AperTO - Archivio Istituzionale Open Access dell'Università di Torino

FoxP3, CTLA-4, and IDO in Canine Melanocytic Tumors

This is a pre print version of the following article:

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1777712> since 2021-03-05T11:59:46Z

Published version:

DOI:10.1177/0300985820960131

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

TITLE PAGE

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

Short running title: Melanoma immunoescape in dogs

AUTHORS: Ilaria Porcellato*, Chiara Brachelente*, Katia Cappelli*, Laura Menchetti*, Serenella Silvestri*, Monica Sforza*, Samanta Mecocci*, Leonardo Leonardi*, Luca Mechelli*

*Department of Veterinary Medicine, University of Perugia, Italy

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

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

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

93 Cytotoxic T lymphocyte antigen (CTLA-4), also known as CD152, is a member of the
94 family of immunoglobulin-related receptors expressed on both activated and
95 regulatory T cells (Tregs), responsible for T cells immune regulation and
96 preservation of a normal immune environment. CTLA-4 binds with high affinity B7
97 ligands (CD80 and CD86) on antigen presenting cells (APCs). This leads to the
98 inhibition of T cell response and cycle (T cell exhaustion), and antagonization of the
99 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

room temperature with 30% H₂O₂ following a standardized protocol.⁵² Immunohistochemistry was performed on serial sections with antibodies against FoxP3 (1:100 dilution; rat monoclonal, Clone FJK-16s; Thermo Fisher, Waltham, Massachusetts, US), CTLA-4 (dilution 1:100; mouse monoclonal, clone F-8; Santa Cruz Biotechnology, Dallas, Texas, US), and IDO (1:50 dilution; rabbit polyclonal; Biorbyt, Cambridge, UK) as previously reported.⁵² Tris-EDTA (pH 9.0) was used to perform heat-induced epitope retrieval for CTLA-4. Positive reaction was revealed with 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark); Mayer's hematoxylin was applied as a counterstain. Reactive canine lymph node was used as a positive control for all the antibodies of this study. Negative controls were run by incubating sections with TBS and omitting the primary antibody and by incubating control-tissue with antibody isotype (only for monoclonal antibodies) to assess the absence of non-specific staining. Positive cells were counted by two operators, in 5 HPF (FN 20), selecting "hot spots" and avoiding areas of necrosis and/or near ulceration; a mean value was then obtained for each case and expressed as the number of positive cells/HPF. The same method was applied for the evaluation of FoxP3, CTLA-4, and IDO positive cells. The expected labeling was nuclear for FoxP3, both membrane and cytoplasmic for CTLA-4, and granular and cytoplasmic for IDO.

RNA extraction and Real Time PCR

Three-to-5 (depending on sample size), 8 µm-thick, sections were cut from paraffin blocks. Normal tissue around the tumor was resected and discarded with the help of a sterile scalpel blade or a sterile needle. RNA extraction was performed with a commercial kit (Invitrogen™ PureLink™, FFPE RNA Isolation Kit) following the 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* GUS β gene (NM_001003191). Primers on reference genes (GUS β , 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 $\Delta\Delta Cq$ 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 Fit Tests. 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 ($\rho=0.709$, $P<0.01$). Also, mitotic count strongly correlated with the protein expression of FoxP3 ($\rho=0.706$, $P<0.01$) and CTLA-4 ($\rho=0.650$, $P<0.01$), and with gene expression of *IDO* ($\rho=0.563$, $P<0.01$).

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 $\geq$ 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.

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

349 cells/HPF value previously reported by our group,⁵² but showed both higher
350 sensitivity and sensibility. Results from our study seem to confirm that FoxP3 could
351 be a major player in mechanisms of immunoescape that favors tumor growth and
352 progression also in dogs, particularly in oral and cutaneous melanomas.

353 Furthermore, the strong correlation between FoxP3 and CTLA-4, together with the
354 moderate correlation with IDO protein expression, may be in the synergic role of
355 these proteins in the establishment of an immunosuppressed tumor
356 microenvironment. A strong correlation was also evidenced between FoxP3 protein
357 expression and mitotic count, accounting for Treg role in favoring tumor growth. At
358 the moment, the markers to identify Tregs are few and often not completely specific
359 also in human medicine,⁵⁶ making the characterization and the definition of the role
360 of this T cell subpopulation, within tumor immune environment, complex and still not
361 completely understood. Also, it must be reminded that the presence of FoxP3⁺ Tregs
362 could be influenced by tumor site, molecular subtype of the tumor, and tumor
363 stage.⁶² Therefore, further investigations should be encouraged to overcome these
364 limits in defining Treg biology and role in cancer, both in veterinary and comparative
365 medicine.

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

374 what is reported in human melanoma.¹¹ However, in the multivariate analysis, CTLA-
375 4 lost its statistical significance, suggesting that CTLA-4 is not an independent
376 predictor. This result, on one hand, is probably due to the association of this marker
377 with other variables, perhaps to be searched within the complexity of the tumor
378 immune environment itself. On the other hand, the association between the protein
379 and gene expression of this marker and the tumor mitotic count, which is considered
380 one of the most affordable prognostic features of canine melanomas,^{3,68} seems to
381 corroborate the hypothesis of the immunosuppressive role of this molecule in
382 melanoma growth. A larger study group should be therefore investigated, to gather
383 more information on the role of this molecule in melanocytic tumors of dogs. To our
384 knowledge, this is the first study that focuses on CTLA-4 within canine melanoma
385 microenvironment, in contrast to previous studies aimed at characterizing the
386 expression of this costimulatory molecule in circulating cells during neoplastic
387 disease and in a healthy subject.⁷¹⁻⁷³ Our results, although preliminary, highlight the
388 presence of this molecule within canine melanoma and open the way to further
389 investigations on the role of CTLA-4-associated pathways in canine oncology.

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

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

ACKNOWLEDGEMENTS:

The authors would like to thank Gianluca Alunni, Valeria Migni and Luca Stefanelli for their precious technical assistance.

1. Alexandrov LB, Nik-Zainal S, Wedge DC, et al. Signatures of mutational processes in human cancer. *Nature*. 2013;500:415–421.
2. Almela RM, Ansón A. A Review of Immunotherapeutic Strategies in Canine Malignant Melanoma. *Vet Sci*. 2019;6:15.
3. Bergin IL, Smedley RC, Esplin DG, Spangler WL, Kiupel M. Prognostic Evaluation of Ki67 Threshold Value in Canine Oral Melanoma. *Vet Pathol*. 2011;48:41–53.
4. Bilir C, Sarisozen C. Indoleamine 2,3-dioxygenase (IDO): Only an enzyme or a checkpoint controller? *J Oncol Sci*. 2017;3:52–56.

- 448 5. Bowlt Blacklock KL, Birand Z, Selmic LE, et al. Genome-wide analysis of
449 canine oral malignant melanoma metastasis-associated gene expression. *Sci*
450 *Rep.*;9:6511.
- 451 6. Brachelente C, Cappelli K, Capomaccio S, et al. Transcriptome Analysis of
452 Canine Cutaneous Melanoma and Melanocytoma Reveals a Modulation of
453 Genes Regulating Extracellular Matrix Metabolism and Cell Cycle. *Sci Rep.*
454 2017;7:6386.
- 455 7. Brochez L, Chevolet I, Kruse V. The rationale of indoleamine 2,3-dioxygenase
456 inhibition for cancer therapy. *Eur J Cancer.*;76:167–182.
- 457 8. Camacho LH. CTLA-4 blockade with ipilimumab: biology, safety, efficacy, and
458 future considerations. *Cancer Med.* 2015;4:661–672.
- 459 9. Carreau N, Pavlick A. Revolutionizing treatment of advanced melanoma with
460 immunotherapy. *Surg Oncol.* 2019;
- 461 10. Carvalho MI, Pires I, Prada J, Greg?rio H, Lobo L, Queiroga FL. Intratumoral
462 FoxP3 expression is associated with angiogenesis and prognosis in malignant
463 canine mammary tumors. *Vet Immunol Immunopathol.* 2016;178:1–9.
- 464 11. Chakravarti N, Ivan D, Trinh VA, et al. High cytotoxic T-lymphocyte-associated
465 antigen 4 and phospho-Akt expression in tumor samples predicts poor clinical
466 outcomes in ipilimumab-treated melanoma patients. *Melanoma Res.*
467 2017;27:24–31.
- 468 12. Chevolet I, Speeckaert R, Haspeslagh M, et al. Peritumoral indoleamine 2,3-
469 dioxygenase expression in melanoma: an early marker of resistance to
470 immune control? *Br J Dermatol.* 2014;171:987–995.
- 471 13. Chevolet I, Speeckaert R, Schreuer M, et al. Characterization of the *in vivo*
472 immune network of IDO, tryptophan metabolism, PD-L1, and *CTLA-4* in

- 473 circulating immune cells in melanoma. *Oncoimmunology*. 2015;4:e982382.
- 474 14. Chikuma S. CTLA-4, an Essential Immune-Checkpoint for T-Cell Activation. In
475 Springer, Berlin, Heidelberg; 2017:1–28.
- 476 15. Collins A V., Brodie DW, Gilbert RJC, et al. The Interaction Properties of
477 Costimulatory Molecules Revisited. *Immunity*. 2002;17:201–210.
- 478 16. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting:
479 from immunosurveillance to tumor escape. *Nat Immunol*. 2002;3:991–998.
- 480 17. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu*
481 *Rev Immunol*. 2004;22:329–360.
- 482 18. Esplin DG. Survival of dogs following surgical excision of histologically well-
483 differentiated melanocytic neoplasms of the mucous membranes of the lips
484 and oral cavity. *Vet Pathol*. 2008;45:889–896.
- 485 19. Field A. *Discovering Statistics Using SPSS*. Sage. London, UK; 2009.
- 486 20. Flemming A. Tumour heterogeneity determines immune response. *Nat Rev*
487 *Immunol*. 2019;19(11):662-663.
- 488 21. Fu Q, Chen N, Ge C, et al. Prognostic value of tumor-infiltrating lymphocytes in
489 melanoma: a systematic review and meta-analysis. *Oncoimmunology*. 2019;8.
- 490 22. Gerber AL, Müntz A, Schlapbach C, et al. High expression of FOXP3 in
491 primary melanoma is associated with tumour progression. *Br J Dermatol*.
492 2014;170:103–109.
- 493 23. Gray A, Grushchak S, Mudaliar K, Kliethermes S, Carey K, Hutchens KA. The
494 microenvironment in primary cutaneous melanoma with associated
495 spontaneous tumor regression: evaluation for T-regulatory cells and the
496 presence of an immunosuppressive microenvironment. *Melanoma Res*.
497 2017;27(2):104-109.

- 498 24. Grosso JF, Jure-Kunkel MN. CTLA-4 blockade in tumor models: an overview
499 of preclinical and translational research. *Cancer Immun.* 2013;13:5.
- 500 25. Hernandez B, Adissu HA, Wei B-R, Michael HT, Merlino G, Simpson RM.
501 Naturally Occurring Canine Melanoma as a Predictive Comparative Oncology
502 Model for Human Mucosal and Other Triple Wild-Type Melanomas. *Int J Mol*
503 *Sci.* 2018;19.
- 504 26. Hitte C, Le Béguec C, Cadieu E, et al. Genome-Wide Analysis of Long Non-
505 Coding RNA Profiles in Canine Oral Melanomas. *Genes (Basel).* 2019;10:477.
- 506 27. Holmgaard RB, Zamarin D, Li Y, et al. Tumor-Expressed IDO Recruits and
507 Activates MDSCs in a Treg-Dependent Manner. *Cell Rep.* 2015;13:412–424.
- 508 28. Iversen TZ, Andersen MH, Svane IM. The targeting of indoleamine 2,3
509 dioxygenase -mediated immune escape in cancer. *Basic Clin Pharmacol*
510 *Toxicol.* 2015;116:19–24.
- 511 29. Jacquelot N, Roberti MP, Enot DP, et al. Predictors of responses to immune
512 checkpoint blockade in advanced melanoma. *Nat Commun.* 2017;8:592.
- 513 30. Jia H, Qi H, Gong Z, et al. The expression of FOXP3 and its role in human
514 cancers. *Biochim Biophys Acta - Rev Cancer.* 2019;1871:170–178.
- 515 31. Jia H, Ren W, Feng Y, et al. The enhanced antitumour response of pimozide
516 combined with the IDO inhibitor L-MT in melanoma. *Int J Oncol.* 2018;53:949–
517 960.
- 518 32. Ko JS. The Immunology of Melanoma. *Clin Lab Med.* 2017;37:449–471.
- 519 33. Koppolu V, Rekha Vasigala V. Checkpoint immunotherapy by nivolumab for
520 treatment of metastatic melanoma. *J Cancer Res Ther.* 2018;14(6):1167-1175.
- 521 34. Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3. *Nat Rev*
522 *Immunol.* 2017;17(11):703-717.

- 523 35. Maekawa N, Konnai S, Takagi S, et al. A canine chimeric monoclonal antibody
524 targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or
525 undifferentiated sarcoma. *Sci Rep*. 2017;7:8951.
- 526 36. Maes RK, Langohr IM, Wise AG, Smedley RC, Thaiwong T, Kiupel M. Beyond
527 H&E. *Vet Pathol*. 2014;51:238–256.
- 528 37. Mahmoud F, Shields B, Makhoul I, et al. Immune surveillance in melanoma:
529 From immune attack to melanoma escape and even counterattack. *Cancer*
530 *Biol Ther*. 2017;18:451–469.
- 531 38. McCall MN, McMurray HR, Land H, Almudevar A. On non-detects in qPCR
532 data. 2014;30:2310–2316.
- 533 39. Melsted WN, Johansen LL, Lock-Andersen J, et al. HLA class Ia and Ib
534 molecules and FOXP3+ TILs in relation to the prognosis of malignant
535 melanoma patients. *Clin Immunol*. 2017;183:191–197.
- 536 40. Meuten DJ, Moore FM, George JW. Mitotic Count and the Field of View Area:
537 Time to Standardize. *Vet Pathol*. 2016;53:7–9.
- 538 41. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer
539 immunoediting and its three component phases—elimination, equilibrium and
540 escape. *Curr Opin Immunol*. 2014;27:16–25.
- 541 42. Monjazeb AM, Kent MS, Grossenbacher SK, et al. Blocking Indoleamine-2,3-
542 Dioxygenase Rebound Immune Suppression Boosts Antitumor Effects of
543 Radio-Immunotherapy in Murine Models and Spontaneous Canine
544 Malignancies. *Clin Cancer Res*. 2016;22:4328–4340.
- 545 43. Munn DH. Indoleamine 2,3-dioxygenase, Tregs and cancer. *Curr Med Chem*.
546 2011;18:2240–2246.
- 547 44. Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of

548 immune responses. *Trends Immunol.* 2013;34:137–143.

549 45. Munn DH, Sharma MD, Johnson TS, Rodriguez P. IDO, PTEN-expressing
550 Tregs and control of antigen-presentation in the murine tumor
551 microenvironment. *Cancer Immunol Immunother.* 2017 2017 Aug;66(8):1049-
552 1058.

553 46. Munn DH, Mellor AL. IDO in the Tumor Microenvironment: Inflammation,
554 Counter-Regulation, and Tolerance. *Trends Immunol.* 2016;37:193–207.

555 47. Namdar A, Mirzaei R, Memarnejadian A, et al. Prophylactic DNA vaccine
556 targeting Foxp3+ regulatory T cells depletes myeloid-derived suppressor cells
557 and improves anti-melanoma immune responses in a murine model. *Cancer*
558 *Immunol Immunother.* 2018;67:367–379.

559 48. Osella-Abate S, Conti L, Annaratone L, et al. Phenotypic characterisation of
560 immune cells associated with histological regression in cutaneous melanoma.
561 *Pathology.* 2019;51:487–493.

562 49. Pasquali S, Chiarion-Sileni V, Rossi CR, Mocellin S. Immune checkpoint
563 inhibitors and targeted therapies for metastatic melanoma: A network meta-
564 analysis. *Cancer Treat Rev.* 2017;54:34–42.

565 50. Passarelli A, Mannavola F, Stucci LS, Tucci M, Silvestris F. Immune system
566 and melanoma biology: a balance between immunosurveillance and immune
567 escape. *Oncotarget.* 2017;8:106132–106142.

568 51. Pelak MJ, Śnietura M, Lange D, Nikiel B, Pecka KM. The prognostic
569 significance of indoleamine-2,3-dioxygenase and the receptors for
570 transforming growth factor β and interferon γ in metastatic lymph nodes in
571 malignant melanoma. *Pol J Pathol.* 2015;66:376–382.

572 52. Porcellato I, Brachelente C, De Paolis L, et al. FoxP3 and IDO in Canine

573 Melanocytic Tumors. *Vet Pathol.* 2019;56:189–199.

574 53. Prendergast GC, Malachowski WJ, Mondal A, Scherle P, Muller AJ.
575 Indoleamine 2,3-Dioxygenase and Its Therapeutic Inhibition in Cancer. *Int Rev*
576 *Cell Mol Biol.* 2018;336:175–203.

577 54. Prouteau A, André C, Prouteau A, André C. Canine Melanomas as Models for
578 Human Melanomas: Clinical, Histological, and Genetic Comparison. *Genes*
579 *(Basel).* 2019;10:501.

580 55. Que Y, Xiao W, Guan Y, et al. PD-L1 Expression Is Associated with FOXP3+
581 Regulatory T-Cell Infiltration of Soft Tissue Sarcoma and Poor Patient
582 Prognosis. *J Cancer.* 2017;8:2018–2025.

583 56. Rodríguez-Perea AL, Arcia ED, Rueda CM, Velilla PA. Phenotypical
584 characterization of regulatory T cells in humans and rodents. *Clin Exp*
585 *Immunol.* 2016;185:281–291.

586 57. Rowshanravan B, Halliday N, Sansom DM. CTLA-4: a moving target in
587 immunotherapy. *Blood.* 2018;131(1):58-67

588 58. Rubel F, Kern JS, Technau-Hafsi K, et al. Indoleamine 2,3-Dioxygenase
589 Expression in Primary Cutaneous Melanoma Correlates with Breslow
590 Thickness and Is of Significant Prognostic Value for Progression-Free Survival.
591 *J Invest Dermatol.* 2018;138:679–687.

592 59. Sakai K, Maeda S, Yamada Y, et al. Association of tumour-infiltrating
593 regulatory T cells with adverse outcomes in dogs with malignant tumours. *Vet*
594 *Comp Oncol.* 2018;16(3):330-336.

595 60. Schmid F, Brodesser D, Reifinger M, et al. Canine oral primary melanoma
596 cells exhibit shift to mesenchymal phenotype and phagocytic behaviour. *Vet*
597 *Comp Oncol.* 2019;17(3):211-220.

- 598 61. Schultheiss PC. Histologic features and clinical outcomes of melanomas of lip,
599 haired skin, and nail bed locations of dogs. *J Vet Diagn Invest.* 2006;18:422–
600 425.
- 601 62. Shang B, Liu Y, Jiang S, Liu Y. Prognostic value of tumor-infiltrating FoxP3+
602 regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep.*
603 2015;5:15179.
- 604 63. Sharma MD, Baban B, Chandler P, et al. Plasmacytoid dendritic cells from
605 mouse tumor-draining lymph nodes directly activate mature Tregs via
606 indoleamine 2,3-dioxygenase. *J Clin Invest.* 2007;117:2570–2582.
- 607 64. Shi L, Meng T, Zhao Z, et al. CRISPR knock out CTLA-4 enhances the anti-
608 tumor activity of cytotoxic T lymphocytes. *Gene.* 2017;636:36–41.
- 609 65. Silvestri S, Porcellato I, Mechelli L, Menchetti L, Rapastella S, Brachelente C.
610 Tumor Thickness and Modified Clark Level in Canine Cutaneous Melanocytic
611 Tumors. *Vet Pathol.* 2019 Mar;56(2):180-188.
- 612 66. Simpson RM, Bastian BC, Michael HT, et al. Sporadic naturally occurring
613 melanoma in dogs as a preclinical model for human melanoma. *Pigment Cell*
614 *Melanoma Res.* 2014;27:37–47.
- 615 67. Smedley RC, Spangler WL, Esplin DG, et al. Prognostic Markers for Canine
616 Melanocytic Neoplasms. *Vet Pathol.* 2011;48:54–72.
- 617 68. Smedley RC, Spangler WL, Esplin DG, et al. Prognostic markers for canine
618 melanocytic neoplasms: a comparative review of the literature and goals for
619 future investigation. *Vet Pathol.* 2011;48:54–72.
- 620 69. Spangler WL, Kass PH. The Histologic and Epidemiologic Bases for
621 Prognostic Considerations in Canine Melanocytic Neoplasia. *Vet Pathol.*
622 2006;43:136–149.

- 623 70. Spangler WL, Kass PH. The histologic and epidemiologic bases for prognostic
624 considerations in canine melanocytic neoplasia. *Vet Pathol.* 2006;43:136–149.
- 625 71. Tagawa M, Kurashima C, Takagi S, et al. Evaluation of costimulatory
626 molecules in dogs with B cell high grade lymphoma. *PLoS One.* 2018;13.
- 627 72. Tagawa M, Maekawa N, Konnai S, Takagi S. Evaluation of Costimulatory
628 Molecules in Peripheral Blood Lymphocytes of Canine Patients with Histiocytic
629 Sarcoma. Bayry J, ed. *PLoS One.* 2016;11:e0150030.
- 630 73. Tagawa M, Yamamoto Y, Shimbo G, et al. Gene and protein expression of a
631 soluble form of CTLA-4 in a healthy dog. *J Vet Med Sci.* 2017;79:871–875.
- 632 74. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH.
633 Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan
634 tissue destruction, revealing a critical negative regulatory role of CTLA-4.
635 *Immunity.* 1995;3:541–547.
- 636 75. Vajaitu C, Draghici CC, Solomon I, et al. The Central Role of Inflammation
637 Associated with Checkpoint Inhibitor Treatments. *J Immunol Res.*
638 2018;2018:4625472.
- 639 76. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-
640 time quantitative RT-PCR data by geometric averaging of multiple internal
641 control genes. *Genome Biol.* 2002;18;3(7):RESEARCH0034.
- 642 77. Wei B-R, Michael HT, Halsey CHC, et al. Synergistic targeted inhibition of
643 MEK and dual PI3K/mTOR diminishes viability and inhibits tumor growth of
644 canine melanoma underscoring its utility as a preclinical model for human
645 mucosal melanoma. *Pigment Cell Melanoma Res.* 2016;29:643–655.
- 646 78. Wolf Y, Bartok O, Patkar S, et al. UVB-Induced Tumor Heterogeneity
647 Diminishes Immune Response in Melanoma. *Cell.* 2019;179:219-235.e21.

648

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

659

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

663

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

666

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

670

671 Figure 5. Box plots of FoxP3, CTLA-4, and IDO, positive nuclei/HPF in the upper
672 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).