3 role has gained attention also in veterinary oncology. A recent study performed on canine osteosarcoma patients has demonstrated that B7-H3 plays a non-immunological role in sustaining tumorigenicity (72). However, no involvement for B7-H3 in OMM has been reported to date. We found that B7-H3 is expressed by CMM12 (**Figure 1A**), a canine OMM cell line (9). CMM12 cells that were treated with an anti-human B7-H3 mAb (376.96), which cross-reacted with the canine molecule, displayed a significant reduction in proliferation (**Figure 1B**), suggesting that B7-H3 downstream signaling potentially sustains OMM cells' proliferative behavior.

Since B7-H3 actively supports tumor cells resistance to chemotherapy (74), we tested the sensitivity of CMM12 to chemotherapy in combination with treatment with the 376.96 mAb. The combinatorial treatment was remarkably more effective than the single treatments alone (**Figure 1B**). To achieve better and more significant results other combinatorial protocols should be tested, using different mAb and doxorubicin concentrations. Nevertheless, these results prompt further investigations to determine the relevance of blocking B7-H3 to increase the efficacy of chemotherapy for OMM treatment, and we are actively working in this direction. The results obtained in the canine setting could be eventually of clinical relevance for human melanoma treatment too.

ANTI-CANCER VACCINES FOR OMM TREATMENT

Therapeutic vaccines against cancer aim to educate the immune system to recognize antigens that are expressed by tumor cells and induce effector immune responses. Several melanoma-associated antigens have been characterized in both humans and dogs, including the disialogangliosides GD2 (75) and GD3 (76–78), tyrosinase (79, 80), gp100 (81), CSPG4 (19, 82), and others.

A number of anti-cancer vaccination strategies targeting these antigens have been tested in companion dogs affected by different tumors, including melanoma (83). These include dendritic cell (DC) vaccines loaded with tumor antigens (84), autologous whole-cell vaccines (81, 85), tumor antigen combined with adjuvants (86) and gene-based vaccination (19, 87); some have already been tested in clinical veterinary trials.

Considering the translational relevance of GD2 and GD3 antigens for both canine and human melanomas, relevant veterinary studies of vaccination against these targets suggested their safety and ability to protect canine melanoma patients when provided as an adjunct to conventional therapies (86, 88). Nevertheless, adjuvants and/or antigen modifications are needed to increase the immunogenicity of the vaccine to be successfully translated in a standard of care (88, 89).

Regarding gene-based vaccination, DNA vaccines against tumor-associated antigens are a promising approach for treating cancer (90, 91). The huge number of DNA-vaccines that have been tested in the last decades in pre-clinical and clinical studies highlights the potential relevance of this strategy for future medical applications. Nevertheless, DNA vaccines face many challenges that till now have prevented their successful translation in the human clinic. Indeed, the first and only FDA-approved DNA vaccine for anti-tumor therapy is ONCEPT (Merial), a xenogeneic DNA plasmid that carries the sequence of human tyrosinase. It was approved for the treatment of dogs affected by locally controlled OMM, since it increased survival times of treated dogs as compared to unvaccinated controls, with no adverse events (92). ONCEPT approval signed the beginning of a new era in treating melanoma in dogs. Nevertheless, some studies have risen controversy around the effectiveness of ONCEPT after its licensing (93, 94).

Whether the human or the veterinary clinical context is concerned, one of the main reasons for the just modest therapeutic effect demonstrated by DNA vaccines in clinical trials could be the strong immunosuppressive condition induced by the tumor. Indeed, a clinically evident tumor triggers several mechanisms of immune suppression that may remain active despite local tumor control. Among them T cell exhaustion and expansion of T regulatory cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). Several approaches could be applied to enhance vaccine efficacy. Recently, a strong consensus for combining cancer vaccines with ICIs, concomitantly or after immunization (95, 96) is emerging. Various combinatorial strategies have been already tested in pre-clinical and clinical studies for different cancers, resulting promising in coupling the benefits of ICIs in overcoming immunosuppression, with the ability of vaccines to prime the antigen-specific cytotoxic response (97–99).

The combination of immunotherapy with local radiotherapy and/or chemotherapy that can induce immunogenic cell death is likewise a favorable way to prompt a more effective systemic anti-tumor immune response (100–102).

As an example, combinatorial approaches encompassing the use of cytokines, STING agonist and/or vaccines have indeed demonstrated enhanced efficacy when combined to radiotherapy in both pre-clinical and clinical studies, achieving improved therapeutic effects on melanoma-derived metastasis (103, 104). Recently the combination of trimodal radiotherapy and intratumoral immunocytokine vaccination has been tested in advanced stage tumor-bearing dogs, including melanoma cases, and has preliminarily showed to induce positive immunomodulatory effects within the primary tumor (100). All these studies provide the proofs of principle of

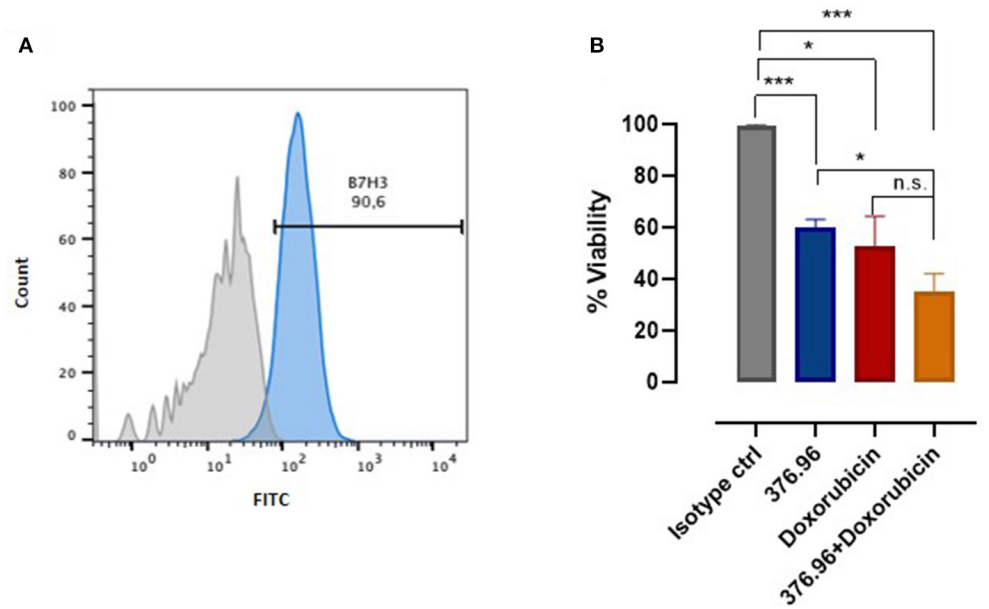

FIGURE 1 | B7-H3 expression and targeting in OMM. **(A)** Flow cytometry analysis of B7-H3 expression on canine CMM-12 OMM cells performed using a FACS Verse (BD Biosciences). The results were analyzed using FlowJo software and representative FACS curves are reported, showing CMM-12 staining using a control IgG1 isotype (filled gray area) and the anti-human B7-H3 mAb 376.96 (filled blue area; 25 μg/ml final concentration). **(B)** Cell proliferation assessed using an MTT assay, as described in (73). The results are expressed as the percentage (mean value ± SEM) of the viability of cells treated with anti-B7-H3 mAb, with respect to cells treated with the control IgG1 isotype, considered as 100%, alone or in combination with doxorubicin at a final concentration of 0.5 μM. Graph shows the results of three independent experiments. Student's *t*-test: $^{*}p < 0.01$; $^{***}p < 0.001$.

combining different strategies to achieve better therapeutic effects, eventually allowing to broaden the percentage of patients who might potentially respond to anti-cancer therapy.

In addition to tumor induced immunosuppression, antigen-loss variants of tumor cells may persist after local tumor control that may escape vaccine-induced immune responses. The selection, as vaccination targets, of not disposable tumor associated antigen(s) with a key role in cancer progression may reduce the risk of variant escape. Similarly, the accurate selection of patients for tumor expression of the target antigen would result into more informative clinical trials (105).

DNA VACCINATION AGAINST THE CSPG4 ANTIGEN

For its features, we have focused our attention on CSPG4, as a promising tumor-associated antigen to target for effective anti-cancer vaccination against both canine and human melanoma.

CSPG4 is a well-established tumor-associated antigen in human melanomas (106–108), with a widespread expression on cancer cells, but absent in normal adult tissues (108–111). It is highly evolutionarily conserved, with over 88% similarity between the human and canine amino acid sequences (9, 10), suggesting a possible overlapping role in these species. It acts as a scaffold for signaling molecules, forming a complex that drives the activation of key transduction pathways that confer the malignant behavior (108, 112–114). Being a cell-surface tumor antigen, CSPG4 represents an ideal target for effective anti-cancer immunotherapy as $CSPG4^{+}$ cancer cells are potentially susceptible to the concomitant attack of vaccine-inducible T cells and antibodies (20, 91).

As for human melanoma patients, CSPG4 overexpression in canine OMM (18) is clinically relevant, since $CSPG4^{+}$ OMM-affected dogs have worse prognosis than those whose tumors do not express the antigen (9, 19). Therefore, we tested the safety and efficacy of anti-CSPG4 immunotherapy, by means of DNA vaccination, in combination with *in-vivo* electroporation (19, 91, 115) in companion dogs affected by naturally occurring $CSPG4^{+}$ OMM. Dogs affected by a CSPG4-negative OMM were not included in the trials, since they could not benefit from the anti-CSPG4-immunotargeting and would risk leading to confounding results regarding the real vaccination efficacy.

Our first trial with a xenogeneic human (Hu)-CSPG4 DNA plasmid, was safe and effective in inducing a humoral immune response, that was linked to significantly prolonged survival in immunized dogs compared to the conventionally treated population (9, 19, 82). Anti-CSPG4 antibodies induced by the vaccination directly down-regulate CSPG4 expression *in-vitro* hampering CSPG4 tumorigenic functions in melanoma cells, suggesting that they could have a beneficial impact on the clinical course of the disease (9, 19, 82). Immunological mechanisms could be foreseen for vaccine-induced antibodies that could be thus effective in eliminating tumor cells through either antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC); this aspect is

currently under investigation. Importantly, patients which received the vaccination displayed delayed metastasis onset, compared to non-vaccinated dogs, which rapidly exhibited metastatic spreading (19). Nevertheless, Hu-CSPG4 xenovaccination induced relatively low-affinity antibodies against dog (Do)-CSPG4 (Riccardo et al., manuscript under revision), thus probably limiting the efficacy of the vaccine (116, 117). We have therefore developed a second-generation vaccine that carries a hybrid human/dog sequence, encoding for a chimeric protein that would result in the induction of a more efficient humoral and cellular immune response than those prompted by the fully xenogeneic or fully homologous ones (117, 118). The first demonstration of the potential advantages of applying a chimeric DNA vaccination against the CSPG4 molecule in veterinary medicine is ongoing in a prospective, multi-centric clinical trial in dogs affected by stage II-IV CSPG4$^+$ OMM (Riccardo et al., manuscript under revision). To improve the efficacy of this anti-cancer vaccine, the possibility of combining anti-CSPG4 DNA vaccination with ICIs for the treatment of OMM could be an interesting new therapeutic option for canine patients' management, being also of precious translational value for the human context.

SMALL EXTRACELLULAR VESICLES AS A NEW TOOL FOR MELANOMA MANAGEMENT

Regardless of the choice of therapy, there is a clear need to identify easily accessible biomarkers that may facilitate the early diagnosis of the disease. In this context, liquid biopsy is emerging as an early, non-invasive, and accessible technique for the accurate molecular profiling of a patient's tumor-derived materials. This technique will likely improve diagnoses, clinical decision making and prognostic accuracy.

Of the tumor-derived materials that can be detected in patient biofluids by means of liquid biopsy, investigations into extracellular vesicles (EVs) are intensifying (119). EVs are lipid-bilayer delimited particles that are naturally shed from cells and are amongst the main key players in cell-cell communication in the TME (120). EVs can carry a heterogeneous variety of biologically active molecules, depending on the cell of origin (121), and play a fundamental role in regulating neoplastic events (122). According to their dimension and biogenesis, we can distinguish EX (ranging from 50 to 150 nm diameter) and microvesicles (ranging from 100 to 1,000 nm diameter), that are emerging as a new frontier in cancer management in humans, with there also being potential impact for dogs (123). The CD9, CD63 and CD81 markers of expression (123, 124) have been usually used as EX biomarkers, although it has to be considered that they are present also on the membrane of other EVs (125, 126), therefore specific markers to strictly discriminate the different subtypes of EVs released by cells are still under discover. For this reason and due to some overlap in size between EX and microvescicles, we will refer more in general to small EVs (SEVs). In oncological patients, SEVs can provide a comprehensive "snapshot" of the tumor status. Knowledge of the proteome of melanoma-derived SEVs is still largely unexplored. However, it has been shown that human-melanoma-cell-derived SEVs-protein cargo differs from normal melanocyte derivatives (120), and potential clinically relevant markers have been identified for isolating circulating SEVs for diagnostic purposes (122). It has been demonstrated that circulating SEVs may provide clinicians with a better overview of dynamic tumor heterogeneity (127), and guide them toward the most appropriate personalized therapeutic approach. Moreover, it has been highlighted the predictive value of circulating SEVs in the melanoma immunotherapy, demonstrating that the monitoring of circulating SEVs-PD-L1 predicts tumor response to treatment and clinical outcome (127).

In canine patients, very few studies have been carried out so far. It has been recently demonstrated that the number of EV isolated from the plasma of dogs with cancer, including melanoma, was higher than in healthy controls (128). On our side, considering that human CSPG4$^+$ melanoma cells release SEVs that carry high levels of CSPG4 (111, 129), we investigated its presence in canine-melanoma-cell-derived SEVs. Sustained levels of CSPG4 were found in CMM12-derived SEVs (**Figure 2**), indicating that circulating-SEV-CSPG4$^+$ may be a potential biomarker for canine CSPG4$^+$-OMM diagnosis and prognosis for anti-CSPG4 immunotherapy.

Additionally, SEVs that were isolated from human-patient plasma have also been found to be enriched in immunoregulatory proteins (111, 129). Although the immunomodulatory role of B7-H3 in canine cancers still needs to be defined, we sought to discern whether soluble B7-H3 in melanoma-derived SEVs

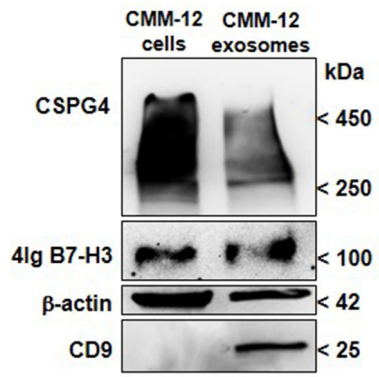

FIGURE 2 | CSPG4 and B7-H3 expression in canine-OMM-cell-derived SEVs. EX purification columns (Exo-spin Midi-Columns, Cell Guidance System, Cambridge, UK) were used to purify enriched EX-SEVs from the supernatant of fetal bovine serum-deprived CMM-12 cells. Briefly, the collected media was centrifuged to remove any cell debris and then incubated with the Exo-Spin Buffer to precipitate SEVs including EX and purified using Exo-Spin midi-column. Eluted SEVs were then ultracentrifuged at 100,000 x g and the pellet was resuspended in RIPA buffer for protein extraction. Representative immunoblot of CSPG4 and B7-H3 of lysates of SEVs is shown. Western Blot analysis for CSPG4 was performed as described in (73), mAb 376.96 was used for B7-H3 detection. CD9 (10626D; Thermo-Fisher Scientific) was used as the SEVs marker and β-Actin (AC-15; Santa Cruz Biotechnology) was used as the protein-loading control.

could be detected and potentially used as a prognostic biomarker for OMM. When CMM12-derived SEVs were analyzed, a high level of B7-H3 was detected (**Figure 2**). This result provides a starting point for investigating the diagnostic and prognostic predictive value of B7-H3 in circulating SEVs in melanoma bearing-dogs, with relevant translational implications for human immunotherapy.

DISCUSSION

Immunotherapy has revolutionized melanoma treatment in humans and successful clinical responses have been obtained. Spurred by achievements in human clinics, veterinary oncologists have started to exploit the possibility of applying this strategy to the treatment of pet cancers.

While several immunotherapeutic approaches for treating OMM in dogs have been translated from human to veterinary clinics, other strategies have been developed in canine patients first, with high translational relevance for humans (19, 45, 79, 80, 82, 130, 131). Anti-CSPG4 DNA vaccination may emerge as a novel therapeutic approach in veterinary medicine to counteract OMM progression (19, 82), with important implications for human melanoma patients. Nonetheless, the therapeutic effectiveness of our, and other, anti-melanoma strategies could be enhanced. In the wake of human findings, treatments using "old" and "new"-generation ICIs together with anti-cancer vaccination hold great promise for the management of melanoma. In this general framework and considering that precision medicine has become a central theme of cancer management, particular focus must be placed on the key role that SEVs may play in the immuno-oncology of melanoma.

However, several aspects of canine immunity are still unexplored, representing a limitation in the development of effective immunotherapies for dogs. For instance, the lack of a deep characterization of the major histocompatibility complex in dogs, the Dog Leukocyte Antigen (DLA) system, limits the possibilities of developing T-cell-based immunotherapies and investigating functional aspects of the anti-tumor T-cell response *in-vitro*. As well, while the properties of the four human IgG subclasses have been well established and it is known that ADCC and CDC are mainly activated by IgG1 or IgG3, the knowledge about both the complement system in dogs and IgG subclasses is still growing. Up to now, four canine IgG subclasses have been identified, and it is suggested that IgG2 subclasses could mainly provide a specific contribution to ADCC and CDC activity (132). Defining more in detail the components of the canine immune system would allow to better assess the functions of vaccine-induced antibodies for tumor cell killing. In conclusion, exploiting the high similarity between canine and human melanomas, the therapeutic advances achieved in both the veterinary and the human clinics can mutually revolutionize the treatment of melanoma patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LT, FC, FR, and PB contributed to the conception of this perspective. LT, FC, and FR wrote the draft of the manuscript. DG, MC, SF, and PB contributed to manuscript revision, read, and approved the submitted version. All authors contributed to the article and approved the submitted version.

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Tumor-Associated Macrophages in Canine Oral and Cutaneous Melanomas and Melanocytomas: Phenotypic and Prognostic Assessment

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The tumor microenvironment is a complex system, where neoplastic cells interact with immune and stromal cells. Tumor-associated macrophages (TAMs) are considered among the most numerically and biologically noteworthy cellular components in tumors and the attention on this cellular population has been growing during the last decade, both for its prognostic role and as a potential future therapeutic target. Melanoma, particularly the oral form, despite being one of the most immunogenic tumors, bears a poor prognosis in dogs and humans, due to its highly aggressive biological behavior and limited therapeutic options. The aims of this study are to characterize and quantify TAMs (using CD163, CD204, Iba1, and MAC387) in canine melanocytic tumors and to evaluate the association of these markers with diagnosis, histologic prognostic features, presence of metastases, and outcome, and to provide preliminary data for possible future therapies targeting TAMs. Seventy-two melanocytic tumors (27 oral melanomas, 25 cutaneous melanomas, 14 cutaneous melanocytomas, and 6 oral melanocytomas) were retrospectively selected and submitted to immunohistochemistry and double immunofluorescence. Double immunolabeling revealed that most $CD163^+$ and $CD204^+$cells co-expressed Iba1, which labeled also dendritic cells. Iba1 was instead rarely co-expressed with MAC387. Nevertheless, the expression of macrophagic markers showed a mild to moderate association among the four markers, except for CD204 and MAC387. The number of $CD163^+$, $CD204^+$, and $MAC387^+$ cells was significantly higher in oral melanomas compared to oral melanocytomas ($p < 0.001$; $p < 0.05$ and $p < 0.01$, respectively), whereas Iba1 was differentially expressed in cutaneous melanomas and melanocytomas ($p < 0.05$). Moreover, CD163, IBA1 and MAC387 expression was associated with nuclear atypia and mitotic count. The number of $CD163^+$cells was associated with the presence of metastases and tumor-related death in oral melanocytic tumors ($p < 0.05$ and $p = 0.001$, respectively).

Keywords: dogs, melanoma, tumor-associated macrophages, CD204, CD163, Iba1, MAC387, tumor-associated macrophages (TAMs)

INTRODUCTION

Tumors are complex ecosystems composed of tumor cells, stromal cells, and immune cells; macrophages are recognized as one of the major components of these ecosystems (1–3). Still, in cancer, the role of this multifaceted cellular population, which serve as nexus between innate and adaptive immunity, is still poorly understood and studies addressing this topic in both human and veterinary medicine are surprisingly few. Actually, most of the oncology studies and large study groups are focused on lymphocytes and on their role in cancer immune response (4–10). Macrophages are a wide-ranging population of immune cells characterized by broad heterogeneity, high plasticity, and remarkable ability to sense changes in the surrounding microenvironment and respond to it. These cells are crucial in the detection, phagocytosis, and destruction of foreign and endogenous material, pathogens, but also cancer cells. Moreover, together with their phagocytic activity, they can participate in adaptive immunity by recruiting lymphocytes and presenting antigens to T cells (11–13).

Probably, the majority of TAMs are recruited macrophages (2), which are derived from circulating monocytes (14) and are in close contact with tumor cells, therefore the definition of tumor-associated macrophages (TAMs). Within the tumor microenvironment, these macrophages, together with myeloid-derived stem cells (MDSCs) are the major players in myeloid immunosuppression, which is believed to be triggered by cancer-related inflammation (15). TAMs can play different roles: promote angiogenesis and lymphangiogenesis, suppress antitumor immunity, particularly by suppressing cytotoxic T cell response, and stimulate cancer cells inducing proliferation, survival and invasion, and metastasis, therefore promoting tumor progression (3, 12).

The presence of TAMs in melanoma and their role in tumor progression and prognosis has been assessed in humans (16–20) and, in the last few years, the potential role of these cells as targets of future immunotherapies has been suggested (4, 21).

Recent studies have demonstrated that the role of TAMs depends on both their subtype (16, 17) and microanatomical localization. Besides, research has been focusing on the polarization of macrophages and also on their transcriptomic signature (2, 3, 14, 22).

In human cutaneous melanoma, high numbers of TAMs, especially when localized within the tumor cell nests, seem to enhance melanoma progression (18). Moreover, it has been shown that the production of midkine (MDK) can educate macrophages toward a tolerant phenotype, promoting $CD8^+$cells dysfunction (20).

Studying TAMs both as prognostic indicators and as therapeutic targets in canine melanocytic tumors seems to be a promising path to follow to increase the current knowledge of their role in these tumors. Indeed, melanocytic tumors, despite being common in dogs, have sometimes unpredictable behavior and no curative available therapies (23–27); therefore, studies investigating additional valid prognostic features and feasible targets for new therapeutic strategies are fundamental.

Investigating TAMs in canine tumors is currently complicated by the limited availability of confirmed markers for canine macrophages to be used also on formalin-fixed and paraffin-embedded tissues.

Iba1 (also known as AIF-1) is a 17-kDa protein, which is specifically expressed by macrophages/microglia and upregulated during the activation of these cells (28). Some studies use Iba1 as a panmyeloid marker (29), whereas in other cases it is hypothesized that Iba1 identifies a M2-like phenotype of macrophages (30). In veterinary medicine, Iba1 has been recently used for the identification of TAMs in different canine tumors (31–33).

The scavenger receptor CD163 is expressed exclusively in macrophages and, less so, in monocytes; the expression of this protein is prompted by anti-inflammatory IL-6 and IL-10, whereas it is downregulated by pro-inflammatory stimuli (34). The expression of CD163 has a robust association with worse overall survival in human epithelial tumors and melanoma (35).

CD204 (also known as Scavenger Receptor-A, SR-A) is a transmembrane protein preferentially expressed on macrophages (36). The expression of this protein has shown a robust association with worse overall survival in epithelial neoplasms and melanoma in humans (35). Both CD163 and CD204 are recognized as possible M2-polarization markers.

Even though a rigorous categorization of macrophages is not possible, being them a continuous in terms of phenotype, a classification of these cells into M1-polarized macrophages (classically activated) and M2-polarized macrophages (anti-inflammatory) can be recognized. The process of polarization is the answer of macrophages to microenvironmental stimuli, by the acquisition of a specific phenotype. M1-polarized macrophages (pro-inflammatory) are critical in bacterial killing, tumor resistance, supporting tumor destruction and antigen presentation, and Th1 response (11). On the other hand, M2-polarized macrophages have anti-inflammatory properties, therefore favoring tumor growth and survival (35).

S100A8/A9 complex, also called calprotectin or MAC387, is expressed in phagocytic cells such as neutrophils, monocytes, dendritic cells, activated macrophages (but not non-activated macrophages), and platelets (37). This complex is intensely upregulated after trauma, in inflammatory processes, and stress (38). In a recent study, higher numbers of infiltrating MAC387-positive cells were found in metastasizing primary melanomas compared to non-metastasizing melanomas (39).

The aims of this study are:

- to characterize phenotypically and quantify TAMs within canine melanocytic tumors, using the currently available canine macrophagic markers IBA1, CD163, CD204, and MAC387 by immunohistochemistry and double immunofluorescence;
- to evaluate the association between the expression of different TAMs' markers with diagnosis, histologic prognostic features, presence of metastases, and outcome.

MATERIALS AND METHODS

Case Selection

Seventy-two cases of canine melanocytic tumors were retrospectively selected from the archive of the Department of Veterinary Medicine of the University of Perugia.

Cases were included based on the following inclusion criteria:

- histological diagnosis of melanocytic tumor;
- in case of amelanotic tumor, diagnosis of melanocytic tumor confirmed by immunolabeling with Melan A and/or PNL2;
- available neoplastic tissue with an area >0.5 cm $^{2;}$
- primary melanocytic tumor.

Cases were excluded if showing sampling or fixation artifacts.

A telephonic interview was conducted with the referring veterinarians to assess the presence of melanoma metastases and the cause of death. The minimum time from diagnosis to follow-up was set at 1 year. Dogs that were euthanized due to bad conditions caused by melanoma (inoperable local disease or metastases), were grouped together with dogs spontaneously dead because of the melanocytic tumor.

Histopathological Examination

All the cases were re-examined by three pathologists (IP, MS, and CB) to confirm the original diagnosis following standard diagnostic criteria (40). Heavily pigmented tumors were bleached, following previously reported protocols (41).

The histological evaluation also included other features: mitotic count, nuclear atypia (40), pigmentation (expressed as a percentage of neoplastic pigmented cells), tumor thickness (42), and presence of melanophages. The first four features are considered significant in the prognosis of melanocytic neoplasms (40).

The presence of melanophages (macrophages containing a variable quantity of melanin pigment) was quantified as follows:

- 0: no melanophages observed in the examined sections;
- 1: small focal aggregates and/or scattered melanophages within the tumor (<5/HPF field);
- 2: multifocal aggregates of >5 melanophages, with areas of the tumor where no melanophages can be seen;
- 3: large multifocal to coalescing aggregates of melanophages.

Western Blotting

Before immunohistochemical analysis, mouse anti-human Iba1 (Merck Millipore, Burlington, MA, US), anti-human CD163 (clone EDHu-1; Bio-Rad; Hercules, CA, US), and anti-human MRS-A/CD204 (clone SRA-E5;Cosmo Bio Co., LTD; Yuseong-Gu, Daejeon, Republic of Korea) antibodies were validated for detection of the canine proteins by Western blotting (WB), as previously reported (43). Forty mg of fresh tissues (lymph node harvested from a dog dead from trauma and placenta collected immediately after a spontaneous delivery) were homogenized in lysis buffer (Cell Signaling; Danvers, MA, US) containing protease inhibitor cocktail (Sigma-Aldrich) and centrifugated for 13,000 × g for 15 min at 4°C. The protein pellet was resuspended in phosphate-buffered saline (PBS) pH 7.4 and quantified using the Bradford assay. 50 μg of total proteins of each tissue were separated by polyacrylamide gel electrophoresis (SDS-PAGE) 12% T or 14% T and then transferred on nitrocellulose membranes. Specific protein bands were detected incubating membranes with the mouse anti-human CD163 (1:500), mouse anti-human CD204 antibody (1:300) or mouse anti-human Iba1 antibody (1:250) O.N. at 4°C and then at room temperature for 90 min with anti-mouse IgG polyclonal antibodies (1:5,000; Cell signaling). Immunoreactivity was evidenced by ECL system (Amersham ECL Prime Western Blotting Detection Reagent; Amersham, Amersham UK). The film images were acquired using a GS-800 imaging systems scanner (Bio-Rad).

TABLE 1 | Protocol details for immunohistochemistry.

Antigen	Clone	Manufacturer	Antigen retrieval	Dilution
CD163	EDHu-1	Bio-Rad	HIER; Tris-EDTA buffer; pH 9.0	1:100
CD204	SRA-E5	Cosmo Bio Co., LTD.	HIER; Tris-EDTA buffer; pH 9.0	1:100
Iba1	MABN92	Merck Millipore	HIER; Tris-EDTA buffer; pH 9.0	1:100
MAC387	M0747	Dako	HIER; Tris-EDTA buffer; pH 9.0	1:250

HIER, heat-induced epitope retrieval.

Immunohistochemistry

From formalin-fixed and paraffin-embedded samples, 5-μm sections were cut and mounted on poly-L-lysine-coated slides, which were then dewaxed and dehydrated. Immunohistochemistry was performed on serial sections with antibodies raised against CD163, CD204 (44), Iba1 (45), and MAC387 (33, 46, 47). In cases with more available histocassettes, the most representative was chosen, avoiding samples with large necrotic areas or ulceration. Positive controls were obtained from canine reactive lymph nodes for all the four antibodies used in this study; negative controls were run omitting the primary antibody and incubating control sections with TBS. Immunohistochemical protocols and antibodies used in this study are summarized in **Table 1**.

For each antibody, positive cells were manually counted in five consecutive HPF starting from hot spots, then the mean value was calculated; positive cells were considered only if they were intratumoral. Positive cells in the peripheral areas of the tumor or in the immediate peritumoral areas were not quantified but the presence of positive cells was recorded.

Double Immunofluorescence

Double immunofluorescence was performed on five selected cases (two oral melanomas and three cutaneous melanomas). We selected Iba1 (as a panmyelocytic marker) and tested its co-expression with CD163, CD204, and MAC387. After dewaxing and dehydrating, the slides were incubated with a 3% H_2O_2 methanol solution. Antigen retrieval was performed as reported for immunohistochemistry and primary mouse

antibodies (CD163, CD204 and MAC387) were incubated overnight at 4°C with the appropriate concentration. After TBS washing, a secondary goat anti-mouse antibody, conjugated with a red fluorochrome (Goat Anti-Mouse IgG H&L - Alexa Fluor® 647, Abcam) and with a 1:200 dilution, was applied for 2 h at room temperature. After a careful wash, a protein block step was performed prior to incubation with an anti-Iba1 antibody conjugated with a green fluorochrome (clone 20A12.1, Alexa Fluor® 488 Conjugate, Merck). Last, a drop of aqueous mounting medium containing DAPI (Abcam) was added and slides were incubated for 5 min at room temperature, before coverslip mounting.

Samples were evaluated using a fluorescent microscope Olympus BX51 equipped with the camera Nikon mod.DS-Qi2Mc. NIS-ELEMENTS D software was used for image acquisition and analysis.

Statistical Analysis

Normality was assessed with a Shapiro–Wilk test for all continuous variables. Descriptive statistics were used to describe data; values are expressed as medians (Mdn) and interquartile range (IQR). Non-parametric tests were used to test hypotheses. The Kruskal-Wallis test and Mann–Whitney U test were performed to assess differences among groups. Correlation analysis was performed using Spearman's test (ρ). Descriptive statistics were performed using Microsoft Excel; other statistical tests were performed with IBM SPSS (version 21).

RESULT

Animals of the Study

The 72 melanocytic tumors retrospectively selected from our archive were obtained from 72 dogs. Twenty-seven cases were diagnosed as oral melanomas, 25 as cutaneous melanomas, 14 as cutaneous melanocytomas, and six as oral melanocytomas. Twenty-nine dogs were female, whereas 42 were male. In one case information on the sex of the animal was not available. The most represented breeds were mixed (25/72; 34.7%), German Shepherd (6/72; 8.3%), Labrador retriever (5/72; 6.9%), Golden retriever (4/72; 5.5%), English setter (3/72; 4.1%) and Yorkshire terrier (3/72; 4.1%).

The mean age of dogs at the moment of the diagnosis was 11 years (±3.1).

Of the 72 cases selected for the study, 55 had a complete follow-up. Nineteen cases were oral melanomas, 19 cutaneous melanomas, 12 cutaneous melanocytomas, and five oral melanocytomas.

Of the oral melanomas, 12 had distant metastases assessed at the moment of death, whereas five had a local relapse of the tumor after surgical excision. Two dogs did not have relapse nor metastases and one was still alive at the moment of the follow-up (>3 years), whereas the other dog died because of bronchomalacia a year later. Five out of six cases diagnosed with oral melanocytoma were still alive and only one case had a slowly growing relapse (>2 years after excision). In the group of cutaneous melanomas, six dogs died because of the tumor and, of these, five out of six had metastatic disease; only one case was euthanized due to the local invasion of a large mass infiltrating the temporo-mandibular region. Of the remaining 13 cases five died for other causes, whereas eight were still alive at the moment of the collection of follow-up data. Among cutaneous melanocytomas, one had metastasis to the local lymph node (diagnosed histologically), but after more than 3 years the dog is still alive and with no residual disease. In this case the authors agreed on the diagnosis of melanocytoma, based on histological features of the tumor and on what reported in human medicine, where nodal metastases can occur in up to 46% of cases of melanocytoma, despite benign behavior (48). Of the other 11 dogs with cutaneous melanocytomas, two died for other causes, whereas nine were still alive at the moment of the collection of follow-up data. Complete clinical information is reported in **Supplementary Table 1**.

Histopathological Variables and Correlation With Outcome

Analysis of the histopathological variables showed a median mitotic count of 13.45 (IQR = 2.34–45.58). A statistical difference was detected among the four subgroups with different histological diagnosis ($p < 0.001$), in particular, oral melanomas (Mdn = 36.27; IQR = 11.7–60.84) statistically differ from oral melanocytomas (Mdn = 0.5; IQR = 0–1.46; $p < 0.001$) and cutaneous melanomas (Mdn = 23.4; IQR = 3.84–44.45) from cutaneous melanocytomas (Mdn = 2.17; IQR = 0–3; $p < 0.001$). Significant difference is lost among oral and cutaneous melanomas ($p = 0.12$) and oral and cutaneous melanocytomas ($p = 0.13$).

Considering all the cases of this study, the median value of pigmented cells was 30% (IQR = 5%−80%). Percentage of pigmented neoplastic cells in oral melanomas had a median value of 5% (IQR = 5%−30%), in cutaneous melanomas of 30% (IQR = 5%−70%), in oral melanocytomas of 92.50% (IQR = 82.50%−95.00%), and in cutaneous melanocytomas of 85% (IQR = 71.25%−100%). A statistically significant difference was observed among the four subgroups ($p < 0.001$); in particular, oral melanocytomas were significantly more pigmented than oral melanomas ($p < 0.001$) and the same was true for the cutaneous counterpart ($p < 0.001$).

Atypia in the present study group had a median value of 45% (IQR = 20%−80%), expressed as a percentage of cells showing signs of atypia on the total neoplastic cells. The median value of atypical cells in oral melanomas was 60% (IQR = 40%−80%) whereas in cutaneous melanomas was of 60% (IQR = 25%−85%). On the other hand, atypia in oral (Mdn = 7.50%; 4.50%–35.00%) and cutaneous melanocytomas (Mdn = 10%; IQR = 5%–26.25%) was lower. Oral melanomas showed a significantly higher percentage of atypical cells when compared to oral melanocytomas ($p = 0.003$) and the same was observed in cutaneous melanomas compared to cutaneous melanocytomas ($p < 0.001$).

Melanophages were graded from 0 to 3 and showed more frequently grade 3 or 2 in oral and cutaneous melanocytomas, whereas they were of a lower grade in the malignant counterpart. A statistical difference was observed in the four groups, with

oral melanocytoma containing more melanophages than oral melanomas ($p = 0.003$) and cutaneous melanocytomas more melanophages than cutaneous melanomas ($p = 0.005$).

All these four histological variables were associated with outcome; in particular, higher mitotic counts and percentages of cellular atypia were associated with tumor-related death ($p < 0.001$ and $p = 0.001$, respectively). On the other hand, a higher percentage of pigmented cells and higher infiltration of melanophages were associated with a better outcome ($p < 0.001$ and $p = 0.001$, respectively). Considering only oral tumors (both melanomas and melanocytomas), atypia and pigmentation maintained their statistically significant association with tumor-related death ($p < 0.05$ for both variables), whereas the significance was lost for the number of melanophages ($p = 0.26$) and for the mitotic count ($p = 0.07$), although both parameters showed a trend. On the other hand, analyzing only cutaneous tumors, three of the four variables maintained their statistical significance (mitotic count: $p < 0.005$; pigmentation: $p < 0.05$; and melanophages: $p = 0.005$), whereas it was lost for atypia ($p = 0.09$). Histological data are reported in **Supplementary Table 1**.

Validation of Antibodies Cross-Reactivity in Canine Tissues

The WB images (**Figure 1**), obtained using anti-human Iba1 and anti-human CD163 antibodies, show a single band corresponding to the expected molecular weight 16 and 130–180 kDa, respectively, in the lymph node lane, whereas the anti-human CD204 antibody recognizes isoforms between 90 and 120 kDa. No bands were detected in the placenta lane for all antibodies tested.

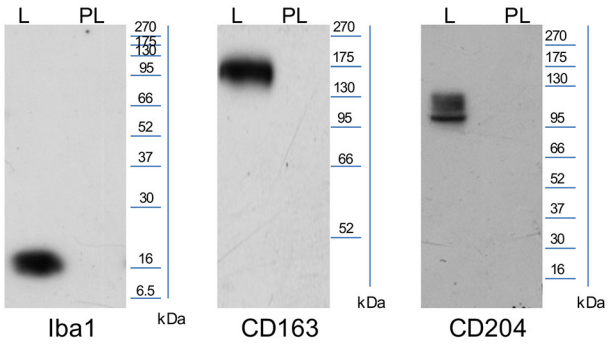

FIGURE 1 | Representative images of WB performed using SDS-page 12% (CD163≃130–180 kDA, CD204≃90–120 kDA) and SDS-page 14% (Iba1≃16 kDa; L, lymph node and PL, placenta).

TAMs Markers Expression and Correlations With Diagnosis, Histopathological Variables, Metastases, and Outcome

Iba1 Expression Is Higher in Cutaneous Melanomas Compared to Cutaneous Melanocytomas

Iba1 was expressed in the cytoplasm of macrophages and monocytes. Cells with prominent cytoplasmic projections were interpreted as dendritic cells. Also, Iba1 labeling was variable in melanophages, with the majority of these cells being weakly or not labeled. The median number of Iba1$^+$cells/HPF was 42.8 (IQR = 29.3–61.31). Kruskal-Wallis test indicated a statistically significant difference among the four diagnosis groups ($p = 0.001$; **Figure 2A**). Iba1$^+$cells/HPF in oral melanomas (Mdn = 51.8; IQR = 40.9–75.3) did not differ statistically from oral melanocytomas (Mdn = 26.3; IQR = 3.05–54.3; $p = 0.76$); on the contrary, a statistical difference was observed between cutaneous melanomas (Mdn = 40.4; IQR = 11.6–59.4; **Figure 3A**) and melanocytomas (Mdn = 2.4; IQR = 0.65–22.5; **Figures 3B,C**; $p < 0.05$).

CD163 Expression Is Higher in Oral Melanomas Compared to Oral Melanocytomas

Immunolabeling for CD163 was observed as strong membranous staining. Cells were morphologically defined as macrophages or monocytes, according to the size of the cell, the amount of cytoplasm, and size and location of the nucleus. Melanophages were variably positive to CD163, showing frequently a weaker immunolabeling compared to smaller monocytes. CD163$^+$macrophages showed a variable morphology, from dendritic, to polygonal or rounded, to spindloid; also, the dimension of positive cells was highly variable, being larger when cells were filled with pigment or vacuoles. CD163$^+$cells were frequently scattered as single elements throughout the tumor; aggregates were instead more frequently observed near areas of necrosis.

The median number of CD163$^+$cells/HPF was 64.25 (IQR = 42.5–84.35) and showed a statistically significant difference among the four diagnosis groups (oral melanoma, cutaneous melanoma, cutaneous melanocytoma and oral melanocytoma; $p < 0.001$; **Figure 2B**). Oral melanomas were characterized by the highest number of CD163$^+$cells/HPF (Mdn = 73.9; IQR = 54.5–108.9; **Figure 3D**), followed by cutaneous melanomas (Mdn = 64.9; IQR = 51.7–83.36), cutaneous melanocytomas (Mdn = 49.6; IQR = 41.1–64.35), and oral melanocytomas (**Figure 3E**; Mdn = 17.4; IQR = 8.1–25.95). Statistical significance increased by running a Mann–Whitney *U*-test comparing oral melanomas to oral melanocytomas ($p < 0.001$), whereas it was lost comparing cutaneous melanomas to cutaneous melanocytomas ($p = 0.77$). Four cases were not examined due to the lack of tissue after serial recuts. In some cases, the presence of extratumoral CD163$^+$cells was observed, in face of a non-infiltrated tumor (**Figure 3F**).

CD204 Expression Is Higher in Oral Melanomas Compared to Oral Melanocytomas

CD204$^+$ cells showed a marked membranous positivity; the immunolabeled elements were cytologically interpreted as macrophages and monocytes, with variable shapes and dimensions. Similarly to CD163, melanophages were often characterized by a faint positivity.

The median number of CD204$^+$cells within the tumors was 41.6 cells/HPF (IQR = 30–63.8). Kruskal–Wallis test did not reveal a statistically significant difference among the four groups, but a trend was identified ($p = 0.058$; **Figure 2C**).

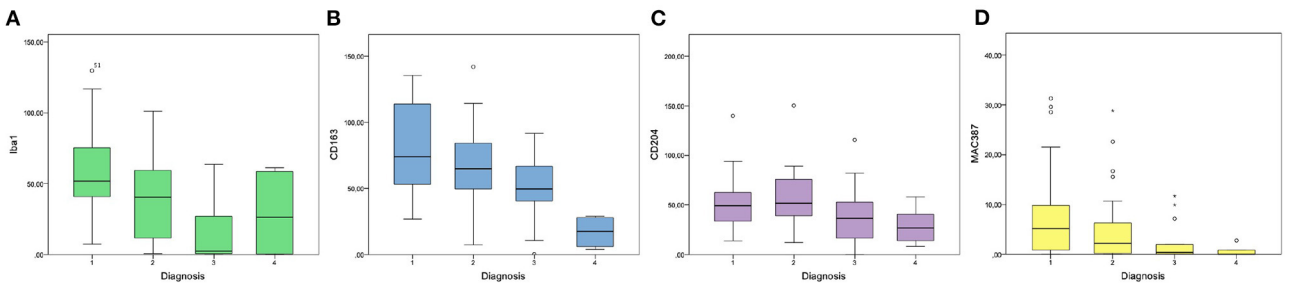

FIGURE 2 | Graphics showing the results of intratumoral Iba1 **(A)**, CD163 **(B)**, CD204 **(C)**, and MAC387 **(D)** cell counts in the four groups of diagnosis (1: oral melanoma; 2: cutaneous melanoma; 3: cutaneous melanocytoma; 4: oral melanocytoma).

Therefore, a Mann–Whitney *U*-test was run comparing oral melanomas to oral melanocytomas and cutaneous melanomas to cutaneous melanocytomas. Oral melanomas showed a statistically significant higher number of $CD204^+$cells/HPF (Mdn = 49.2; IQR = 33.5–62.6; **Figure 3G**), when compared to their benign counterpart (Mdn = 26.7; IQR = 16.4–37.75; **Figure 3H**; $p < 0.04$). On the contrary, the difference was not statistically significant between cutaneous melanomas and melanocytomas ($p = 0.77$; Mdn = 51.8; IQR = 39.3–74.45 and Mdn = 36.4; IQR = 18.45 and 50.2, respectively). Interestingly, CD204 immunoreactivity in cells showing evidence of phagocytosis was frequently observed within malignant tumors (**Figure 3I**).

MAC387 Expression Is Higher in Oral Melanomas Compared to Oral Melanocytomas

MAC387 expression was observed as a strong, diffuse cytoplasmic immunolabeling in both mononucleated cells (interpreted as macrophages) and polymorphonucleated cells, mostly neutrophils. Monocytes were observed mostly in deeper areas of the tumor and were few (**Figure 3J**). Melanophages were invariably negative for MAC387; whereas intravascular neutrophils and monocytes showed strong immunolabeling (**Figure 3K**). Superficial areas with ulceration were usually heavily infiltrated by neutrophils (**Figure 3L**).

The mean number of MAC387 was 2.06 cells/HPF and was significantly different among the four diagnosis groups ($p < 0.05$; **Figure 2D**). In particular, oral melanomas marker expression (Mdn = 5.17; IQR = 0.85–9.78) was significantly higher than in oral melanocytomas (Mdn = 0; IQR = 0–0.6; $p < 0.01$). Instead, a significance was not reached between cutaneous melanomas (Mdn = 2.19; IQR = 0.21–6.27) and melanocytomas (Mdn = 0.4; IQR = 0–1.91; $p = 0.76$).

Correlations Between TAMs Markers Expression and Histopathological Variables

CD163 expression showed a moderate positive correlation with the expression of CD204 and Iba1 ($p < 0.001$; $\rho = 0.472$ and $\rho = 0.521$, respectively). The correlation with MAC387 was instead weak ($p < 0.01$; $\rho = 0.329$). A moderate positive correlation was observed also with prognostic histologic markers of nuclear atypia ($p < 0.001$; $\rho = 0.416$) and mitotic count ($p < 0.001$; $\rho = 0.446$). A weak negative correlation was instead observed with pigmentation ($p < 0.01$; $\rho = -0.368$) and with the presence of melanophages ($p < 0.05$; $\rho = -0.265$).

The expression of CD204, instead, showed a positive weak-to-moderate correlation with Iba1 expression ($p = 0.001$; $\rho = 0.391$); a weak expression was observed also with nuclear atypia and mitotic count ($p < 0.05$; $\rho = 0.251$ and $\rho = 0.276$, respectively).

Iba1 expression was moderately correlated with nuclear atypia ($p < 0.001$; $\rho = 0.403$) and had a weak-to-moderate correlation with the mitotic count ($p = 0.001$; $\rho = 0.373$). A moderate negative correlation was seen with pigmentation and melanophages ($p < 0.001$; $\rho = -0.481$ and $\rho = -0.410$, respectively).

MAC387 showed a moderate positive correlation with mitotic count ($p < 0.001$; $\rho = 0.428$) and a weak correlation with nuclear atypia ($p < 0.01$; $\rho = 0.310$). A weak negative correlation was seen with pigmentation and melanophages ($p = 0.001$ and $p < 0.05$; $\rho = -0.368$ and $\rho = -0.288$, respectively).

The results of the correlation analysis are reported in **Table 2**.

The correlation with tumor thickness was assessed only for cutaneous tumors. No correlation was observed with CD163 and CD204, whereas a moderate positive correlation was present with Iba1 and MAC387 ($p < 0.05$; $\rho = 0.450$ and 0 $\rho = 0.451$, respectively).

Correlations Between TAMs Markers Expression and Metastases and Outcome

Considered altogether, Iba1, CD163, and MAC387 expression was significantly associated with tumor-related death associated with death due to melanoma ($p < 0.01$ for Iba1 and $p < 0.05$ for CD163 and MAC387). Furthermore, Iba1 expression was associated with the presence of metastases ($p < 0.05$), and, although not statistically significant, CD163 expression showed a trend ($p = 0.73$).

When we performed the analysis on oral tumors and cutaneous tumors separately, CD163 confirmed its association with metastases and with death for melanoma in the oral group ($p < 0.05$ and $p = 0.001$, respectively). On the other hand, none of the markers tested was associated with metastases or death for melanoma in the group of cutaneous tumors. A mild trend was observed for CD204 ($p = 0.067$).

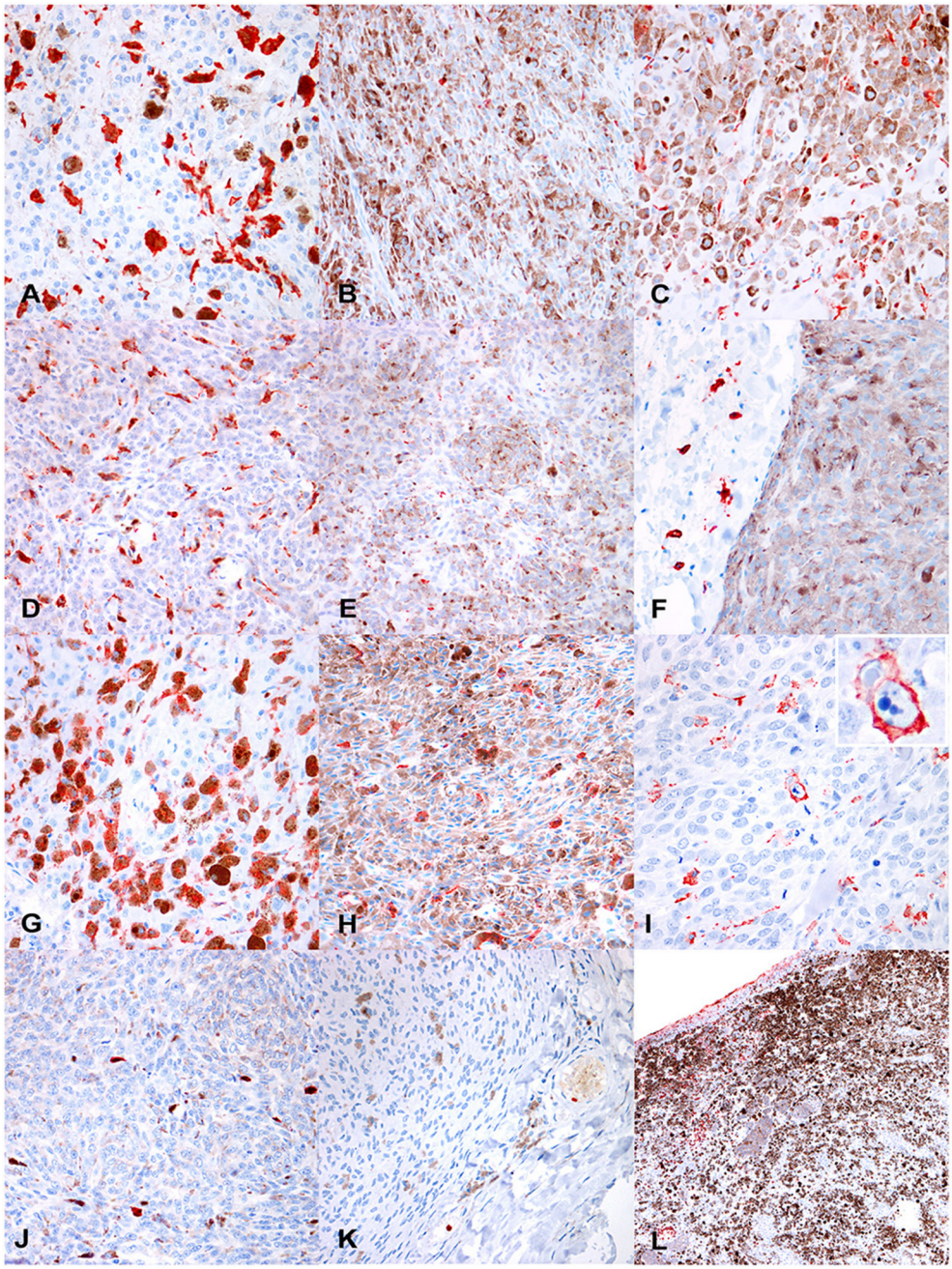

FIGURE 3 | (A) Canine, cutaneous melanoma. Numerous intratumoral, large, Iba1$^+$cells. Non neoplastic cells containing melanine (melanophages) are often faintly positive or negative (400×, AEC and hematoxylin). **(B)** Canine cutaneous melanocytoma. Rare intratumoral IBA1$^+$ cells (400×, AEC and hematoxylin). **(C)** Canine cutaneous melanocytoma. In some tumors, Iba1$^+$cells were smaller and with a more spindloid shape (400×, AEC and hematoxylin). Immunohistochemical results. **(D)** Canine, oral melanoma. Disseminated intratumoral CD163$^+$cells, that show a polygonal-to-spindloid morphology (400×, AEC and hematoxylin). **(E)** Canine, cutaneous melanocytoma. Scattered CD163$^+$ cells (400×, AEC and hematoxylin). **(F)** Canine, cutaneous melanocytoma. Extratumoral CD163$^+$ cells, not infiltrating the tumor (400×, AEC and hematoxylin). **(G)** Canine, oral melanoma. Numerous, disseminated, intratumoral CD204$^+$cells, that show a variable morphology, from rounded to elongated (400×, AEC and hematoxylin). **(H)** Canine, oral melanocytoma. Scattered CD204$^+$cells among neoplastic cells. **(I)** Canine, cutaneous melanoma. Occasionally, CD204$^+$cells, showed phagocytic activity (detail in the inset; 400×, AEC and hematoxylin). **(J)** Canine, oral melanoma. Occasional MAC387$^+$cells, in deeper areas of the tumor far from areas of ulceration (400×, AEC and hematoxylin). **(K)** Canine, cutaneous melanocytoma. Occasional intravascular MAC387$^+$cells, likely migrating into the tissue, whereas no MAC387$^+$cells can be seen into the tumor (400×, AEC and hematoxylin). **(L)** Canine, oral melanoma. Numerous MAC387$^+$cells (both macrophages and neutrophils) are present near superficial ulceration (200×, AEC and hematoxylin).

TABLE 2 | Correlation analysis (Spearman rank correlation coefficient, ρ).

	CD204	Iba1	MAC387	Nuclear atypia	Mitotic count	Pigmentation	Melanophages
CD163	0.472**	0.521**	0.329**	0.416**	0.446**	−0.368**	−0.265*
CD204	1.000	0.391**	0.184	0.251*	0.276*	−0.209	−0.069
Iba1		1.000	0.268*	0.403**	0.373**	−0.481**	−0.410**
MAC387			1.000	0.310**	0.428**	−0.368**	−0.288*
Nuclear atypia				1.000	0.597**	−0.319**	−0.299*
Mitotic count					1.000	−0.671**	−0.611**
Pigmentation						1.000	0.831**

*$p < 0.05$. **$p < 0.01$.

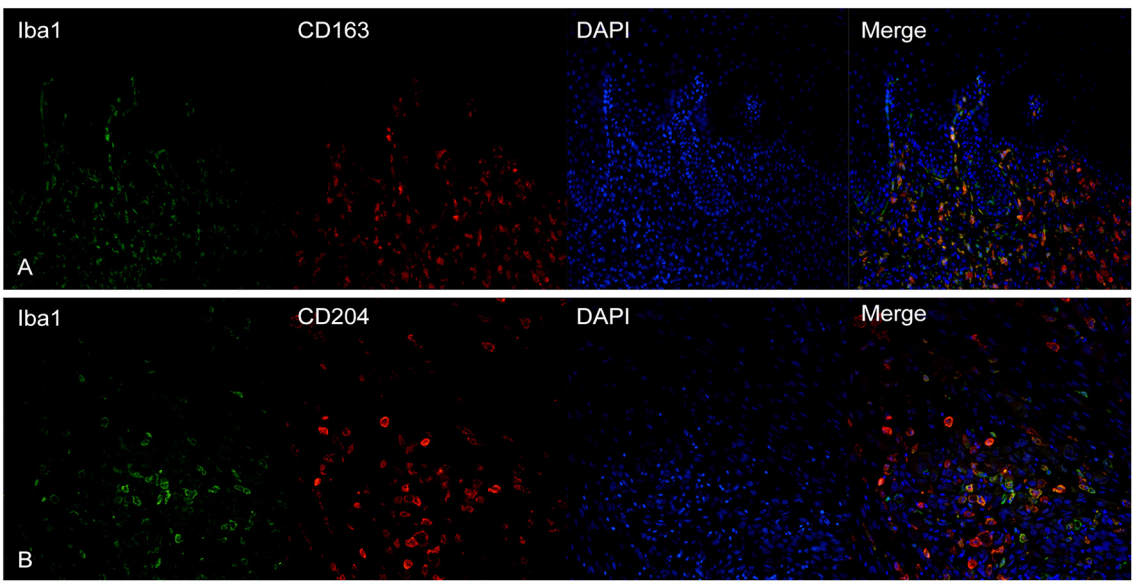

FIGURE 4 | (A) Canine, oral melanoma. Colocalization of Iba1 (green) and CD163 (red) shows a partial co-expression of the two markers. Intraepithelial dendritic cells are negative for CD163 but positive for Iba1. **(B)** Canine, oral melanoma. A partial overlap of Iba1 (green) and CD204 (red) was observed. Nuclear counterstain was performed with DAPI.

TAMs Markers Co-Expression

CD163 showed a frequent co-expression with Iba1, but the two cellular populations did not overlap completely (**Figure 4A**). Large polygonal cells with abundant cytoplasm, interpreted as macrophages, often showed a marked positivity for CD163 but were negative for Iba1. Instead, cells with dendritic morphology, as well as intraepithelial dendritic cells, were positive for Iba1 but negative for CD163.

CD204 and Iba1 showed a co-expression profile similar to CD163-Iba1 and, also in this case, cells with a macrophagic morphology, were usually positive for CD204 but not for Iba1 (**Figure 4B**). Intraepithelial dendritic cells were variably positive, being instead strongly positive for Iba1.

MAC387 showed a variable co-expression with Iba1; usually, scant double-labeled Iba1-MAC387^{+}cells were seen within the tumor (**Figure 5A**). On the other hand, near superficial areas of ulceration, double-positive cells increased (**Figure 5B**).

DISCUSSION

The tumor microenvironment is a rich and complex system, where neoplastic cells interact with immune cells and stroma; TAMs are considered among its most numerically and biologically significant cellular components (1, 2).

The present study focuses for the first time on TAMs in canine melanocytic tumors using different macrophagic markers, namely Iba1, CD163, CD204, and MAC387. The correlation of these markers' expression with prognostic histologic features, presence of metastases, and outcome are also assessed.

Overall, we observed a moderate association between CD163 and CD204, CD163 and Iba1 and a weak-to-moderate association between CD204 and Iba1. These results seem to indicate an association and at least a partial overlap of the expression of these markers, as confirmed by the co-expression analysis through double immunofluorescence. MAC387 has been considered a reliable macrophagic marker for years in veterinary medicine

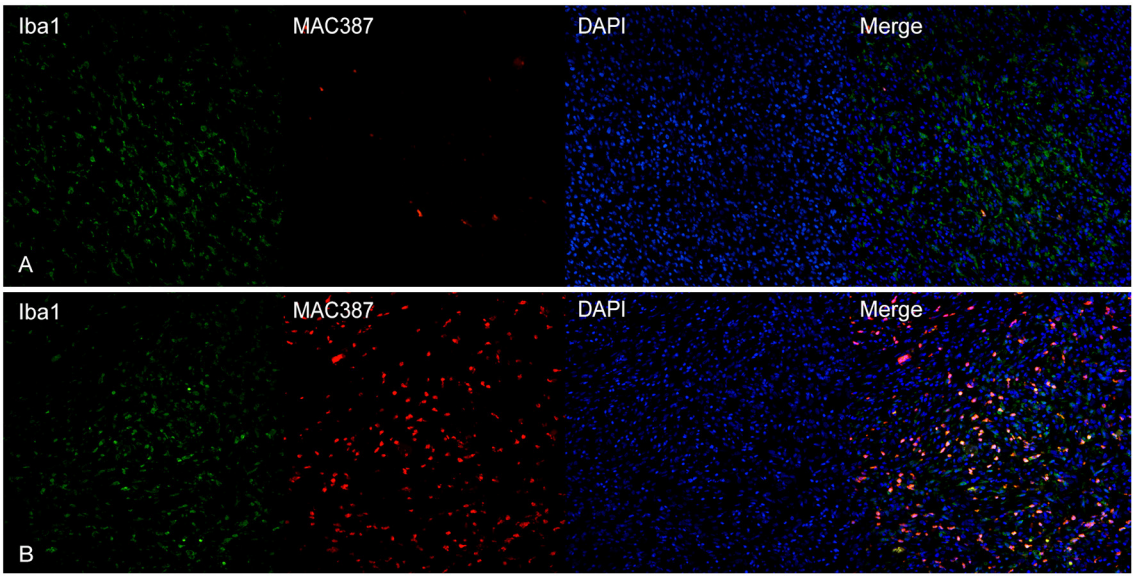

FIGURE 5 | (A) Canine, oral melanoma. Colocalization of Iba1 (green) and MAC387 (red). In melanomas, MAC387$^+$cells were few and often did express also Iba1. **(B)** Canine, cutaneous melanoma. In areas near superficial ulceration, the number of cells co-expressing Iba1 and MAC387 was elevated. Nuclear counterstain was performed with DAPI.

(49, 50); however, our present results show that this protein is expressed only on a small percentage of intratumoral macrophages and that therefore it should not be considered a sensitive marker for this cellular population. The expression of this marker is likely limited to recently recruited macrophages, as reported in other studies (51). Double labeling showed an increase of the presence of a population of cells co-expressing MAC387 and Iba1 near areas of active inflammation (near superficial ulcers), together with neutrophilic infiltration. This finding may indicate a possible M1-polarization of macrophages expressing MAC387, and support the hypothesis of a recent migration of these cells to the affected tissue (51, 52).

In the present study, the number of CD163$^+$cells/HPF was significantly higher in oral melanomas when compared to oral melanocytomas; moreover, a moderate positive correlation was observed with nuclear atypia and mitotic index, together with a negative correlation with pigmentation and the presence of melanophages. Taken together, these results seem to indicate an increase of CD163$^+$cells in association with malignant prognostic markers (nuclear atypia and mitotic index), and an inverse correlation with pigmentation, usually associated with a better outcome (53).

CD163 expression was also higher in melanomas with evidence of metastasis and in tumors from dogs that died because of the tumor. These results are similar to human melanoma, where CD163 expression has shown a robust association with worse overall survival (18, 35). An increase in CD163 expression is similarly reported in other high-grade canine tumors, such as lymphomas and gliomas (33, 46). CD163 is considered a marker for M2-polarized macrophages, hence, the presence of numerous CD163$^+$cells supports the hypothesis of an activation of immunosuppression and immunoescape mechanisms within the tumor microenvironment also in canine melanomas, as we previously hypothesized by testing the expression of IDO, Foxp3, and CTLA-4 (41, 54).

CD204 was significantly more expressed in oral melanomas when compared to the benign counterpart, but this difference was not observed in the cutaneous melanocytic tumors; overall, a mild correlation with nuclear atypia and mitotic index was present. These results seem to indicate that also this marker may be associated with prognosis, as reported in other studies in both humans and dogs (17, 31, 33, 35), but further studies with a larger cohort with a complete follow-up are needed to substantiate these results in canine melanomas.

Iba1 expression showed a significant difference between cutaneous melanomas and melanocytomas, but not in the oral counterpart and was moderately correlated with nuclear atypia. This marker is considered a pan-myeloid in humans and has shown a negative impact on survival in patients with glioblastoma, despite not reaching a statistical significance (29); moreover, Iba1 has been suggested as a prognostic marker and a possible therapeutic target in human hepatocellular carcinomas (30). In veterinary medicine, a linear correlation between Iba1 expression and the mitotic count has been observed in canine soft tissue sarcomas (32). As a matter of fact, the role of Iba1$^+$cells in tumor microenvironment has not been completely elucidated, but its favoring function in a pro-tumor immune microenvironment and as a negative prognostic factor seems likely.

Last, MAC387 was more expressed in oral melanomas compared to melanocytomas and was moderately associated with the mitotic count. This finding may be associated with

the presence of ulceration, which was more common in oral melanomas compared to the benign counterpart, where numerous cells co-expressing MAC387 and Iba1 were observed by means of double immunofluorescence. Despite performing cell counts far from areas of ulceration, the presence of these cells may be justified by these events, even if ulceration is not immediately adjacent. Anyway, it is important to stress that, in general, the number of MAC387$^+$ cells was noticeably lower when compared to the other markers. The exact role of MAC387$^+$ macrophages is still not clear; in some studies, they are reported as M1-polarized macrophages (33, 55), whereas some other results seem to address them as an M2-like population (39).

The lack of statistical significance evidenced between cutaneous melanomas and cutaneous melanocytomas for CD163, CD204 and MAC387 expression, may reflect the unpredictable behavior of the first group of tumors. Also, it can be hypothesized that some tumors, histologically classified as cutaneous melanomas, should be reclassified as melanocytomas, also based on the biological behavior. Hence, a review of the histological prognostic features (40) for cutaneous melanocytic tumors might be considered, in order to increase their accuracy.

The partial co-expression of TAM markers evidenced by our study also confirms the complexity of TAMs within canine melanocytic tumors, particularly within the malignant ones. Therefore, further investigation is recommended to better identify the roles of each macrophagic subpopulation and to assess their use as prognostic markers. M2 markers are usually associated with worse overall survival in human neoplastic diseases, but also the anatomical region may influence these associations (27). This may partially explain the differences we observed between oral and cutaneous tumors and deserves further investigation.

The present study underlines the potential prognostic role of TAMs in canine melanocytic tumors.

The main limits that we encountered are due to the lack of specific macrophagic markers in dogs; for instance, CD68, which is considered a pan-macrophagic marker in humans, does not seem suitable for canine FFPE samples. To understand and define the universe of TAMs, it is necessary to complement these morphological studies with biomolecular approaches and our group is currently working on the phenotypic and proteomic characterization of macrophages and histiocytes in canine melanocyte tumors.

Another problem is associated with the growing difficulty in having homogeneous groups to perform survival analysis. Regarding this, some of our groups were not numerically homogeneous (i.e. oral melanocytomas). However, this reflects a real epidemiological difference and confirms the need to collect more data on these rare categories of melanocytic tumors. Different therapeutic approaches can deeply alter the results of survival analyses; therefore, we decided to analyze the prognostic significance of TAMs markers only in association with the presence of metastasis and on the outcome.

The results of this study confirm that TAMs may represent an interesting target for future immunotherapeutic approaches in dogs; moreover, these data may be useful for comparative studies, particularly with human oral melanoma, for which the canine counterpart is recognized as a valuable spontaneous model (24, 56–59). CD163 expression has shown a significantly different profile and potential prognostic significance in oral melanomas and melanocytomas, suggesting an immunosuppressive and pro-tumorigenic role of this cellular subpopulation. Moreover, the functional characterization of CD163$^+$ and CD204$^+$ macrophages in oral melanocytic tumors and of Iba1$^+$ cells in cutaneous tumors may help to deepen our knowledge of the immune pathways involved in melanoma progression and mediated by TAMs. Overall, this study shows that TAMs in canine melanocytic tumors deserve further investigations, not only as prognostic biomarkers, but also as future feasible therapeutic targets for both canine and human disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the study was performed on archive FFPE material harvested for diagnostic or curative scopes. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

IP, MS, and CB conceived the presented idea, classified, and selected the tumor cases. IP wrote the manuscript. LM supervised the findings of this work. AG, IB, and AM performed the experiments on tumor tissues. EC and AT validated the antibodies by western blotting. IP, MS, IB, and AM performed cell counting. IP performed the statistical analysis. All authors discussed the results and contributed to the final manuscript.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.878949/full#supplementary-material

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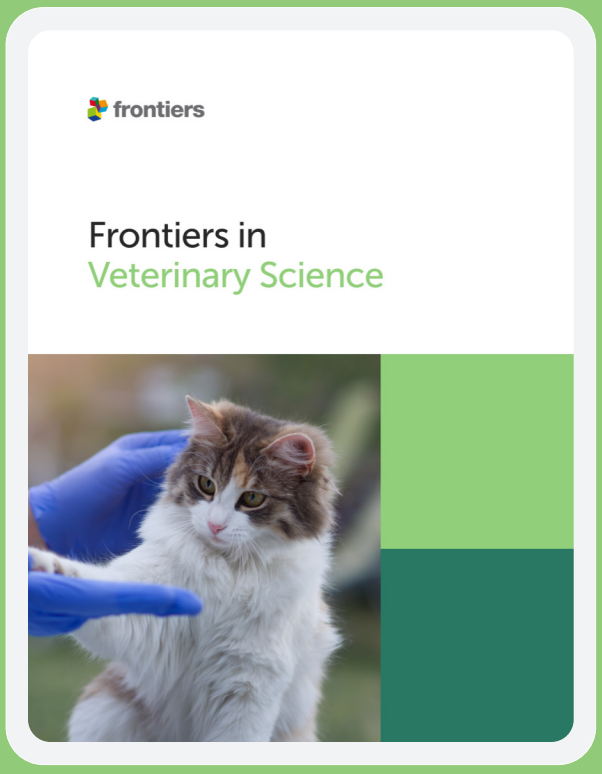

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