Detection of PD-1, PD-L1 and CTLA-4 by RNA *in situ* hybridization in canine oral melanocytic neoplasms and their microenvironment

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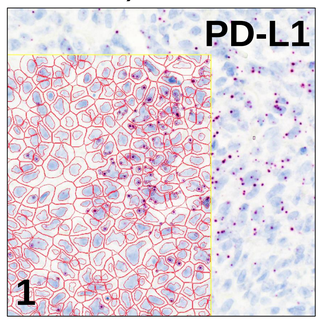
Introduction

Immune checkpoint pathways can be co-opted by cancer cells to evade immune destruction. The programmed death-1 (PD-1)/ programmed death ligand-1 (PD-L1) and cytotoxic T-lymphocyte antigen-4 (CTLA-4) checkpoints mainly act as regulators of T cells functions. Immunotherapy with monoclonal antibodies blocking PD-1/PD-L1 axis and CTLA-4 may restore and improve anti-tumor immunity and mediate durable tumor regression for various cancer types in humans, especially melanoma.

The aim of this study was to assess the expression of PD-L1, PD-1, and CTLA-4 in canine oral melanocytic neoplasms, to gain insight into their role and evaluate their potential prognostic value.

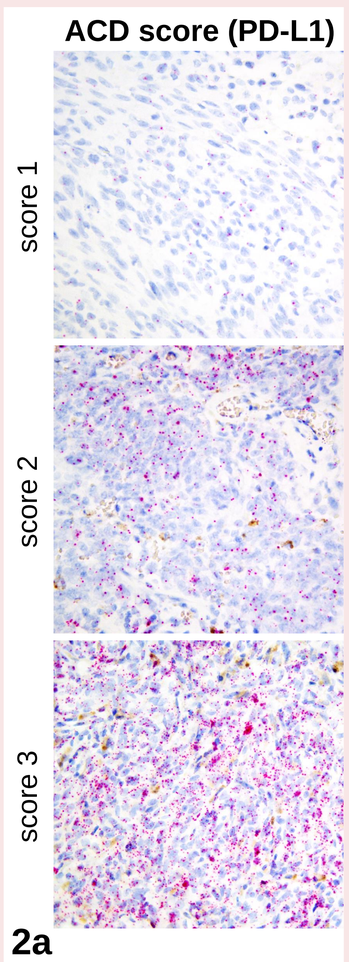
Materials and Methods

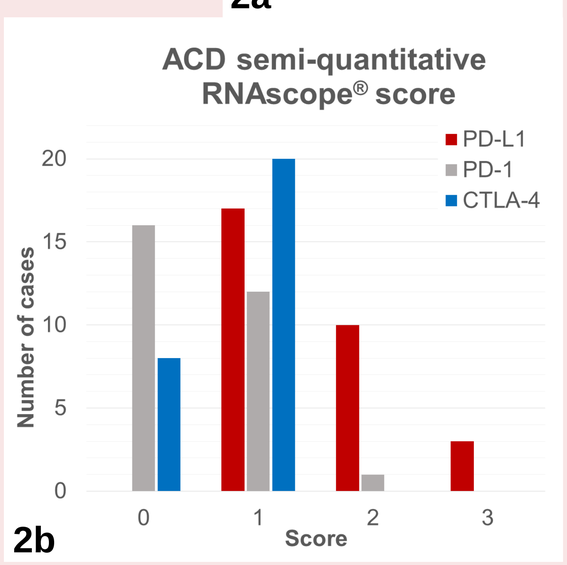
Automated RNAscope® *in situ* hybridization (ISH; ACD, Newark, CA) was performed in 24 oral melanomas and 5 melanocytomas using anti-canine PD-L1, PD-1, CTLA-4 probes. Positive signals (red cytoplasmic punctate dots or clusters) in tumor cells were scored (0-4) according to the ACD semi-quantitative scoring system and assessed by image analysis (QuPath) using “cell/ subcellular detection” functions (**Fig. 1**) on randomly selected annotations. Results were correlated to clinico-pathological features, including the mitotic count, nuclear atypia, Ki67 index, CD3+/CD20+ tumor-infiltrating lymphocytes (TILs) grade, and outcome.

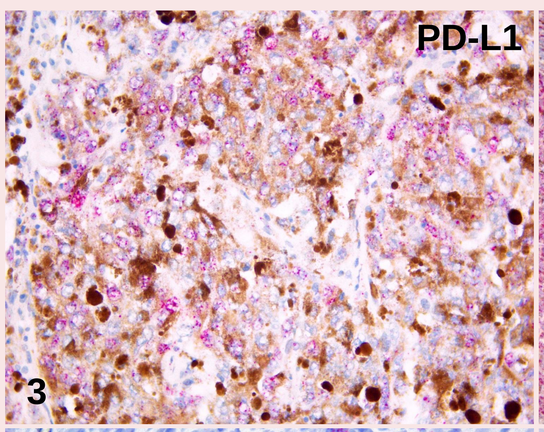


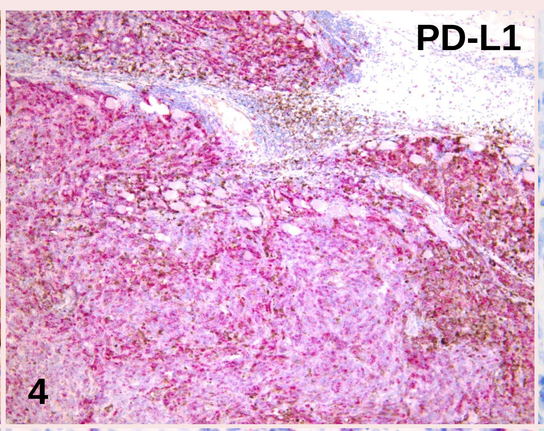
Results

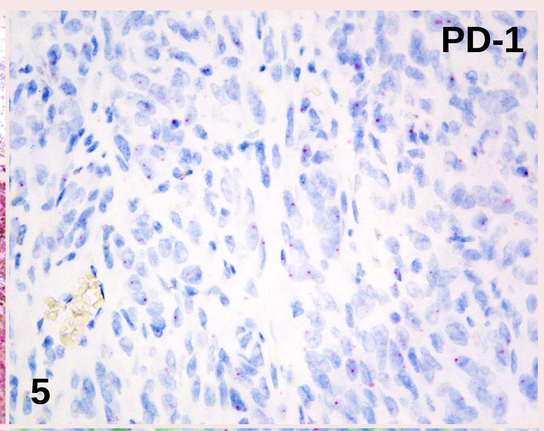
- PD-L1 was expressed in all melanocytic neoplasms (score 1-3) (**Fig. 2; 3**), with multifocal (20 cases) or diffuse (9 cases) pattern of distribution/intensity. PD-L1 expression was usually higher at the host-tumor interface (**Fig. 4**, areas with score 4).
- PD-1 signals were detected in 13/24 melanomas (score 1-2) (**Fig. 2b; 5; 4**), with multifocal (11 cases) or diffuse (9 cases) pattern; PD-1 was not detected in melanocytomas.
- CTLA-4 was expressed in 18/24 melanomas and 2/4 melanocytomas (score 1) (**Fig. 2b; 6**), with multifocal (8 cases) or diffuse (12 cases) pattern.
- Results of the ACD semi-quantitative score (**Fig. 2**) and the automated analysis with QuPath (ratio counted spots/cell) for PD-L1 and PD-1 were correlated ($p < 0.01$; Wilcoxon test).
- PD-L1 and PD-1 scores in tumor cells tended to increase with higher TILs grade.
- PD-L1, PD-1 and CTLA-4 were variably expressed by TILs (**Fig. 6**, arrows**; 7**) and tumor-associated macrophages (**Fig. 8**, dual PD-L1 RNAscope® + Iba-1 immunohistochemistry).
- There was no association among RNAscope® scores and the overall survival or disease free interval. However, scores of PD-1≥1 were associated with mitotic count ≥4 ($p < 0.01$; Fisher Exact test) and nuclear atypia ≥30% ($p < 0.5$; Fisher Exact test).

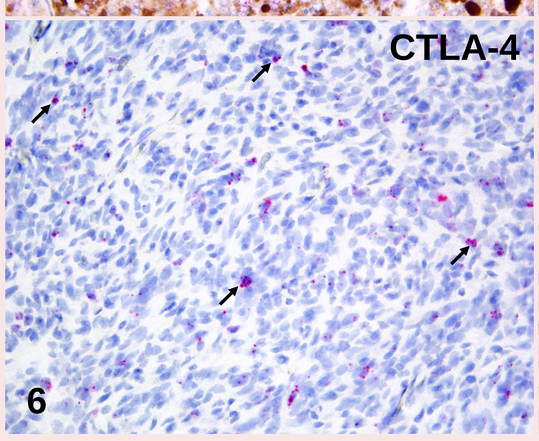


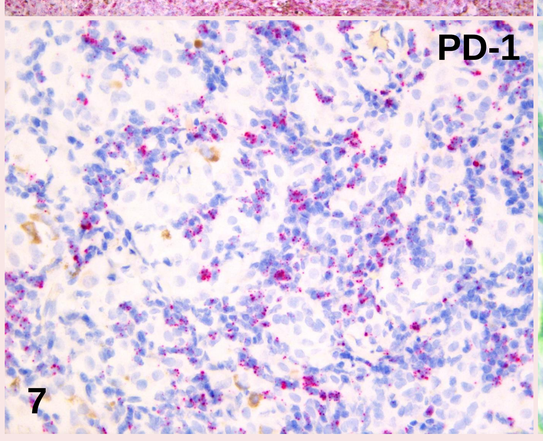


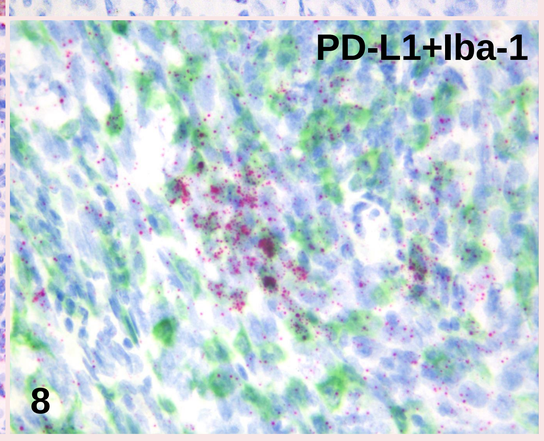












Discussion and conclusions

In this study, we observed that PD-L1/PD-1 and CTLA-4 are expressed by canine melanocytic tumor cells and their microenvironment (TME), supporting them as key players in the immune evasion of these neoplasms.

RNAscope® ISH is more specific and sensitive compared to immunohistochemistry (IHC) for immune checkpoints, since reactive antibodies and standardized procedures are scarcely available for dogs.

PD-L1 overexpression appears to be one of the main mechanisms by which tumor cells escape their elimination by tumor-specific PD-1-expressing TILs in the TME. The PD-1/PD-L1 interaction inhibits T cell proliferation, survival and effector functions. PD-L1 expression in tumor cells can be intrinsic or induced by inflammatory signals, which may reflect the correlation of PD-L1 score with TILs grade and explain the different expression patterns.

The expression of PD-1 and CTLA-4 in tumor cells, other than TILs, has never been reported in canine melanomas, while it has been described in human melanoma cells, as well as in other solid tumors, and might represent scarcely known mechanisms of tumor immune evasion.

PD-L1/PD-1 and CTLA-4 expression by tumor cells do not seem to have prognostic value, although higher PD-1 values are associated with known unfavorable prognostic factors.

Our study represents a first step for the possible development of immunotherapy strategies in dogs, also as a potential preclinical model for humans.

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