Immunohistochemical analysis of programmed death-ligand 1 expression in equine sarcoids

Jennifer Benvegnen, Bettina De Breuyn, Vinzenz Gerber, Sven Rottenberg, Christoph Koch

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1	Immunohistochemical analysis of programmed death-ligand 1 expression in					
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4	Jennifer Benvegnen ^{1, 2} , Bettina De Breuyn ¹ , Vinzenz Gerber ² , Sven Rottenberg ¹ , Christoph					
5	Koch ²					
6						
7	¹ Institute of Animal Pathology, Vetsuisse Faculty, University of Bern, Länggassstrasse 122,					
8	3012 Bern, Switzerland					
9	² Swiss Institute of Equine Medicine (ISME), Department of Clinical Veterinary Medicine,					
10	Vetsuisse Faculty, University of Bern, and Agroscope, Länggassstrasse 124, 3012 Bern,					
11	Switzerland					
12						
13	Corresponding author ¹ :					
14	Jennifer Benvegnen					
15	ISME Pferdeklinik Bern					
16	Länggassstrasse 124					
17	3012 Bern					
18	+4131.631.22.43					
19	e-mail: j.benvegnen@hotmail.com					
20	Abstract					

¹ Present adress: Route de Bettens 25A, 1306 Daillens, SWITZERLAND

The aim of the study was to assess the expression of the immune checkpoint inhibitor programmed death-ligand 1 (PD-L1) in equine sarcoids (ES). PD-L1 is expressed by various cancer cells to block T cell-mediated elimination of tumor cells.

Antibodies targeting human PD-L1 were tested by immunohistochemistry (IHC) for their cross-reactivity with equine PD-L1 using formalin-fixed, paraffin-embedded (FFPE) tissues.

Our results do not support an important role of PD-L1-mediated immune evasion in ES disease, and hence do not offer a rationale for anti-PD-1/PD-L1-based immunotherapy against ES.

29 Keywords: Equine sarcoids, immune checkpoint inhibition, immune evasion,

30 immunohistochemistry, PD-L1

31 **1. Introduction**

Equine sarcoids (ES) are the most common tumors in equids and account for more than half of all skin tumors in this species [1,2]. Treatment is often challenging due to the notoriously high propensity for tumor recurrence [3], the lack of tissue-sparing treatment options and effective systemic or prophylactic treatment modalities.

In human medical oncology, a novel therapeutic approach has recently achieved remarkable successes: targeting of the immune checkpoint inhibitor programmed death-ligand 1 (PD-L1) or programmed cell death protein 1 (PD-1) [4,5]. It has resulted in tumor eradication in cancer patients previously thought to be incurable (*e.g.* advanced melanoma or lung cancer).

The physiologic role of immune checkpoints is to maintain self-tolerance and protect tissues from self-damage, for instance while responding to an infection [6–8]. The dysregulation of these immune checkpoint proteins can be observed in various cancers, and represents an important mechanism for tumor cells to evade the immune system [6,9]. PD-L1 is expressed

by various cancer cells to block T cell-mediated elimination of the tumor cells by binding to
programmed cell death protein 1 (PD-1) at the surface of T lymphocytes. This mechanism of
immune evasion can be prevented by using specific antibodies against PD-L1 or PD-1 [6].

The aim of this study was to establish an immunohistochemistry (IHC) staining protocol to assess PD-L1 expression in ES-derived and other equine tissue samples. As an additional control to investigate the cross-reactivity of the antibody for equine PD-L1, we also tested mandibular lymph node as lymph nodal tissue has been described to contain immune cells expressing PD-L1 in humans [10].

We hypothesized that (transformed) equine fibroblasts derived from ES tumors express PD-L1. Substantial expression would support PD-L1 as a mechanism of immune evasion in ES disease and consequently, immunotherapy directed against PD-L1 would be a reasonable approach for the treatment of ES tumors.

56 **2. Material and Methods**

Lesional tissue samples from ten horses presented for surgical removal of histologically confirmed ES tumors were investigated. Samples were obtained from seven geldings, two mares and one stallion of different breeds (one Shetland pony, one Rocky mountain horse, one American Quarter horse, one Arabian, one Friesian, one Swiss Warmblood, one Franches-Montagnes and one mixed breed) and included ES tumors of different gross morphology (three fibroblastic, four verrucous, two nodular and one mixed). These samples were retrieved from the ISME (Swiss Institue of Equine Medicine) tumor tissue bank.

Samples of equine placenta from a Franches-Montagnes mare and samples of an equine
mandibular lymph node from a Selle-Français mare were collected from patients of the ISME.
All owner gave their informed written consent.

PD-L1 is expressed at the surface of villous syncytiotrophoblasts and cytotrophoblasts of the
placenta to confer the fetomaternal tolerance [11]. Human placenta tissue samples, used as
positive control, were obtained from the Translational Research Unit (TRU) of the University
of Bern.

A rabbit polyclonal antibody (orb158130, Biorbyt, UK) was used, which reacts with human and mouse PD-L1, and is predicted to cross-react with the horse antigen. Comparison of the equine PD-L1 amino acid sequence using BLAST (<u>https://blast.ncbi.nlm.nih.gov</u>) revealed an 80% homology to human PD-L1.

As a positive control for the IHC protocol on FFPE slides the cytokeratine AE1/AE3 antibody
(monoclonal mouse α-human, Dako, M3515) was used at a dilution of 1:50. Negative controls
were created by omitting the primary antibodies.

Four-µm FFPE tumor sections were dried on silane-coated slides. The slides were 78 subsequently deparaffinizaded and re-hydrated. Antigen retrieval was performed in citrate 79 80 buffer (pH 6.0), by boiling for 10 minutes in the microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes. Bovine serum albumin, also 81 at a concentration of 3%, was subsequently added. The primary antibody (orb158130, 82 Biorbyt, dilution 1:200, incubation at room temperature) was applied overnight. The 83 secondary biotinylated antibody (Dako, K0675) + System HRP (Dako, K0675) were added. 84 Between every step the slides were washed in 0,05% Tween Tris-buffered saline 85 (TBS/Tween) solution at pH 7.6 (Dako, S3006). DAB (3,3'-diaminobenzidine 86 87 tetrahydrochloride) was used for the staining. In a final step, slides were counterstained with 88 hematoxylin. IHC protocol is shown in detail in **Table 1**.

The stained slides were assessed by a board-certified pathologist (SR) using light microscopy,
inspecting the sections with x4, x10, x20 and x40 objectives. A semiquantitative scoring

system was applied for the assessment of cell staining. A positive cell was defined as a cell
exhibiting a membranous signal alone or a membranous and cytoplasmatic signal. The
following scoring system was used: grade 0: 0%, grade 1: <10%, grade 2:10-25%, grade 3:
25-50%, and grade 4: >50% of cells staining positive for PD-L1.

95 **3. Results**

96 Positive (cytokeratin-staining) and negative controls (primary antibody omitted) yielded97 expected results, thus highlighting the technical accuracy of the experiment (data not shown).

PD-L1-staining of equine placental tissue yielded a membrane and a cytoplasmic signal 98 99 (Figure 1), consistent with the staining of the human placenta samples. Although the signal was somewhat weaker than on the human placenta, the result suggests that the orb158130 100 antibody does detect PD-L1 in horses. This result was corroborated in the horse lymph node, 101 102 showing a few clearly positive cells, especially in the area of lymph follicle but also in others areas. The signal was mainly granular and localized in the membrane and the cytoplasm. In 103 7/9 examined ES tumors, less than 10% of the tumor cells exhibited a positive signal. Only in 104 2/9 ES tumors 25-50% of the cells showed a positive signal for PD-L1 (score 3+). The results 105 are summarized in Table 2. 106

107 **4. Discussion**

Based on the findings of this study, ES-derived, transformed equine fibroblasts do not appear to consistently express PD-L1. In fact, most of the cells of the analyzed ES tumors stained negative for the protein PD-L1. In 7/9 of the examined ES tumor samples, less than 10% of the tumor cells tested positive for PD-L1 expression. In contrast, cells in equine and human placental samples consistently expressed PD-L1. Similarly, cells in lymph tissue showed a granular membranous and cytoplasmic signal.

Although the number of examined tumors is low, this pilot experiment suggests that PD-L1 is 114 115 not generally expressed by ES-derived transformed equine fibroblasts and therefore PD-1 blockade is unlikely a general mechanism of immune evasion in ES disease. However, PD-L1 116 is not only expressed by tumor cells but also by T- and B-lymphocytes and dendritic cells that 117 infiltrate the tumor microenvironment [12]. Reportedly, the predominant immune cell 118 populations which infiltrate ES tissues are macrophages and monocytes [13], but also T-cells 119 [14]. In our study, we focused on the quantification of PD-L1-positive transformed fibroblasts 120 that are readily differentiated from immune cells, based on morphological criteria. As a small 121 percentage of immune cells was also PD-L1-positive, it would be interesting to characterize 122 the precise nature of these immune cells in ES in the future. 123

In human medicine, overexpression of PD-L1 has been observed in some cancers, including 124 melanomas [15]. Three equine melanomas were included in the study and in two lesions about 125 30-50% of tumor cells stained positive for PD-L1. In this study, human placenta delivered at 126 birth was used as a positive control. In human placentas the expression of PD-L1 is highest in 127 the second and third trimester of pregnancy [16]. In equine placenta, endometrial cups are 128 present in the uterine wall from day 40 and up to day 150 of gestation, but regress after day 129 70. Given the particular role of endometrial cups in the development of the fetal immune 130 system, staining of the epithelium of endometrial cups may differ in earlier gestation stages in 131 equids compared to humans. 132

The similarities in the PD-L1 staining pattern between human and equine positive control tissues found in this comparative analysis suggest that the antibody used in the described protocol is indeed valid for PD-L1 detection in equine tissues, albeit it is not an unequivocal proof for PD-L1-specificity of the antibody in the horse. As equine keratinocytes also stained positive in five out of nine slides with ES-derived tissues, unspecific cross-reactivity of the antibody cannot be ruled out and is the most likely explanation for this observation.

Alternatively, BPV-DNA may more consistently lead to the expression of PD-L1 in infected 139 140 equine keratinocytes compared to BPV-transformed equine fibroblasts. Whereas BPV completes an infectious life-cycle within the epidermal keratinocytes of its natural bovine host 141 142 and leading to the production of countless infectious virions [17], the BPV-infection in the equine host is generally considered "non-productive" or "abortive" [18,19]. More recently, 143 however, BPV-DNA has also been detected in keratinocytes of ES tissues [20], albeit at much 144 lower levels compared to those found in BPV-transformed equine fibroblasts. Nonetheless, 145 these findings combined with reports of ES disease-transmission in the absence of a bovine 146 source for BPV [21] give reason to belief that the BPV-infection in ES-affected horses may 147 not be entirely "abortive". Likewise, it may be speculated that a BPV-infection may induce 148 PD-L1 expression in equine keratinocytes but not in fibroblasts. Thus, this would be an 149 explanation for the positive staining for PD-L1 of keratinocytes associated with ES-lesions, in 150 151 the absence of positive staining of fibroblasts, as observed in this study.

Ideally, the specificity of an antibody should be tested in isogenic cell lines or tissues containing a wild-type and a knockout of the gene of interest. Using CRISPR-cas9 technology it would be possible to induce Pd-ll knockouts in cultured cells that normally express this receptor and thereby generate such controls. Another approach would be the expression of the equine Pd-ll cDNA in cells that are PD-L1 negative. However, a disadvantage of this approach is that non-physiologically high expression levels are reached and that it remains unclear whether the antibody detects physiologically relevant protein levels.

159 While our results do not support PD-L1 as a potential therapeutic target, it is not the only 160 protein able to downregulate the immune response. Many immunosuppressive mechanisms 161 have been documented in the tumor microenvironment, including the recruitment of 162 regulatory T cells (Tregs) [22] or myeloid derived suppressor cells (MDSC) [23], production 163 of IL-10 [24] and TGF β [25] or expression of other immune checkpoint regulators, such as

164 CTLA-4 (cytotoxic T lymphocyte antigen) [26,27]. In this study, we focused on PD-L1, but 165 further studies are needed to evaluate potential mechanisms of immune evasion and the 166 presence of other negative regulatory molecules in the microenvironment of ES tumors.

167 **5.** Conclusion

In this experiment, we observed positive PD-L1 staining in equine placental and lymph 168 follicle cells, but only in low numbers of ES-derived equine fibroblasts. This suggests that this 169 PD-L1 is not regularly expressed in ES and PD-L1 blockade does not serve as an important 170 mechanism of immune evasion in this form of neoplasia. However, these findings need to be 171 confirmed in a greater number of samples and using antibodies that are more thoroughly 172 validated for applications in equine-derived tissues. Nonetheless, PD-L1 is not only expressed 173 by tumor cells but also by immune cells that infiltrate the tumor microenvironment, and 174 future research may reveal the precise nature of the immune cells within ES tissues that 175 express PD-L1 and unravel their role in ES disease. Finally yet importantly, preliminary 176 177 results obtained on equine melanomas indicate the potential of a targeted therapy of this tumor type with anti-PD-L1 antibodies. 178

179 Declarations

180	-	List of abbreviations: PD-L1: programmed death-ligand 1, PD-1: Programmed cell

- 181 death 1, ES: Equine sarcoid, IHC: Immunohistochemistry, FFPE: Formalin-fixed,
- 182 paraffin-embedded, DAB: diaminobenzidine tetrahydrochloride, HRP: Horseradish
- 183 peroxidase, MDSC: myeloid derived suppressor cells, CTLA-4: cytotoxic T
- 184 lymphocyte antigen, TGF β : Transforming growth factor beta
- Ethics approval and consent to participate: ethical approval / animal use permission
 obtained nr. BE 110/15
- 187 Declarations of interest: none

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- Authors 'Contributions: SV and CK conceived this study. JB and BDB carried out the
- 193 experiment, SV performed the histological examination. JB drafted the manuscripts.
- 194 CK was a major contributor in writing the manuscript. VG revised the manuscript. All
- authors read and approved the final manuscript
- 196 Acknowledgements: Not applicable
- 197

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274 Tables

Table 1: Immunohistochemistry protocol used in the study

Deparaffination and Hybridation	
Melting paraffin in the oven at 60°C	➤ 10min
Xylol	▶ 10min
Xylol	▶ 10min
Ethanol 100%	➤ 2min
Ethanol 100%	➤ 2min
Ethanol 95%	➤ 2min
Ethanol 70%	➤ 2min
Distilled water	➤ 2min
Antigen- Retrieval	
10mM Citrate Buffer pH 6.0	10min boiling in microwave
Room temperature cooling	➤ 5min
Distilled water	➢ 2x 5min
Endogenous peroxidase block	
H_2O_2 3% (in water)	➤ 20min
Distilled water	➤ 2x 5min
Wash with Dako washing buffer (S3006)	> 5min
Biotin blot	
Block endogenous protein with bovine	➤ 30min
serum albumin 3% (BSA) in PBS	
Wash with Dako washing buffer (S3006)	➤ 2x5min
Primary antibody	
PD-L1 in Ab diluent (S0809, Dako)	Dilution 1 :200, overnight incubation
Wash with Dako washing buffer (S3006)	➤ 3x5min
Secondary antibody	
Universal secondary antibody (K0675,	➤ 30min
Dako)	➤ 3x5min
Wash with Dako washing buffer (S3006)	
	➤ 30min
HRP-conjugat streptavidin (K0675, Dako)	
	➤ 3x5min
Wash with Dako washing buffer (S3006)	
	➤ 4min
DAB	
	> 5min
Distilled water	Few seconds
Hematoxylin couterstain	> 5min
Running tap water	➤ 5min
Distilled water	
Mounting	

276

277

- **Table 2:** Summary of the number of slides per score. One equine sarcoid was excluded of the
- study because most of the tissue was destroyed after the immunohistochemistry procedure.

280

Score	0	1+	2+	3+	4+
Human placenta					1
Equine placenta					1
Lymph node		1		J	
Equine Sarcoids					
Verrucous	1	1		1	
• Nodular		2	Q.		
Fibroblastic	1	1	5	1	
 Mixed (verrucous and fibroblastic) 	1				

281

282

283 Figure



- Figure 1: Equine sarcoid, negative (no programmed death-ligand 1 staining) and positive
- controls. Note that the signal is consistently weaker in equine tissues compared to human
- 287 tissues.

Highlights

- Establishment of an IHC protocol for equine PD-L1 using FFPE material
- Preliminary finding indicate that equine sarcoid-derived tissues do not frequently express PD-L1
- The low expression levels of PD-L1 in equine sarcoid-derived tissues argue against PD-L1 contribution to immune evasion in this neoplastic disease

Sontral Pression

Declarations of interest: none

Journal Prevention

Ethical approval / animal use permission obtained nr. BE 110/15

Journal