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Programmed-cell death ligand 1 (PD-L1) expression in equine sarcoids and squamous cell carcinoma

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Abstract

Background: Sarcoids and squamous cell carcinomas (SCCs) are the most concerning equine oncological diseases. Both tumors are challenging to manage due to their invasive behavior and high prevalence of recurrences. Furthermore, SCCs have a propensity to metastasize. Programed cell-death ligand 1 (PD-L1) has been one of the main therapeutic targets for immunotherapy in various human tumors. PD-L1 research in equine tumors is scarce and more efforts are necessary to understand the potential of this biomarker as a therapeutical target.

Aim: Evaluate the immunohistochemical expression of PD-L1 in equine sarcoids and SCC.

Methods: Thirteen equine tumors (seven sarcoids and 6 SCCs) were tested by immunohistochemistry and evaluated semi quantitatively to assess the percentage of positive cells.

Results: None of the sarcoids presented PD-L1 expression. Regarding SCC, 2 tumors presented <10% of labeled cells; 2 tumors presented 10%–25% of labeled cells and 2 tumors presented 25%–50% of labeled cells. There were statistically significant differences between sarcoids and SCC regarding the expression of PD-L1.

Conclusion: Our results point to the fact that PD-L1 could be a potential therapeutic target against SCC, and also encourage in-depth studies in this area, with larger sample sizes.

Keywords: Equine, Immunotherapy, PD-L1, Sarcoid, SCC.

Introduction

Equine sarcoids and squamous cell carcinomas (SCC) are the most common cutaneous tumors in horses. Both represent challenging oncological diseases due to their aggressive nature and the sensitive locations where they typically emerge (Taylor and Haldorson, 2012; Funiciello and Roccabianca, 2020; Ogłuszka *et al*., 2021).

There is a wide range of therapeutical options available, however, many clinical cases end up in frustration for both clinicians and owners, due to the high propensity for recurrence of these tumors (Surjan *et al*., 2014; Funiciello and Roccabianca, 2020). In addition, most treatments have merely local effects, with only a few systemic treatments being reported (Goodrich and Semevolos, 2000; Palmer, 2002; Hewes and Sullins, 2009). Sarcoids are characterized by their high invasiveness into surrounding tissues and by becoming more aggressive after manipulation (e.g., after biopsy or surgery) (Funiciello and Roccabianca, 2020). SCCs are also known for their invasive behavior, but even worse, for their propensity to metastasize (Taylor and Haldorson, 2012). Therefore, efforts need to be made to find new systemic therapies, since the local therapeutical options available still have limitations that need to be overcome. In human oncology, immunotherapy is one of the most important areas of research and one of the newest therapeutical approaches, presenting good success rates (Hanks, 2022). Immune checkpoints refer to a set of pathways that regulate the immune system's action preventing autoimmunity and maintaining self-tolerance (Kythreotou *et al*., 2018; Hanks, 2022). Tumors can promote immune checkpoint dysregulation to escape from anti-tumor effects of immune cells, leading to tumor immune evasion, one of the hallmarks of cancer (Kythreotou *et al*., 2018; Hanks, 2022).

Programmed cell-death 1 (PD-1) and programmed cell-death ligand 1 (PD-L1) immune checkpoint has been one of the main targets of immunotherapy. Some tumors overexpress PD-L1 being capable of inhibiting the action of tumor-infiltrating lymphocytes

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that express PD-1. As so PD-1/PD-L1 blockade can be used as an effective therapeutical option (Maekawa *et al*., 2014; Patel and Kurzrock, 2015; Kythreotou *et al*., 2018;Stevenson *et al*., 2021; Maekawa *et al*., 2021;).
PD-L1 expression studies in equine oncology are still scarce. With the exception of equine melanomas, where recent reports showed promising results (Ganbaatar *et al*., 2020; Pimenta *et al*., 2023), the few existing works on equine sarcoids and SCC do not seem to have such favorable results (Benvegnen *et al*., 2021; Porcellato *et al*., 2021). As so, further studies are necessary in this field, with this work pretending to evaluate the immunohistochemical expression of PD-L1 in equine sarcoids and SCC.

Materials and Methods

Tissue samples

This study included formalin-fixed paraffin-embedded samples of equine sarcoids and SCC, with a previous histological diagnosis.

Clinical information

Clinical information was collected, namely age, gender, breed, coat color, and tumor mass localization. However, complete reports were not available for all horses.

Immunohistochemistry

The immunohistochemical technique was performed using a commercial detection system (NovoLink Polymer Detection System; Novocastra, Leica Biosystems Newcastle, UK), according to the manufacturer's instructions. 3 µm tissue sections were dewaxed in xylene, and hydrated through a series of alcohol solutions, ending in tap water. Citrate buffer solution (0.01 M pH 6.0 ± 2) was used for microwave antigen retrieval (1 cycle of 5 minutes at 750 W). After antigen retrieval, bleaching was performed. Endogenous peroxidase was blocked using 3% hydrogen peroxide for 5 minutes and endogenous protein blocking was also performed for 5 minutes. Primary antibody Anti-PD-L1 (ab233482, Abcam) was diluted 1:200 in phosphate-buffered saline and incubated at 4°C overnight. After that, slides were incubated with a secondary antibody. Immunostaining was visualized by incubation with 3,3' – diaminobenzidine tetrahydrochloride (DAB) chromogen. Slides were counterstained with Gill's hematoxylin.
To evaluate the cross-reactivity and specificity of the antibody, BLAST (*https://blast.ncbi.nlm.nih.gov*) was used, which revealed an 84.76% homology between the antibody amino acid sequence and equine PD-L1 amino acid sequence, being highly predictive of cross reaction.

Immunohistochemical evaluation

The evaluation of PD-L1 extension of labeling was performed by two independent pathologists (IP, JP). Positivity was indicated by membranous or membranous and cytoplasmatic brown labeling. Equine placenta was used as positive control (Fig. 1), and equine kidney as negative control (Fig. 2). PD-L1 staining was scored accordingly to Benvegnen *et al*. 2021 as: 0 – negative, 1) <10% labeled cells; 2) 10%–25% labeled cells; 3) 25%–50% labeled cells and 4) >50% labeled cells (Benvegnen *et al*., 2021).

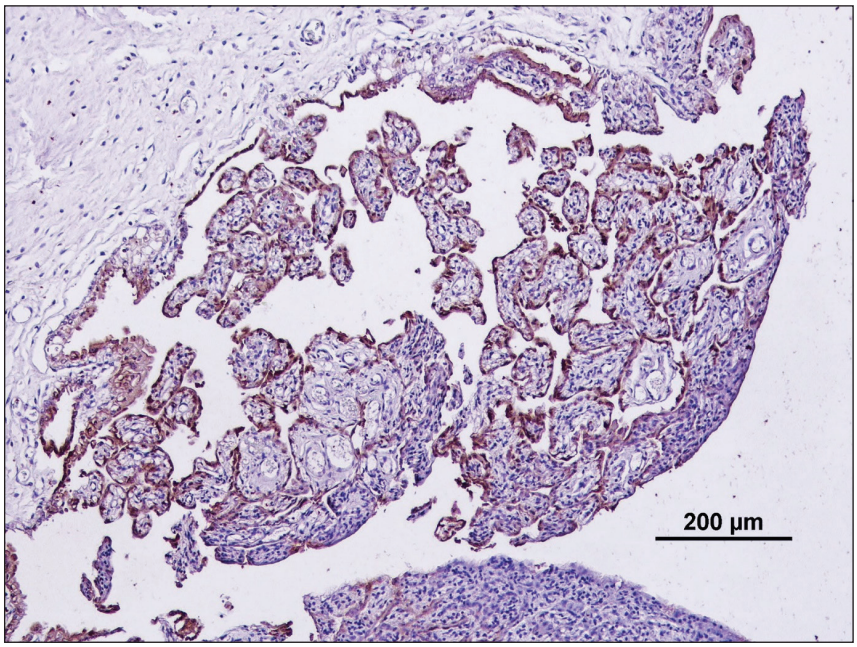

Fig. 1. PD-L1 positive control (equine placenta). Membranous staining is possible to see. Brown staining was visualized due to DAB chromogen and counterstaining Gill's hematoxylin (200 µm).

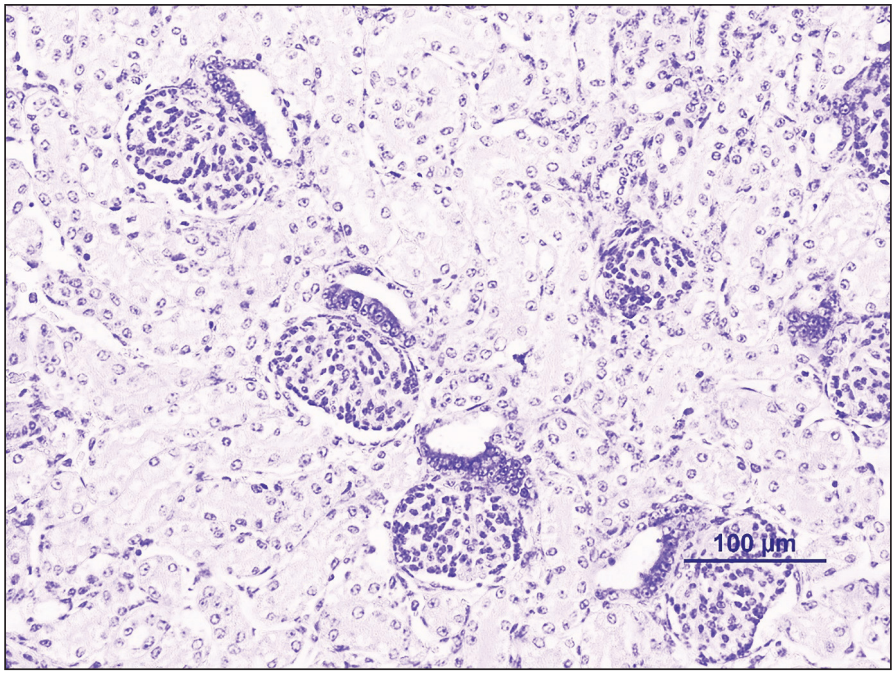

Fig. 2. PD-L1 negative control (equine placenta). No staining is visible (100 µm).

Statistical analysis

To evaluate if there were differences in medians of PD-L1 expression between sarcoids and CCE, Mann-Whitney test was performed. Qui-square (X^2) test of independence was performed to evaluate whether PD-L1 staining was associated with the clinical features collected. Results were considered statistically significant when $p < 0.05$.

Ethical approval

Not needed for this study.

Results and Discussion

Thirteen horses were included in this study, with a median age of 12.3 ± 6.9 years old (the youngest with 3 and the oldest with 20) being 9 males and 4 females. Seven horses were Pure-bred Lusitano, one Warmblood, two crossbred and no information was available for 3 other horses. Regarding localization, tumors were found in the testicular region (n = 1 sarcoid), perianal region (n = 1 SCC), neck (n = 1 sarcoid), eye (n = 1 SCC), and abdomen (n = 1 sarcoid). Regarding coat color, two horses were bay, one brown, and 1 grey.

Seven tumors were sarcoids and six were SCC. Regarding sarcoids, 3 tumors presented only cytoplasmatic labeling (Fig. 3) and 4 tumors did not present any expression. Thus, all sarcoids were considered negative (Table 1). Regarding SCC, 2 tumors presented <10% of labeled cells; 2 tumors presented 10%–25% of labeled cells and 2 tumors presented 25%–50% of labeled cells (Table 1). All tumors presented both membranous and cytoplasmatic labeling. Figures 4 and 5 represent PD-L1 staining in equine SCC. There was no association between PD-L1 staining and clinical features. There was a statistically significant difference between sarcoids and SCC regarding PD-L1 expression ($p = 0.001$).

Regarding clinical features, the lack of clinical information about most horses included in this study did not allow us to perform an in-dept analysis of this information which may have contributed to the statistical result obtained.

Benvegnen *et al*., 2021 evaluated PD-L1 expression by immunohistochemistry in 9 equine sarcoids and found that 3 were negative, 4 had less than 10% of labeled cells and 2 had 25%–50% of labeled cells. Although the results are not favorable either, there was some positivity to report. Anyway, our work seems to corroborate the mentioned results that did not support PD-L1 as a future therapeutical target for equine sarcoids. Although our sample and of the aforementioned study are small, the results are similar, highlighting the fact that PD-L1 may not play an important role in the progression of these tumors and therefore may not be an interesting therapeutic target. However, more studies with larger samples are needed to corroborate or refute these results.

Porcellato *et al*., 2021 studied PD-L1 immunostaining in 17 equine SCC, reporting that only one presented expression. The results of the present study are quite different. Although our sample is smaller, all tumors (6/6) presented some degree of positivity. These findings rekindle the curiosity to carry out new studies about equine SCC to better understand the potential of PD-L1 on these tumors.

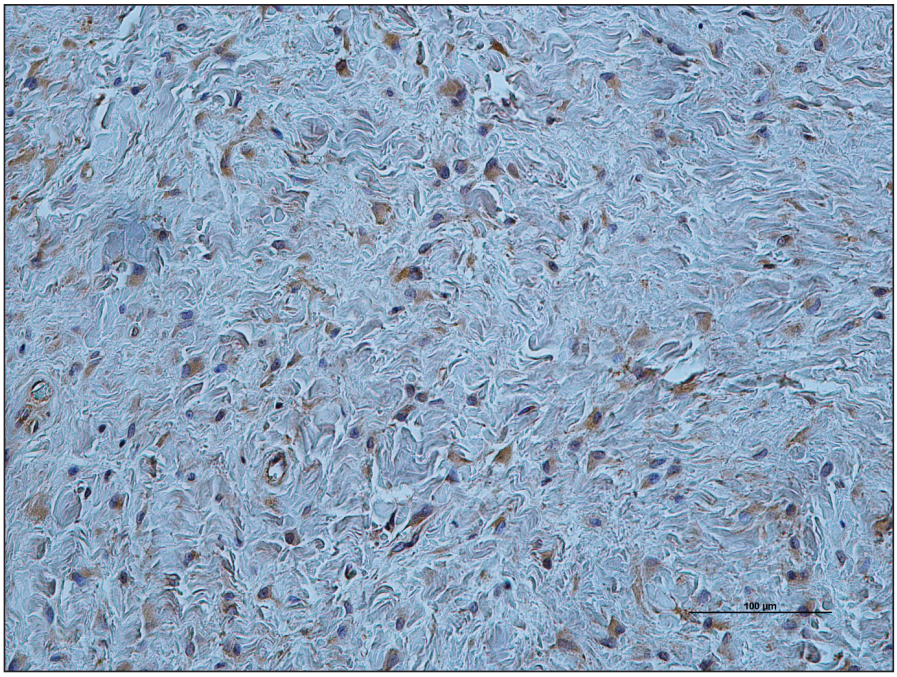

Fig. 3. PD-L1 cytoplasmatic staining in equine sarcoid (100 µm). Brown staining was visualized due to DAB chromogen and counterstaining Gill's hematoxylin.

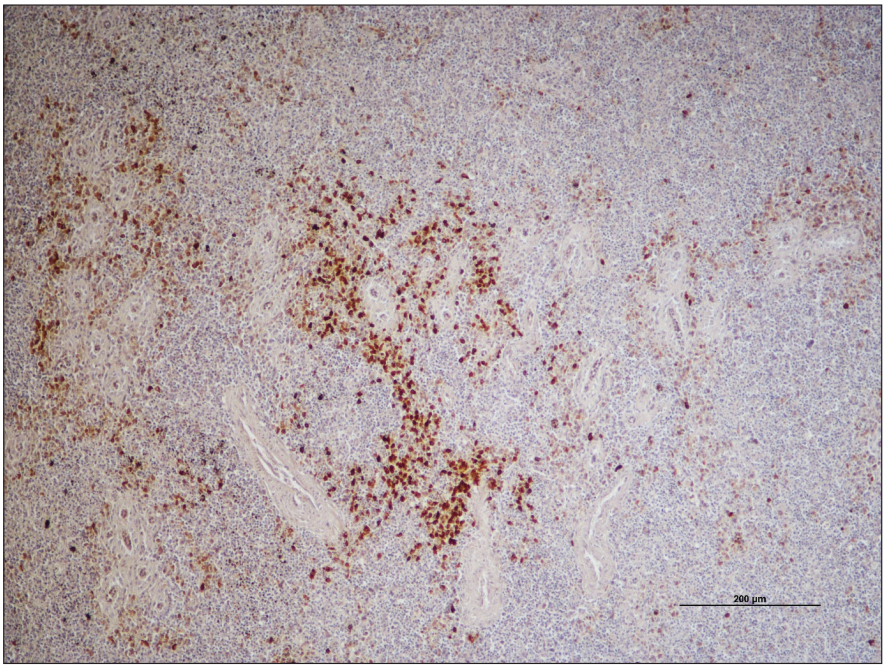

Fig. 4. Partially diffuse (25%–50%) staining of tumor cells in an equine SCC (200 µm). Brown staining was visualized due to DAB chromogen and counterstaining Gill's hematoxylin.

An important point of discussion between our work and the two aforementioned studies is that in the 3 articles, the PD-L1 antibody used is different, which likely contributes to obtaining different results. However, the fact that the 3 studies contained small sample sizes means that their results cannot be considered absolute and need to be confirmed by further studies. Nevertheless, in future studies, it will be important to correctly validate the antibody for these tumors and species, to obtain more reliable results.

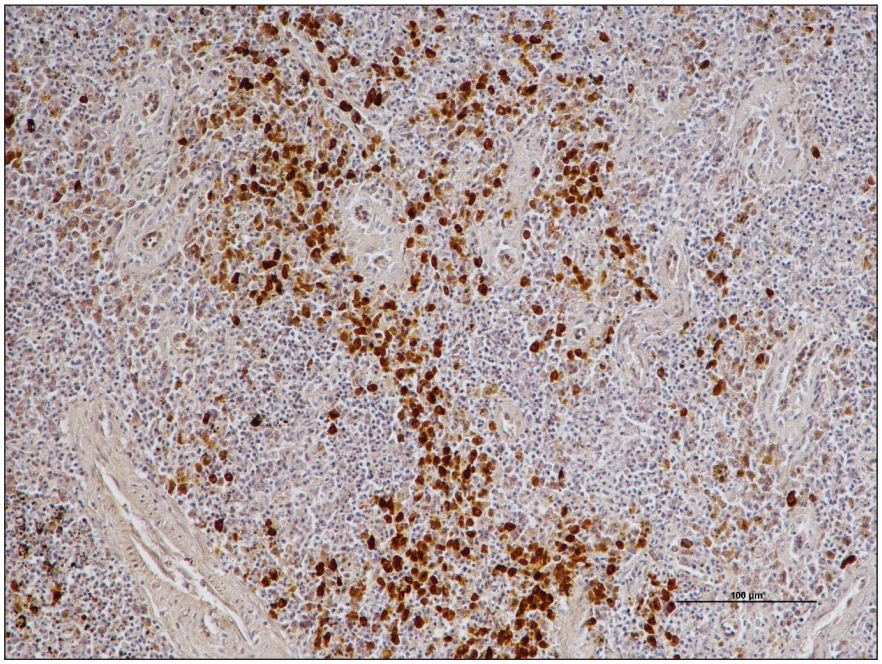

Fig. 5. Higher magnification of figure 2, where membranous and cytoplasmatic labeling of tumors cells is possible to see (100 µm). Brown staining was visualized due to DAB chromogen and counterstaining Gill's hematoxylin.

Table 1. Distribution of extension of labeled cells between sarcoids and SCC.

Tumor type	Extension of labelled cells					Total
	0%	<10%	10%–25%	25%–50%	>50%	
Sarcoids	7					7
SCC		2	2	2		6

In canine SCC, studies about immunohistochemical evaluation of PD-L1 are also scarce and the results are not consensual. Maekawa *et al*., 2016 evaluated five SCCs and reported that none of the tumors expressed PD-L1. Although the sample size is similar to ours, these results contrast with what we find in the horse, since all equine SCC showed some degree of PD-L1 positivity. In contrast with the aforementioned study, Maekawa *et al*., 2021 studied twenty canine SCCs from which 18/20 demonstrated PD-L1 expression. 16/20 presented more than 50% of labeled cells and 2/20 presented between 1%–49% of labeled cells. The fact that 80% of tumors carried more than 50% of labeled cells sharply contrasts with our results since none of the equine SCC tested, showed such a high percentage of PD-L1 extension of labeling. Regarding human medicine, studies on PD-L1 are quite solid and advanced in various types of tumors including SCC, in which PD-1/PD-L1 blocking therapy is already implemented with a good success rate (Han *et al*., 2020).

Regarding equine melanomas, the third most common tumor type of horses, there are already two published studies about PD-L1 expression, which present more positive tumors and higher extensions of labeling than tumors of the present work. Furthermore, an *in vitro* evaluation of PD-1/PD-L1 blockade effect on equine melanoma cells was already performed, reporting that equine melanoma cells showed that cytokine production by peripheral blood mononuclear cells was enhanced which may be a sign of immune system reactivation. These advances stimulate the curiosity to explore these immunotherapies in equine oncology, an area that clinically relies on purely local therapies and lacks systemic therapies.

Although some articles have reported a differentiated expression of PD-L1 at the invasive front of tumors and in zones with high mitotic activity (Tsutsumi *et al*., 2017; Ullah *et al*., 2020), in the present study the immunolabeling of PD-L1 was focal and random throughout the tumor. However, given the low number of samples analyzed, it is not possible to draw conclusions about this result.

Future studies including a follow-up of equine SCC cases would be interesting since the human literature reports that PD-L1 may have a role in SCC metastatic

spread and so it may be a candidate prognostic marker. According to our findings, although PD-L1 may have a role as a potential therapeutical target in SCC, regarding sarcoids the results are disappointing.

Acknowledgments

None.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors' contribution

JP (José Pimenta), JP (Justina Prada), IP, and MC contributed conception and design of the study; JP (José Pimenta), JP (Justina Prada), and IP performed all the laboratory assays; JP (José Pimenta) wrote the first draft of the manuscript; JP (José Pimenta), JP (Justina Prada), IP and MC critically revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Data availability

All data supporting the findings of this study are available within the manuscript.

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