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Title: The immune microenvironment of canine melanocytic tumors: an investigation on immunoescape pathways

Short running title: Melanoma immunoescape in dogs

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ABSTRACT

Despite recent promising immunotherapy strategies in human melanoma, studies on the immune environment of canine melanocytic tumors are few. In humans, the activation of immunosuppressive cell subpopulations such as T regulatory cells (Tregs), expressing the factor forkhead box protein P3 (FoxP3), the engagement of surface receptors with immunosuppressive functions, namely cytotoxic T lymphocyte antigen (CTLA-4), and the secretion of molecules inhibiting lymphocytes functions, as indoleamine-pyrrole 2,3-dioxygenase (IDO), are recognized, among others, as immunoescape mechanisms that allow tumor growth and progression.

Aim of our study is to investigate the expression of these immunosuppression markers in canine melanocytic tumors, and to evaluate their possible involvement in tumor biology and progression.

Fifty-five formalin-fixed and paraffin-embedded canine melanocytic tumors (25 oral melanomas; 20 cutaneous melanomas; 10 cutaneous melanocytomas) were selected to investigate the expression of FoxP3, CTLA-4 and IDO by immunohistochemistry and qRT-PCR.

These markers showed a high gene and protein expression in oral melanomas. FoxP3 protein expression was associated with an increased hazard of death (univariate: $P<0.001$; multivariate $P<0.05$). Both gene and protein expression of CTLA-4 was associated with a worse prognosis (univariate: 0.001 and <0.05, respectively). Also, IDO gene and protein expression was associated with an increased hazard of death both at univariate and multivariate analysis ($P<0.001$).

FoxP3, CTLA-4 and IDO likely play a role also in canine melanoma immunoescape and progression; moreover, the expression of these molecules could be a helpful

prognostic tool in canine melanoma and, by comparative approach, could pave the way to future immunotherapeutic approaches in dogs.

KEYWORDS

Melanoma, immunosuppression, FoxP3, CTLA-4, Indoleamine-pyrrole 2,3-Dioxygenase, dogs, prognosis.

Human melanoma is recognized as one of the most immunogenic tumors; it has been shown that melanoma cells bear a high mutational burden compared to other malignancies, being able to acquire also hundreds of mutations per megabase.[1,32,50] Recently, the tumor heterogeneity has been demonstrated to further contribute to the determination of the host immune response, being also better than tumor mutational burden in predicting immunotherapy outcome.[20,78] Despite melanoma immunogenicity, the host immune response is not effective in controlling tumor progression, being the tumor itself able to model the immune response to its own benefit through the process of immunoediting.[16] It is currently believed that the interplay between the tumor and the immune system can be ideally divided in three phases; the first one described, the elimination phase, allows the development of a tumor specific immunity, providing the host, by homing of specific CD4+ and CD8+ cells, with the capacity to eliminate the tumor. The equilibrium phase is characterized by a dynamic balance between tumor cell variants that survived the elimination phase and the host immune system, whereas during the escape phase selected tumor cells can avoid immune detection and elimination.[17,41] The role of different immune cellular populations in the process of immunoediting has been widely investigated in human medicine. This led to the development of new strategies of

immunotherapy, particularly by immune checkpoint blockade, that targets the natural immune system of the host, improving or restoring protective immune functions, as well as inhibiting immunosuppressive pathways activated during the escape phase. This type of tumor treatment has been applied to different types of cancer, especially melanoma, resulting in good and durable response, even in patients with metastatic disease.[9,37]

The transcription factor forkhead box protein P3 (FoxP3) is involved in ensuring immune homeostasis,[34] but is also a key-molecule associated with suppression of the activity of cytotoxic T cells in tumor immune response.[30,34] The presence of $FoxP3^+$ Tregs has been associated with a negative prognosis both in human melanoma and other solid cancers.[22,39,55] A recent study also showed that Tregs were less numerous in areas of melanoma regression, confirming their potential role in the establishment of an immunosuppressive environment.[23] Moreover, in a murine melanoma model it was recently shown that a selective FoxP3 depletion achieved through vaccination leaded to the depletion of myeloid-derived stem cells (MDSCs), the reduction of tumor growth, and the improvement in survival rates,[47] supporting the role of Tregs in tumor progression and growth. The presence of $FoxP3^+$ Tregs has been also reported in canine tumors, included melanoma.[10,52,59]

Cytotoxic T lymphocyte antigen (CTLA-4), also known as CD152, is a member of the family of immunoglobulin-related receptors expressed on both activated and regulatory T cells (Tregs), responsible for T cells immune regulation and preservation of a normal immune environment. CTLA-4 binds with high affinity B7 ligands (CD80 and CD86) on antigen presenting cells (APCs). This leads to the inhibition of T cell response and cycle (T cell exhaustion), and antagonization of the binding of the T cell-stimulating receptor, CD28.[14,15,57,64,75] The importance of CTLA-

4 in immune response was stated when a fatal autoimmunity was observed in CTLA-4-deficient mice due to the release of self-reactive T cells, indicating CTLA-4 as a negative regulator of T cell response.[74] Besides, anti-tumor immunity is predominantly mediated by T cells and CTLA-4 has been shown to play a pivotal role in cancer-associated immunoediting, particularly in the escape phase.[64,75] The persistent antigen exposition by melanoma cells and the chronic stimulation of the immune system seems to be critical in the hyperactivation of inhibitory checkpoints on immune cells such as CTLA-4, resulting in a negative feedback on cytotoxic T cells (Figure 1).[24] For its functions, CTLA-4 is also the target of Ipilimumab, an immune checkpoint inhibitor, that proved to be useful in melanoma treatment.[8,33] Another pathway that could contribute to peripheral tolerance and therefore to cancer immunoescape, is mediated by indoleamine-pyrrole 2,3-dioxygenase (IDO), an enzyme with immunosuppressive properties that is postulated to impair the antitumor immune response also in melanoma.[53] IDO can be produced by MDSCs, dendritic cells (DCs), macrophages, and tumor cells themselves, and it is believed to inhibit effector T-cells by depleting tryptophan within the tumor microenvironment.[27,44,46] Tryptophan catabolites (such as L-kynurenine) can suppress the proliferation of activated T cells and, at the same time, both promote the differentiation and activation of Tregs and the CTLA-4 expression.[13] Tregs, in turn, suppress the activation and function of other leukocytes, contributing to the establishment of an immunosuppressive environment and also stimulate IDO production and activation (Figure 1).[43] The inhibition of IDO, in combination with other immunotherapeutic drugs, leads to an improvement in response rate during melanoma therapy.[7] IDO blockade can in fact reduce tumor growth, intratumoral immunosuppression, and stimulate robust systemic antitumor effects.[28,31,42]

During the last years, growing evidences point at canine melanomas, particularly the mucosal ones, as a possible predictive preclinical model for human melanoma,[25] but further studies are recommended to better characterize the canine disease, also on the immunological front.

Aim of this study is to retrospectively investigate the presence of mechanisms of immunoescape and immunosuppression in canine melanocytic tumors, through the analysis of FoxP3, CTLA-4, and IDO gene and protein expression and to gain more information on the possible similarities with human melanoma immunology.

MATERIALS AND METHODS

Case selection

The retrospective case selection had to meet the following inclusion criteria:

- a histological diagnosis of melanoma or melanocytoma,[68] with immunohistochemical positivity for Melan-A and/or PNL2;
- availability of follow-up information;
- a minimum time to follow-up of 365 days.

Mitotic count was assessed, following a proposed standardized method.[40] A telephonic survey was conducted with the referring veterinarians, to collect data on the clinical tumor staging, the follow-up, the presence of local recurrence, and the cause of death. Disease-free and overall survival were calculated from the day of the sample registration in our Department.

Immunohistochemical labeling and evaluation

Samples were cut into 5 µm sections, mounted on poly-L-lysine coated slides, dewaxed and rehydrated. Heavily pigmented tumors were bleached overnight at

150 room temperature with 30% H_2O_2 following a standardized protocol.[52]
151 Immunohistochemistry was performed on serial sections with antibodies against
152 FoxP3 (1:100 dilution; rat monoclonal, Clone FJK-16s; Thermo Fisher, Waltham,
153 Massachusetts, US), CTLA-4 (dilution 1:100; mouse monoclonal, clone F-8; Santa
154 Cruz Biotechnology, Dallas, Texas, US), and IDO (1:50 dilution; rabbit polyclonal;
155 Biorbyt, Cambridge, UK) as previously reported.[52] Tris-EDTA (pH 9.0) was used to
156 perform heat-induced epitope retrieval for CTLA-4. Positive reaction was revealed
157 with 3-amino-9-ethilcarbazole (Dako, Glostrup, Denmark); Mayer's hematoxylin was
158 applied as a counterstain. Reactive canine lymph node was used as a positive
159 control for all the antibodies of this study. Negative controls were run by incubating
160 sections with TBS and omitting the primary antibody and by incubating control-tissue
161 with antibody isotype (only for monoclonal antibodies) to assess the absence of non-
162 specific staining. Positive cells were counted by two operators, in 5 HPF (FN 20),
163 selecting "hot spots" and avoiding areas of necrosis and/or near ulceration; a mean
164 value was then obtained for each case and expressed as the number of positive
165 cells/HPF. The same method was applied for the evaluation of FoxP3, CTLA-4, and
166 IDO positive cells. The expected labeling was nuclear for FoxP3, both membrane
167 and cytoplasmic for CTLA-4, and granular and cytoplasmic for IDO.
168
169 *RNA extraction and Real Time PCR*
170 Three-to-5 (depending on sample size), 8 µm-thick, sections were cut from paraffin
171 blocks. Normal tissue around the tumor was resected and discarded with the help of
172 a sterile scalpel blade or a sterile needle. RNA extraction was performed with a
173 commercial kit (Invitrogen™ PureLink™, FFPE RNA Isolation Kit) following the
174 manufacturer's instructions. Residual genomic DNA was removed from the total RNA

by DNase I amplification grade (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's specifications. RNA quantity was evaluated by means of both NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and Qubit 2.0 Fluorometer (Life Technologies, MA, USA). Total RNA (500 ng) was reverse transcribed using the SuperScript® VILO™ Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's specifications. Successful reverse transcription was confirmed by PCR amplification of the *Canis familiaris* GUSβ gene (NM_001003191). Primers on reference genes (GUSβ, HMBS) and on genes of interest were designed on available sequences using the Primer-BLAST suite (Table 1). Whenever possible, primers were located in different exons or at exon–exon junction to minimize inaccuracies due to residual genomic DNA contamination. For each primer pair, a preliminary qRT-PCR assay was performed on a bulk of samples and amplification of non-specific products or primer-dimer artifacts and efficiency (E) were assessed. The qRT-PCR reactions were carried out on CFX96 Touch instrument (BioRad, Hercules, CA) as previously described.[6] Data analysis was carried out with Bio-Rad CFX Manager software (ver. 3.2.2). To analyse gene expression stability of HKGs, geNorm algorithm, included on CFX Manager software (vers. 3.2.2), was applied.[76] geNorm provides a ranking of the tested genes, considering their expression stability, selecting reference genes according to the stability measure M (average pairwise variation of each gene against all others). The expression ratio of the genes of interest was normalized to the relative abundance of the two reference genes using the ΔΔCq method. Non-detects were imputed with GenexPro software (ver.6) to avoid introducing bias as previously reported.[38]

Cell line validation statement

Cell line validation work has not been conducted due to no cell lines use in this study.

Statistical Analysis

Diagnostic graphics were used to test assumptions and outliers. We analyzed distributions within the categorical variable “breed” using Chi-Square Goodness of FitTests. Differences in number of positive cells and mRNA expression of IDO, FoxP3, and CTLA-4 between diagnoses were analyzed using Kruskal Wallis and Mann-Whitney tests. Values were expressed as medians (Mdn) with interquartile range (IQR). Correlations were evaluated by using Spearman rank correlation coefficient (ρ). Correlation was defined as high when absolute value of $\rho>0.5$, medium when ρ ranged from 0.3 to 0.5, and low when $\rho<0.3$.[19] We used the Life Table method to determine survival probabilities. The differences of survival rate according to diagnosis were evaluated by Kaplan–Meier curve and log-rank test. We used the Cox proportional hazards model to evaluate the influence of parameters on survival. All variables with $P<0.05$ on the univariate analysis were entered into the multivariate model and adjusted for age and mitotic count. We used variance inflation factors (VIF) to identify multicollinearity.[19] The prognostic significance of each variable was expressed as hazard ratio (HR) with corresponding 95% confidence intervals (CIs) and P values. Finally, we used the receiver operating characteristic (ROC) analysis to assess the diagnostic accuracy of the parameters and their cut-off for predicting survival. Optimal cut-off values were determined as points on the curve closest to (0, 1) and by the Youden index. Then, dichotomous variables for each parameter were created based on their cut-off and submitted to

Cox regression adjusting for age and mitotic count. All statistical analyses were performed using SPSS 25.0 software (IBM, SPSS Inc., Chicago, IL, USA) and statistical significance was set at $P \leq 0.05$.

RESULTS

Sample population and mitotic count

The final caseload was represented by 25 oral melanomas, 20 cutaneous melanomas, and 10 cutaneous melanocytomas. Thirty-five dogs were male (6/35, 17.1% were neutered) and 19 were female (6, 31.6% were spayed). For one animal the age was unknown. Most of the dogs were mixed breed (19/55, 33.9%; $P<0.001$), followed by German Shepherd and Dachshund (5/55, 8.9% each), Labrador Retriever (4/55, 7.1%), and Boxer (3/55, 5.4%). The median age was 11 years (range, 1–16 years). Follow-up at the end of the study ranged from 365 to 1615 days, with a median follow-up of 653 days. More than 60% of the study population had a follow-up time longer than 2 years at the end of the study.

The 6-month and 1-year estimated survival probabilities are shown in Table S1 (Supplemental material). Median survival time for mucosal melanoma was 240 days (IQR= 77-433 days), while it was not reached for cutaneous melanoma. Log-rank test showed lower survival time for dogs with mucosal melanoma compared to dogs with cutaneous melanoma ($P=0.005$). No deaths or recurrences were recorded for the cases of melanocytoma.

Mitotic count was higher in oral melanomas (Mdn= 42, IQR=32-61) than in cutaneous melanomas (Mdn= 14, IQR=7-50, $P<0.05$) and melanocytomas (Mdn= 1, IQR=0-2, $P<0.001$).

Immunohistochemistry

FoxP3, CTLA-4, and IDO proteins were expressed in all melanomas, both oral and cutaneous, while some cutaneous melanocytoma samples were completely negative. Immunohistochemical labelling revealed that the number of FoxP3-positive nuclei/HPF (Figure 2, A) was higher in oral melanomas, than cutaneous melanomas and cutaneous melanocytomas. Similarly, the number of CTLA-4 positive nuclei/HPF (Figure 2, B) was higher in oral melanomas than in cutaneous melanomas and cutaneous melanocytomas. The number of IDO positive nuclei/HPF was higher in oral melanoma than in cutaneous melanomas and cutaneous melanocytoma. Results are summarized in Figure 5.

qRT-PCR

Both *GUSβ* and *HMBS* genes displayed a relatively high stability with M values of 0.4, far below the accepted limit of 1.5.[76] The expression level of the three genes was also associated with the histological diagnosis ($P<0.001$). *IDO* and *CTLA4* gene expression was significantly up-regulated in the group of oral melanomas compared to cutaneous melanomas ($P<0.05$) and cutaneous melanocytomas ($P<0.001$). The expression of *FOXP3* was instead upregulated in both oral and cutaneous melanomas, when compared to cutaneous melanocytomas ($P<0.001$). Results of gene expression are summarized in Figure 5.

Correlations between parameters

All the examined parameters, both evaluated with immunohistochemistry and qRT-PCR, showed positive correlations between them and with the mitotic count (Table S2, Supplemental material). A strong correlation was observed between $FoxP3^{+}$

cells/HPF and CTLA-4$^+$ cells/HPF (ρ=0.709, P<0.01). Also, mitotic count strongly correlated with the protein expression of FoxP3 (ρ =0.706, P<0.01) and CTLA-4 (ρ =0.650, P<0.01), and with gene expression of *IDO* (ρ =0.563, P<0.01).

Prognostic significance of IDO, FoxP3, and CTLA-4

In the entire cohort, the univariate Cox analysis (Table 2) showed an increased hazard of death in association with an increased expression of IDO and CTLA-4 (P<0.05), both at the protein and mRNA level. The expression of FoxP3 was associated to the hazard of death only when evaluated by immunohistochemistry (P<0.01). Death due to melanocytic tumor was also related to mitotic count (P<0.01) and animal's age (P<0.05). A multivariate model adjusted for age and mitotic count was built, including IDO, FoxP3, and CTLA-4 positive cells/HPF, *IDO* mRNA, and *CTLA4* mRNA. This model showed that only IDO (P<0.01) and FoxP3 (P<0.05) protein expression remained significant, holding constant the other predictors.

We investigated the sensibility and specificity associated to the IDO$^+$ value of 14.7 cells/HPF (optimal cut off reported in our previous study);[52] results shown a 57% sensibility and 79% specificity, in face of an 82% sensitivity and 68% specificity associated with a cut-off value of 8.4 resulted as optimal in the present study group. Table 3 shows the results of Receiver Operator Characteristic analysis. The highest area under the curve (AUC) was found for FoxP3$^+$ cells/HPF (AUC=0.849; P<0.001), followed by *CTLA4* mRNA (AUC=0.802; P<0.001) and *IDO* mRNA (AUC=0.798; P<0.001).

Then, dichotomous variables were created for each parameter based on their optimal cut-offs (< or ≥ of the cut-off) and analyzed by Kaplan-Meier survival curves (Figure 6) and Cox models adjusting for age and mitotic count (Table 4). All these

variables, except *CTLA4* mRNA, had a prognostic value related with survival independently of age and mitotic count ($P<0.05$; Table 4). In particular, FoxP3 ≥ 6.9 cells/HPF had a significantly higher hazard of death (HR=12.20, 95%CI=2.37-62.72).

DISCUSSION

In human medicine, the growing number of studies on the characterization of melanoma immune environment led to the successful use of immunotherapy, particularly by targeting PD-1 and CTLA-4.[49] In veterinary medicine, the studies on cancer immunity and on the application of immunotherapy are still few.[2,35,52] Canine cutaneous melanomas are usually benign and surgical resection is typically curative, still their behavior can be quite unpredictable, since highly aggressive forms are observed.[18,61,69] On the other hand, mucosal melanomas, particularly oral melanomas, show a malignant behavior with a predisposition to the development of metastasis to lymph nodes and lungs, similarly to human melanomas.[67] At now, different studies suggest the dog as a valuable spontaneous preclinical model in melanoma research, particularly since the common canine oral form has been demonstrated to share numerous similarities with the more rare human disease.[54,66,77]

While different aspects of canine melanoma biology have been investigated, [5,6,25,26,29,60,65] the immune environment interacting with this type of tumors is still largely unknown. Therefore, our study aims at investigating the immune environment of canine melanocytic tumors to acquire further information on the mechanisms of immunosuppression and evasion possibly involved in tumor progression; targets of our investigation are in particular FoxP3, CTLA-4, and IDO.

The survival time within our group of dogs with oral melanomas showed a mean survival time of 240 days, calculated from the moment of the submission of the sample for histological diagnosis. The mean overall survival within our group is higher then what is described in previous literature, with reports of a mean survival time of 147 days.[70] This result is probably due to a growing attention of the owners towards the health of their dogs, and therefore to an early diagnosis, and also probably because labial melanomas, which are reported to have a longer survival time, were included within our oral melanoma group. This result endorses the necessity for further studies to better characterize oral and mucocutaneous canine melanocyte biology, together with melanoma behavior in association with different site of origin of the primary tumor. Moreover, a detailed description of the anatomical site of origin of the tumor should be provided by the clinician/surgeon at the moment of the submission of the tissue sample for histopathological analysis, in order to gain more precise data to define the prognostic significance of the primary tumor location. FoxP3 is an intracellular molecule involved in Tregs development and function, and considered, at now, their most specific marker.[62] In our study, FoxP3 immunohistochemical expression was associated with a higher hazard of death for melanoma, also in the adjusted model. Nevertheless, *FOXP3* gene expression was bordering significance at the univariate Cox analysis (P=0.069), probably due to the use of FFPE material for mRNA extraction, which could have caused a partial degradation of nucleic acids. The survival analysis, based on the cut-off value, indicated that the hazard of death was 12 times higher in dogs with FoxP3≥6.9 cells/HPF. These results seem to confirm that, also in dogs, a higher infiltration of $FoxP3^{+}$ cells is associated with a worse prognosis, similarly to what reported in human melanomas.[21,48] The aforementioned cut-off value was similar to the 6.1

cells/HPF value previously reported by our group,[52] but showed both higher sensitivity and sensibility. Results from our study seem to confirm that FoxP3 could be a major player in mechanisms of immunoescape that favors tumor growth and progression also in dogs, particularly in oral and cutaneous melanomas. Furthermore, the strong correlation between FoxP3 and CTLA-4, together with the moderate correlation with IDO protein expression, may be in the synergic role of these proteins in the establishment of an immunosuppressed tumor microenvironment. A strong correlation was also evidenced between FoxP3 protein expression and mitotic count, accounting for Treg role in favoring tumor growth. At the moment, the markers to identify Tregs are few and often not completely specific also in human medicine,[56] making the characterization and the definition of the role of this T cell subpopulation, within tumor immune environment, complex and still not completely understood. Also, it must be reminded that the presence of $FoxP3^+$ Tregs could be influenced by tumor site, molecular subtype of the tumor, and tumor stage.[62] Therefore, further investigations should be encouraged to overcome these limits in defining Treg biology and role in cancer, both in veterinary and comparative medicine.

During the last few years, immunotherapies with monoclonal antibodies directed against CTLA-4, together with anti-PD1, have revolutionized the treatment of patients with advanced melanoma in human medicine, but still the presence and the role of CTLA-4-expressing cells have been limitedly explored in dogs and in veterinary oncology. In the present study, we found evidence of the expression of this molecule in canine melanocytic tumors. Our results indicate that both CTLA-4 immunohistochemical and gene expression were associated with the histological diagnosis and with an increased hazard of death (univariate analysis), similarly to

what is reported in human melanoma.[11] However, in the multivariate analysis, CTLA-4 lost its statistical significance, suggesting that CTLA-4 is not an independent predictor. This result, on one hand, is probably due to the association of this marker with other variables, perhaps to be searched within the complexity of the tumor immune environment itself. On the other hand, the association between the protein and gene expression of this marker and the tumor mitotic count, which is considered one of the most affordable prognostic features of canine melanomas,[3,68] seems to corroborate the hypothesis of the immunosuppressive role of this molecule in melanoma growth. A larger study group should be therefore investigated, to gather more information on the role of this molecule in melanocytic tumors of dogs. To our knowledge, this is the first study that focuses on CTLA-4 within canine melanoma microenvironment, in contrast to previous studies aimed at characterizing the expression of this costimulatory molecule in circulating cells during neoplastic disease and in a healthy subject.[71–73] Our results, although preliminary, highlight the presence of this molecule within canine melanoma and open the way to further investigations on the role of CTLA-4-associated pathways in canine oncology.

The enzyme IDO can be expressed by different types of cells, in particular MDSCs, DCs, macrophages, but also tumor cells. This enzyme acts both on APCs and T cells, causing immune suppression and therefore facilitating cancer development.[4] Our results show that IDO immunohistochemical expression was an independent predictor of mortality, both at univariate and multivariate Cox proportional regression analysis. The optimal cut-off value for IDO immunohistochemical expression was set at 8.4 cells/HPF, compared to the 14.7 cells/HPF value evidenced in our previous study. In addition to the different characteristics of the sample population, this incongruity can also be explained by the different percentages of sensitivity and

399 specificity associated with this new cut-off. Indeed, in the present study, the lowest
400 cut-off was associated with a higher sensitivity (82%) in the prediction of death of the
401 dog due to melanoma. Indeed, by setting the IDO cut-off at 14.7 cells/HPF in the
402 case series of the present study, the specificity improves, reaching the value
403 indicated in our previous study (79%), but it is not being balanced by an adequate
404 sensitivity (57%). The greater accuracy indicated by the higher AUC of the present
405 study group, together with the higher number of cases with a complete follow-up (55
406 vs 52), suggests that the lower cut-off should be preferred. The role of IDO in canine
407 melanoma seems to be similar to what reported in humans, where IDO protein
408 expression is considered to have a prognostic role in both cutaneous melanoma and
409 nodal metastases.[12,51,58] Gene expression, on the other hand, was significant only at
410 the univariate Cox analysis. The loss of significance could be due to the high
411 variability of mRNA expression detected by qRT-PCR; the possible explanation of
412 this result could be, as for *FOXP3*, the use of FFPE material to retrieve mRNA. In
413 fact, even though numerous studies are based on this protocol for studies of gene
414 expression, fresh-frozen tissue should be preferred to avoid partial mRNA
415 degradation.[36] Our results seem to confirm that IDO is involved in canine tumor
416 immunoescape and progression. Also, IDO could be implicated in the activation of
417 Treg cells within canine melanoma microenvironment, as indicated by the moderate
418 correlation between the variables and also by other authors in different models.[45,63]
419 The results of the multivariate analysis confirm that the mitotic count, the number of
420 IDO^+ cells/HPF, and of $FoxP3^+$ cells/HPF can be considered independent prognostic
421 factors, suggesting that, if further confirmed by prospective studies, these markers
422 could be useful in the oncological evaluation of canine melanocytic tumors.

Taken together, the results from our study seem to confirm the presence of immunosuppressive tumor microenvironment mechanisms controlled by FoxP3, CTLA-4 and IDO, also in canine melanoma, particularly in the most aggressive oral form. After this retrospective investigation, prospective studies on fresh/frozen tissue aiming at the confirmation of these results, also including and extending our investigation to other immune populations and to metastatic lesions, have been planned. Further investigations on the immune environment of canine melanocytic tumors should be stimulated, aiming both at a better characterization of canine melanoma biology and immune environment, also for comparative purposes, and at a future possible employment of immunotherapeutic strategies also in the canine species.

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Figure 1. Immunosuppressive interplay within melanoma microenvironment. MCs: melanoma cells; MDSCs: myeloid-derived stem cells; DCs: dendritic cells; TAMs: tumor associated macrophages; IDO: indoleamine 2,3 dioxygenase; CTLA-4: cytotoxic T-lymphocyte-associated protein 4. IDO is produced by MDSC, DCs, macrophages, and neoplastic cells. IDO can stimulate CD4+ cells to differentiate into Tregs, causing an upregulation of FoxP3 and can also activate mature Tregs. Tregs, in turn, can induce IDO. The persistent antigen exposition by melanoma cells and the chronic stimulation of the immune system, together with IDO production, seems to be critical in the hyperactivation of CTLA-4, which leads to peripheral cell tolerance.

Figure 2. Cutaneous melanoma, haired skin, dog, case n.33. Immunohistochemistry for forkhead box P3 (FoxP3) in a sample of oral melanoma, with disseminated cells within the tumor. AEC and hematoxylin.

Figure 3. Cutaneous melanoma, haired skin, dog, case n.44. Scattered intratumoral CTLA-4^{+} cells (arrows). AEC and hematoxylin.

Figure 4. Oral melanoma, oral mucosa, dog, case n.16. Peripheral infiltration of IDO^{+} cells with granular cytoplasmic reactivity (likely macrophages). AEC and hematoxylin.

Figure 5. Box plots of FoxP3, CTLA-4, and IDO, positive nuclei/HPF in the upper panels, and *FoxP3*, *CTLA-4*, and *IDO* mRNA in the lower panels according to

673 diagnosis. ns=not significant, *P<0.05, **P<0.01, ***P<0.001 (multiple comparisons
674 by Mann-Whitney tests).
675
676 Figure 6. Kaplan-Meier survival curves by optimal cut-off values of IDO (8.4
677 cells/HPF), FoxP3 (6.9 cells/HPF), and CTLA-4 (2.2 cells/HPF), and *FOXP3* (35.9
678 mRNA expression level), *CTLA4* (10.1 mRNA expression level), and *IDO* (22.7
679 mRNA expression level).