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Development of monoclonal antibodies targeting canine PD-L1 and PD-1 and their clinical relevance in Canine Apocrine Gland Anal Sac Adenocarcinoma

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Abstract: Canine apocrine gland anal sac adenocarcinoma (AGASACA) is an aggressive canine tumor originating from the anal sac glands. Surgical resection, with or without adjuvant chemotherapy, represents the standard of care for this tumor, but the outcome is generally poor particularly for tumors diagnosed at an advanced stage. For this reason, novel treatment options are warranted, and few recent reports suggested an activation of the immune checkpoint axis in canine AGASACA. In our study we developed canine-specific monoclonal antibodies targeting PD-1 and PD-L1. Forty-one AGASACAs with complete clinical and follow-up information were then analyzed by immunohistochemistry for the expression of the two checkpoint molecules (PD-L1 and PD-1) and the presence of tumor infiltrating lymphocytes (CD3 and CD20), evaluated within the tumor bulk (intratumor) and in the surrounding stroma (peritumor). Seventeen AGASACAs (42%) expressed PD-L1 in a range between 5% and 95%. Intratumor lymphocytes were predominantly CD3+ T-cells and positively correlated with the number of PD-1+ intratumor lymphocytes (ρ=0.36; p=0.02). Peritumor lymphocytes were a mixture of CD3+ and CD20+ cells with a variable PD-1 expression (range 0-50%). PD-L1 expression negatively affected the survival only in the subgroup of dogs treated with surgery alone (n=14; 576 vs 235 days). The presence of a heterogeneous lymphocytic infiltrate and the expression of PD-1 and PD-L1 molecules support the relevance of the immune-microenvironment in canine AGASACAs and the potential value of immune checkpoints as promising therapeutic targets.

Keywords: Dog; Apocrine gland anal sac adenocarcinoma; PD-1; PD-L1, Tumor-infiltrating lymphocytes; Immunohistochemistry.

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1. Introduction

Apocrine gland anal sac adenocarcinoma (AGASACA) is a relatively rare skin neoplasm, representing approximately 17% of perianal tumors of dogs [1]. It is

characterized by an aggressive biological behavior, with a high rate of metastasis affecting the locoregional lymph nodes and, less frequently, distant sites. The median survival time ranges from 1 to 2 years, depending on the clinical stage [1–13]. Prognostic factors include regional lymph node metastasis at the time of diagnosis, tumor and regional lymph node size, histological pattern, necrosis and lymphovascular invasion [14–18]. Conversely, the prognostic significance of hypercalcemia remains controversial and Ki67 proliferation index did not demonstrate a prognostic value [18,19]. Surgical excision represents the best treatment option for AGASACAs without distant metastasis [2]. Chemotherapy and radiotherapy are also employed as adjuvant and/or palliative treatments, and toceranib phosphate showed effective results in prolonging survival time [3–13,20]. Immunotherapeutic approaches have not been explored yet.

In several canine cancers, the immune checkpoint programmed death-1 (PD-1) and its ligand PD-ligand 1 (PD-L1), were recently suggested as prognostic markers as well as promising therapeutic targets [21–27]. The interaction between PD-1 and PD-L1 is physiologically involved in the immune response regulation, resulting in the activation of inhibitory signals, responsible of a reduced production of antibodies and cytokines by the immune cells [28]. In oncology, the PD-1/PD-L1 axis activation induced by PD-L1-expressing tumor cells interacting with PD-1-expressing tumor-infiltrating lymphocytes (TILs), is one of the most studied immune evasion strategies played by cancer, paving the way for the development of immunotherapeutic approaches based on the blockade of these molecules [28]. Recently, the expression of PD-L1 was investigated by immunohistochemistry (IHC) in various canine tumors, among which 95% of the analyzed AGASACAs (19/20) expressed the immune checkpoint molecule, suggesting the activation of the axis [26]. However, these results were limited to a small number of cases and the association with clinico-pathological data was not considered.

In this study, we developed canine-specific monoclonal antibodies targeting PD-1 and PD-L1. The expression of the two immune checkpoint molecules was evaluated by IHC in 41 surgically resected AGASACAs. The immunophenotypic characterization of TILs was also performed. Furthermore, the correlation of IHC results with clinico-pathological features and follow-up data was investigated, in order to elucidate the prognostic role of the immune microenvironment and to provide new therapeutic insights.

2. Materials and Methods

Study population

The AGASACA cases diagnosed and treated at the Veterinary Teaching Hospital (VTH) of the University of Turin, in a spanning period of 10 years (2011-2021) were considered for the study. Only dogs that underwent surgical excision of the primary tumor and of regional lymph nodes when found enlarged on CT scan evaluation, were included in the study, regardless of the administration of adjuvant chemotherapy. Dogs diagnosed with stage IV disease before the surgery or having concurrent severe illness that could significantly reduce the survival time were excluded.

For each dog, signalment, primary tumor side, tumor size, clinical signs at presentation, presence of hypercalcemia (ionized calcium >1.45 mmol/L), presence of ileo-sacral lymphadenopathy, complete blood analysis, pre- and post-contrast whole body computed tomography, type of treatment (surgery with or without adjuvant chemotherapy), and surgical and post-surgical complications were recorded. Follow-up data were obtained from the follow-up visits at the VTH and/or by telephone contact with owners or referring veterinarians. The disease-free interval (DFI) was calculated as the interval between surgery and local recurrence or metastatic spread, and the survival time (ST) as the time from surgery until death for any cause.

Histological analysis

All surgically removed AGASACAs were histologically confirmed and the following morphological features were recorded [19]: predominant tumor pattern (solid, rosettes/tubules and papillary), necrosis (absent or present), inflammatory infiltration (absent or present), status of surgical margins, lympho-vascular invasion (absent or present), cellular pleomorphism (anisokaryosis and anisocytosis), mitotic count and Ki67 index evaluated by IHC.

Generation of anti-PD-1 and anti-PD-L1 monoclonal antibodies

Anti-PD-1 and anti-PD-L1 monoclonal antibodies were generated by Moravian-Biotechnology Ltd. (Brno, Czech Republic). BALB/c mice were hyper-immunized with canine recombinant PD-1 and PD-L1 proteins (with a 6xHis, and Fc tag, respectively; Sino Biological Europe GmbH, Eschborn, Germany). For both targets, splenocytes from hyper-immunized mice were fused with the non-producing mouse myeloma cell line SP2. Supernatants of selected hybrids were screened using dot-blot on nitrocellulose membrane coated with recombinant canine PD1 (Sino Biological Europe GmbH, Eschborn, Germany) or PD-L1 (KingFisher Biotech Inc, Saint Paul, MN, USA) [29]. Reactive supernatants were further validated by ELISA, western blotting and flow cytometry.

ELISA – PD-L1

Recombinant PD-L1-FC fusion protein (Sino Biological Europe GmbH, Eschborn, Germany) was diluted in carbonate coating buffer (pH 9.6) at 1 µg/mL and a 96-well plate (Costar) was coated with 50 ng of protein overnight at 4°C. Wells were subsequently washed three times with PBST and blocked with blocking buffer (PBS with 2% BSA) for 1 hour at room temperature. Blocking buffer was removed and serial dilutions of antibodies (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 – starting from 1mg/mL) were added and incubated overnight at 4°C. Wells were then emptied, washed three times with PBST and incubated for 1 hour at room temperature with goat anti-mouse HRP conjugated antibody (Dako) diluted in PBS with 2% BSA at 1:1000. After washing the wells, Pierce ECL (Thermo Fisher Scientific, Oxford, UK) was added to the wells and the plate was read using BioTek Synergy plate reader. Data were subsequently analyzed using GraphPad Prism 9.4 (GraphPad, San Diego, CA, USA).

ELISA – PD-1

Recombinant PD-1-FC fusion protein (Sino Biological) was diluted in carbonate coating buffer (in-house; pH 9.6). A 96-well opaque white plate (Costar) was coated overnight at 4°C with 50ng of protein per well. Wells were subsequently washed four times with 400µl of PBST 0,05% (the same for all the following washes) and blocked with blocking buffer (BB; PBST with 3% BSA) for 90min at room temperature (RT). Blocking buffer was removed and serial dilutions of the antibody (purified stock in PBS) were prepared (1000, 100, 10, 1 ng/mL, equal to 100, 10, 1, 0.1 ng/well) and added to plate, 100µl per well. The plate was incubated for 1h in RT. Wells were emptied, washed, and incubated for 1 hour at room temperature with a rabbit anti-mouse polyclonal HRP conjugated antibody (Dako P0260) diluted in BB at 1:1000. After washing the wells, Pierce ECL (Thermo Fisher) was added to the wells and the plate was read immediately using Fluoroskan Ascent plate reader (Thermo Fisher), Luminescence mode and integration time 300ms. Data was analyzed using GraphPad Prism 9.4 (GraphPad, San Diego, CA, USA).

Western Blotting

Western blotting (WB) for PD-L1 analysis was performed using recombinant PD-L1-Fc fusion protein (Sino Biological Europe GmbH, Eschborn, Germany) and cell lysates of K9TCC, K9TCC-SH (both kindly provided by Prof. Deborah Knapp, Purdue University, USA), and DH82 (ATCC). K9TCC, K9TCC-SH and DH82. All cell lines were selected

based on previous publications, which have shown that the cell lines expressed PD-1 and/or PD-L1 [27,30,31]. In addition, the K9TCC and K9TCC-SH were considered having different levels of expression of EGFR [32]. EGFR is known to affect PD-L1 stability through modifications in glycosylation levels, allowing to investigate antibody bindings to different patterns of PD-L1 glycosylation [33].

Thirty micrograms of cell lysates were separated on Novex NuPage 4-12% SDS-PAGE gel (Thermo Fisher Scientific, Oxford, UK) and transferred to PVDF membrane using iBlot transfer system (Thermo Fisher Scientific, Oxford, UK). Membranes were then blocked with Intercept® (TBS) Blocking Buffer (Li-cor, Cambridge, UK) and incubated with PD1 1.1 or PD-L1 3.1 antibodies at 1:1000 (PD-1 1.1) or 1:200 (PD-L1 3.1) concentration overnight. After the incubation, membranes were washed and incubated with IRDye® 800CW Donkey anti-Mouse (Li-cor, Cambridge, UK). The membrane was subsequently washed with PBST and PBS. The membrane was imaged using Odyssey imaging system (Li-cor, Cambridge, UK).

Flow cytometry

Cell lines used for WB, were also utilized in flow cytometry (FC) experiments. Cells were expanded in DMEM/F12 (K9TCC, K9TCC SH) or DMEM (DH82, OSA31) media (both from Gibco, Thermo Fisher Scientific, Oxford UK) supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Oxford UK) and Penicillin-Streptomycin (Gibco, Thermo Fisher Scientific, Oxford UK). After reaching confluency, cells were detached using 0.25% Trypsin-EDTA (Gibco, Thermo Fisher Scientific, Oxford UK), counted and $5x10^5$ cells were used for each antibody. For all cell lines, PD-L1 antibodies 1.1, 2.1 and 3.1 were used at concentration of 1:100 diluted in PBS with 2% BSA and incubated for 1 hour. Subsequently, cells were washed with PBS with 2% BSA and incubated with 1:1000 dilution of FITC conjugated anti-mouse polyvalent immunoglobulin secondary antibody (Sigma, Merck KGaA, Darmstadt, Germany), which binds all antibody subclasses. Cells incubated with secondary antibody were used as controls. PD-1 antibody was directly conjugated to APC fluorophore using a commercially available kit (Abcam, Cambridge, UK) and did not require a secondary antibody. Unstained cells were used as controls for PD-1 antibody.

For DH82 cell line, as it expresses Fc receptor, cells were incubated with Fc Receptor Binding Inhibitor (eBioscience, ThermoFisher Scientific, Oxford,UK) for 30 min. Cells were then washed, resuspended in staining buffer and flow cytometry was performed using BD LSR Fortessa flow cytometer (Becton Dickinson, Wokingham, UK). Data were subsequently analyzed using FlowJo (FlowJo LLC, Ashland, OR, USA).

In addition a K9TCC transiently overexpressing PD-1 was generated via transfection of this cell line with a custom-build pcDNA3.1 plasmid vector encoding for canine PD-1 (GeneArt, ThermoFisher Scientific, Oxford,UK). Cells were transfected with the vector using lipofectamine (Gibco, ThermoFisher Scientific, Oxford, UK) and selected using Hygromycin (ThermoFisher Scientific, Oxford, UK). Transfection was confirmed using flow cytometry as described above.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4 μm thick FFPE sections using VECTASTAIN® Universal Quick HRP Kit, Peroxidase, R.T.U. from Vector Laboratories Inc. (Burlingame, CA), following the manufacturer's instructions. Sections were incubated with 0.3% H_2O_2 for endogenous peroxidase activity blocking, and heat-induced antigen retrieval was then performed in citrate buffer (pH 6.0) (for anti-PD-L1, anti-PD-1, anti-CD20 primary antibodies) or in Tris-EDTA buffer (pH 9.0) (for anti-CD3 primary antibody) at 98°C for 20 minutes. After non-specific antibody binding blocking performed with Normal Horse Serum 2.5%, sections were incubated with the following primary antibodies: anti-CD3 (diluted 1:50; Dako, Nowy Sącz, Poland), anti-CD20 (diluted 1:1000; Moravian-Biotechnology) and anti-PD-1 (diluted 1:100) for 2h at room

temperature, and anti-PD-L1 (diluted 1:10) overnight at 4°C. Signal was detected using Vectastain Elite ABC kit and ImmPACT DAB from Vector Laboratories Inc. (Burlingame, CA). In each immunohistochemical run positive controls were included (canine lymph node for anti-CD3 and anti-CD20; canine tonsil for anti-PD-1; canine diffuse large B-cell lymphoma known to have high PD-L1 mRNA expression by RNAscope for anti-PD-L1). Negative controls were prepared by replacing the primary antibody with an irrelevant one in all cases.

Immunohistochemical scoring

IHC stained slides were independently evaluated by 2 pathologists (L.M. and L.L.) and discordant results were reviewed to reach a consensus.

TILs were evaluated on CD3- and CD20-stained slides and separately scored within the tumor bulk (i.e. intratumor lymphocytes) and in the surrounding stroma (i.e. peritumor lymphocytes). Briefly, intratumor lymphocytes were counted in 4 microscopic fields at 200x and a score was arbitrarily assigned according to the following criteria: score 0 = < 10 positive lymphocytes; 1 = 10-25 positive lymphocytes; 2 = 26-60 positive lymphocytes; 3 = > 60 positive lymphocytes. Peritumor lymphocytes were scored from 0-3 according to the number of lymphocytic aggregates in the tumor stroma, regardless of their immunophenotype and counted in 10 fields at 4x magnification ("peritumor lymphocyte total score"; score 0 = ≤ 1 aggregates; 1 = 2-6 aggregates; 2 = 7-14 aggregates; 3 = ≥ 15 aggregates). In addition, the proportion of CD3 and CD20 positive lymphocytes in peritumor aggregates was recorded.

Similarly, PD-1-expressing lymphocytes were separately scored in intratumor and peritumor areas. For intratumor PD-1+ lymphocytes, the same scoring system described above for CD3 and CD20 was applied. For peritumor lymphocytes, the percentage of PD-1+ cells was assessed, and a semiquantitative score was assigned as follow: 0 = <5%; 1 = 5-24%; 2 = 25-49%; 3 = ≥50%.

For PD-L1 evaluation, the percentage and the intensity (0= negative; 1= mild; 2= moderate; 3= marked) of positive tumor cells was assessed.

Statistical analysis

All statistical analyses were performed using R software. The association of clinico-pathological features with the IHC results was explored. Fisher's exact test was used for categorical variables, while continuous variables were tested by means of Student t-test or Wilcoxon rank-sum test, according to the results of Shapiro–Wilk test previously performed to assess the normality of the distribution. The correlation between the IHC results was investigated by Spearman rank correlation test. Survival analysis was conducted using *survival* and *survminer* packages. Univariate Cox proportional-hazards model was used to test the impact of the following variables on DFI and ST: age, sex, breed, weight, presence of lymph nodes metastasis at the time of the diagnosis, presence of hypercalcemia, tumor size (major diameter measured after formalin fixation), type of treatment (adjuvant chemotherapy in addition to surgery), local recurrence, occurrence of regional or distant metastasis, histopathological features and IHC results. A cut-off of $p \leq 0.05$ was used to screen the variables to be included in the multivariate analysis. Kaplan-Meier curves for categorical variables were drawn and compared by means of log-rank test.

3. Results

Study population

Forty-one dogs were included in the study (Table S1). Twenty-three (56.1%) were purebred dogs, including Labrador retrievers (n=9), Border collies (n=3), Czechoslovakian wolfdogs (n=3), German shepherds (n=2). Thirty-two (78%) dogs were female (5 intact; 27 spayed) and 9 (22%) were male (4 intact; 5 neutered). Median age at diagnosis was 11 years (range: 5-15 years) and median body weight was 24 kg (range: 7-40 kg).

Fifteen dogs (36.6%) were hypercalcemic at presentation and 3 (7.3%) had a bilateral tumor mass. The median tumor size (major diameter measured after formalin fixation) was 3.5 cm (range: 0.5-10 cm). Thirty-one (75.6%) dogs had sublumbar lymphadenopathy at presentation, confirmed as metastatic AGASACA by histopathology in all cases. Fourteen dogs (34.1%) were treated only by surgical resection of the primary tumor and regional lymph nodes. Twenty-seven dogs (65.8%) received adjuvant chemotherapy, which consisted of toceranib phosphate (n=25), carboplatin (n=1) and melphalan (n=1).

Generation and validation of anti-PD-1 and anti-PD-L1 monoclonal antibodies

For PD-L1 antibodies, three clones were considered for further validation as they detected PD-L1-FC recombinant protein in the dot blot. The clones were called 1.1 (IgG3), 2.1 (IgG2a) and 3.1 (IgM). All the three clones detected PD-L1-FC in ELISA (Figure S1 A-C) but none did in the FC assay against cells constitutively expressing PD-L1 (Figure S1 D-F, representing the 3.1 clone). In a WB against PD-L1-FC fusion protein, only one clone (IgM PD-L1 3.1) reacted and a single band of approximately 56 kDa was detected (Figure S2 A). Hence, only the IgM PD-L1 3.1 clone was used for further analysis and tested in WB against the three canine cell lines (K9TCC, K9TCC-SH and DH82), producing a dominating band of approximately 34 kDa and some weaker bands above it, likely representing different post-translational forms of PD-L1 [34] (Figure S2 B).

For PD-1 antibodies, one clone, designated 1.1 (IgG2a), was chosen, which detected rcPD-1-His in the dot blot. PD-1 1.1 detected PD-1-FC in ELISA in a dose-dependent manner, within a range of 0.1-100 ng/well (Figure S3 A). When the antibody was tested by flow cytometry against K9TCC and K9TCC SH cells, constitutively expressing canine PD-1, positive cells were identified (Figure S3 B, C). In addition, increased fluorescent signal was detected in K9TCC cells overexpressing PD-1 (K9TCC-PD1 OE; Figure S3 D). In WB the antibody produced a dominant band, approximately 70 kDa in size, with additional, weaker bands underneath (Figure S4). Similarly to PD-L1, PD-1 was heavily glycosylated. Interestingly, in DH82 cell line a single, weaker band at approximately 36 kDa was found, which is close to the size of deglycosylated PD-1 (Figure S4).

Histopathology and immunohistochemistry

Histopathological features and IHC scores for each dog are reported in Table S1. TILs were evaluated by IHC for CD3 (T lymphocytes) and CD20 (B lymphocytes), in addition to the immune checkpoint molecule PD-1.

Intratumor lymphocytes were interspersed with tumor cells in the tumor bulk and were predominantly CD3+ T cells, present in variable number: 2 cases scored 0; 15 cases scored 1; 14 cases scored 2; 10 cases scored 3 (Figure 1A). CD20+ intratumor lymphocytes were rarely detected (all cases scored 0) (Figure 1B). In 13 cases (31.7%), intratumor lymphocytes also expressed PD-1 in a low percentage (score=1), and a positive correlation between the number of CD3+ and PD-1+ intratumor lymphocytes was detected (ρ=0.36; p=0.02) (Figure 1C).

Regarding peritumor lymphocytes total score, 1 case scored 0, 11 cases scored 1, 20 cases scored 2 and 9 cases scored 3. By IHC, peritumor lymphocytes were a mixture of CD3+ and CD20+ cells, often distributed in nodular aggregates, composed of a variable proportion of T and B lymphocytes (Figure 1D and 1E). Generally, CD3+ cells predominated over CD20+ cells, with a CD3+/CD20+ cell ratio ≥ 1 in 98% (40/41) of the cases. In 61% (25/41) of the cases, a variable proportion of peritumor lymphocytes also expressed PD-1 (11 with score 2; 14 with score 3) (Figure 1F). Local recurrence was significantly associated with a low peritumor lymphocyte total score (p=0.009) and a low CD3+/CD20+ ratio in peritumor lymphocytes (p=0.034).

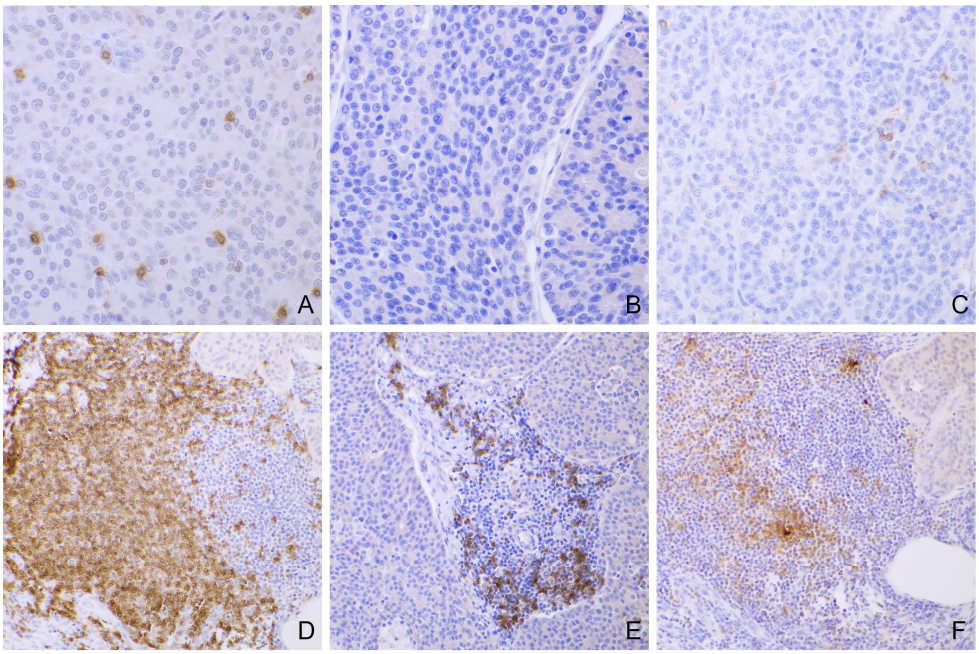

Figure 1. Tumor infiltrating lymphocytes in canine AGASACAs. (A) Numerous intratumor CD3+ lymphocytes (case #22; 40x magnification). (B) Absence of intratumor CD20+ lymphocytes (case #22; 40x magnification). (C) Scattered intratumor PD-1+ lymphocytes (case #22; 40x magnification). (D, E, F) Peritumor lymphocytes in nodular aggregates composed of a mixture of CD3 (D), CD20 (E) and PD-1 (F) positive lymphocytes (case #5; 20x magnification).

Seventeen AGASACAs (42%) expressed PD-L1 in a range between 5% and 95% of tumor cells, with mild to moderate intensity (Figure 2). Interestingly, a single case (case #36) was characterized by a regional area of undifferentiated tumor cells, displaying highly infiltrative behavior, spindeloid morphology and increased cellular atypia. These cells had diffuse and intense expression of PD-L1. No associations between PD-L1 expression and other IHC scores were identified.

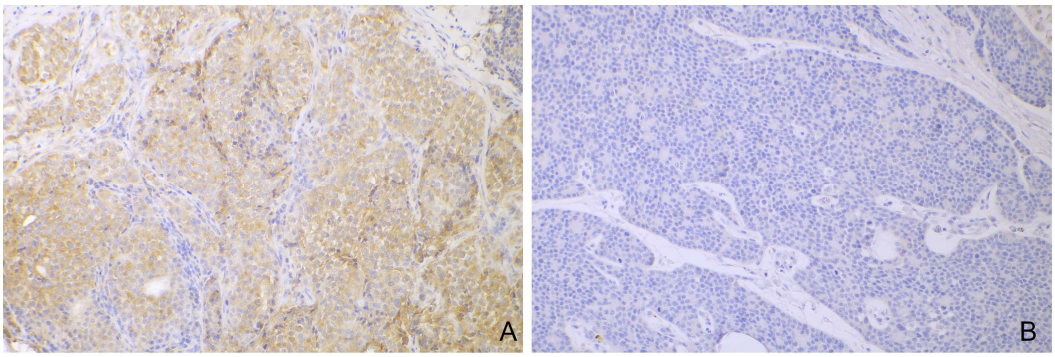

Figure 2. PD-L1 expression in canine AGASACAs (20x magnification). (A) Representative case with diffuse and moderate expression of PD-L1 (case #18). (B) Negative case (case #20).

Survival analysis

At the time of data analysis, 5 dogs (12.2%) were alive after 39 to 819 days, 22 (53.7%) had died for causes attributable to AGASACA (range: 128-1237 days), 14 (34.1%) for unrelated causes (range: 108-1434 days) and one was lost to follow-up (598 days). Eight dogs (19.5%) experienced metastatic disease to regional (ileosacral) lymph nodes after the primary surgery, while 11 dogs (26.8%) developed distant metastasis to various organs, including distant lymph nodes, spleen, liver, and lungs. The median DFI of the whole population was 474 days (range: 0-1237 days), and median ST was 472 days (range: 108-1434 days). By univariate Cox proportional-hazards analysis, dogs with metastatic regional lymph nodes at presentation had a significantly shorter DFI (369 vs 940 days; p=0.046) (Table S2). Body weight, local recurrence, and histological presence of lymphovascular invasion and tumor necrosis negatively affected the ST, however only body weight and lymphovascular invasion maintained statistical significance in multivariate analysis (p=0.027 and p=0.012, respectively) (Table S2).

TILs and immune checkpoint molecules expression did not influence the outcome in the whole population. When the subgroup of dogs treated by surgery alone (n=14) was considered, tumor expression of PD-L1 was associated with a significantly shorter ST compared to cases that tested negative for the marker (576 vs 235 days; p=0.022) (Figure 3; Table S2).

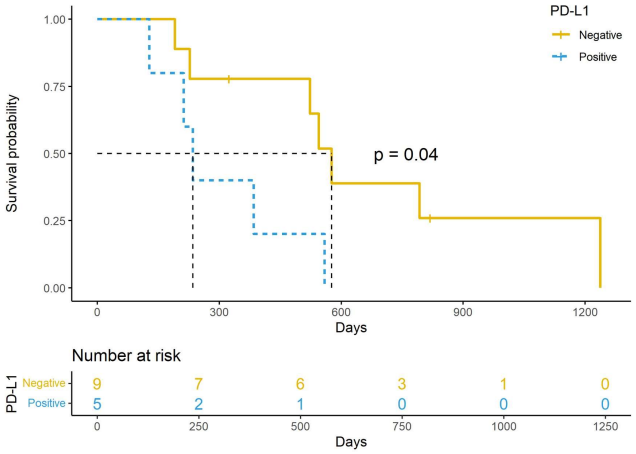

Figure 3. Kaplan–Meier curves of survival time of 14 dogs with AGASACA treated with surgery alone.

4. Discussion

Deciphering the interaction between the tumor and the patient's own immune system has led to the development of cancer immunotherapies, which recently revolutionized the paradigm of cancer treatment in human oncology. In this context, adaptative immunity, and intratumor lymphocytes in particular, was shown to play a key role in the antitumor immune response [35]. Based on the degree of lymphocytic infiltration and molecular signatures, tumors can be classified in three different phenotypes (inflamed, immune-excluded, immune-desert), that are strongly associated to the response to immunotherapeutic treatments, with inflamed (hot) tumors being more likely to benefit from the administration of checkpoint inhibitors [36]. Given the recognized role of several canine tumor types as a spontaneous model for human oncology, there is an increasing interest in characterizing the immune landscape of these tumors. Osteosarcoma, oral malignant melanoma, lymphoma, and mammary carcinomas belong to canine tumors presenting a hot immune phenotype [22,37].

To the best of our knowledge, the immune microenvironment of AGASACA has not been fully characterized so far, but a recent study suggested a possible association, even

if not statistically significant, between inflammatory cell infiltration and outcome [19]. In addition, transcriptome analysis confirmed a "hot" immune signature [38].

Here, in order to further characterize the AGASACA at the molecular level, we have developed two new antibodies against canine ICs highly relevant to cancer in both humans and dogs: PD-1 and PD-L1. Preliminary validation revealed that the PD-1 monoclonal antibody detects its target in WB, ELISA, and FC experiments. Among the three developed PD-L1 clones, all were effective in ELISA, none in FC, and only the 3.1 clone in WB. Upon basic validation, the antibodies PD-L1 3.1 and PD-1 1.1 were used to assess target expression on the canine AGASACA samples. The antibodies will be further characterized in future to assess their potential as drug candidates.

In the present study, the presence and phenotype of TILs was evaluated by IHC in 41 surgically resected AGASACAs and a high degree of lymphocytic infiltration, both in peritumoral and intratumor location, was observed. Moreover, while peritumor lymphocytes were a mixture of T- and B-cells, intratumor lymphocytes were almost exclusively represented by CD3+ T-cells. These findings support an inflamed immunophenotype at least for a subset of cases; in fact, contrary to the immune-excluded phenotype, which is characterized by lymphocytic infiltrates limited to the tumor stroma, inflamed tumors are defined by the presence of T-cells intermingled with neoplastic cells [36]. Even if molecular investigations were beyond the aim of our study, the immunohistochemical characterization of TILs suggests a "hot" phenotype of canine AGASACAs and encourage further investigations in this direction.

Cancer immunotherapy in veterinary oncology is still at early stages of development and application. Some successful examples are represented by the clinical implementation of autologous vaccines in canine lymphoma [39] and anti-CSPG4 electro-vaccination in canine melanoma [40]. Another early but promising example is represented by the empty cowpea mosaic virus treatment in inflammatory mammary carcinoma [41]. Only few preliminary studies, by using specific canine anti-PD-L1 and anti PD-1 antibodies for the treatment of oral malignant melanoma, demonstrated the potential value of immune checkpoint blockade in dogs [24,26]. To evaluate if canine AGASACA is a possible candidate for immunotherapy, the study population was tested by IHC with monoclonal antibodies specifically targeting canine PD-1 and PD-L1, and a subset of TILs expressed PD-1 in variable proportions. These were localized both within the tumor bulk (i.e., intratumor) and in the surrounding stroma (i.e., peritumor); moreover, in intratumor areas the number of PD-1+ lymphocytes positively correlated with the number of CD3+ cells. Although no prognostic value of PD-1 expression by TILs was demonstrated, the enrichment of the tumor microenvironment with PD-1-positive lymphocytes might support their role as a possible target for the development of immunotherapeutic strategies.

To date, the expression of PD-L1 at the protein level has been investigated in a variety of canine tumors. The most comprehensive studies, which included preliminary clinical trials or PD-L1 association with clinico-pathological features and/or outcome, focused on melanoma, osteosarcoma, and mammary carcinoma [21,25,26,42]. In addition, two studies [26,43] tested the expression of PD-L1 by IHC in a limited number but wide range of canine tumors, demonstrating its expression in several tumor types, including 19 out of 20 of the tested AGASACAs. Among these, most of the tumors expressed PD-L1 in more than 50% of the tumor cells. Similarly, in the present study, the expression of PD-L1 by 17 out of 41 AGASACAs was demonstrated, with variable percentages of positive tumor cells. Lack of standardized methods to assess PD-L1 expression by tumor cells in veterinary medicine might have contributed to the lower proportion of positive AGASACAs observed in our study compared to the previous one [26]. In this context, it is also worth to mention the variability observed in human oncology, when different antibody clones and staining platforms are used to assess PD-L1 tumor proportional score [44]. Interestingly, a single case in this study had a regional tumor area displaying morphological features of undifferentiated carcinoma with highly invasive growth

pattern; this area was also characterized by diffuse and intense positivity for PD-L1. Given the morphological features and the early recurrence and regional metastasis observed in this dog, this finding might suggest an association between PD-L1 activation and tumor aggressive behavior.

Survival analysis for the whole cohort showed that body weight, local recurrence, and histological presence of lymphovascular invasion and tumor necrosis negatively affected the outcome, as previously described [18,19]. A shorter DFI was also observed in dogs with nodal metastasis at presentation.

PD-L1 expression was associated with a poorer outcome only in the subgroup of dogs treated with surgery alone (i.e., without medical treatment). This result indicates that, similarly to other canine tumors [21,23] PD-L1 expression in AGASACAs influenced tumor behavior, affecting the ST, thus representing a potential prognostic biomarker. However, due to the limited patient number, this preliminary result needs to be interpreted with the consideration of potential bias and limitations. Future studies, on a larger cohort of patients to confirm this result are needed. The lack of association between PD-L1 expression and survival when the whole cohort was considered might be explained by the influence that adjuvant treatment with toceranib phosphate has on survival [4,12,13]. In our study, we did not observe a clear impact of chemotherapy treatment on survival, but since the chemotherapy protocol was not standardized, it is possible that the adjuvant treatment might have represented a confounding factor when the whole population was considered, potentially obscuring the impact of other variables on the outcome. To our knowledge, the direct effect of toceranib phosphate on PD-L1 expression in canine cancer has not been investigated so far . However, there is some evidence for induction of PD-L1 destabilization and degradation by sunitinib, a tyrosine kinase inhibitor similar to toceranib, leading to a decreased expression of this molecule on tumor cells [45].Although not yet confirmed, the effect of toceranib might be similar.

5. Conclusions

In conclusion, our study showed a high and heterogenous expression of immune checkpoint molecules and the presence of a lymphocytic infiltration in canine AGASACAs. PD-L1 was also associated with the outcome in a subset of patients, indicating a prognostic significance for this marker. Even if in our cohort TILs were not associated with the outcome, the high level of lymphocytic infiltration suggests a hot immune phenotype of this tumor. Taken together, these results show the relevance of the immune-microenvironment in canine AGASACAs, indicating that the stimulation of anti-cancer immunity might represent a valuable treatment approach for this tumor type and deserve more in-depth investigations.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: PD-L1 antibody validation: ELISA and FC; Figure S2: PD-L1 antibody validation: Western Blot; Figure S3: PD-1 antibody validation: ELISA and FC; Figure S4: PD-1 antibody validation: Western Blot; Table S1: Clinicopathological features; Table S2: Survival.

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Conflicts of Interest: BV is a consultant for Moravian-Biotechnology, who developed the PD-1 and PD-L1 antibodies used in this study. The company had no role in the design, execution, interpretation or writing of the study. All other authors declare no conflict of interest.

References

1. Polton, G.A.; Brearley, M.J. Clinical Stage, Therapy, and Prognosis in Canine Anal Sac Gland Carcinoma. *J Vet Intern Med* **2007**, *21*, 274–280, doi:10.1892/0891-6640(2007)21[274:cstapi]2.0.co;2.
2. Barnes, D.C.; Demetriou, J.L. Surgical Management of Primary, Metastatic and Recurrent Anal Sac Adenocarcinoma in the Dog: 52 Cases. *J Small Anim Pract* **2017**, *58*, 263–268, doi:10.1111/jsap.12633.
3. Wouda, R.M.; Borrego, J.; Keuler, N.S.; Stein, T. Evaluation of Adjuvant Carboplatin Chemotherapy in the Management of Surgically Excised Anal Sac Apocrine Gland Adenocarcinoma in Dogs. *Vet Comp Oncol* **2016**, *14*, 67–80, doi:10.1111/vco.12068.
4. Potanas, C.P.; Padgett, S.; Gamblin, R.M. Surgical Excision of Anal Sac Apocrine Gland Adenocarcinomas with and without Adjunctive Chemotherapy in Dogs: 42 Cases (2005–2011). *Journal of the American Veterinary Medical Association* **2015**, *246*, 877–884, doi:10.2460/javma.246.8.877.
5. Valenti, P.; Menicagli, F.; Baldi, A.; Barella, G.; Catalucci, C.; Attorri, V.; Spugnini, E.P. Evaluation of Electrochemotherapy in the Management of Apocrine Gland Anal Sac Adenocarcinomas in Dogs: A Retrospective Study. *Open Vet J* **2021**, *11*, 100–106, doi:10.4314/ovj.v11i1.15.
6. Meier, V.; Besserer, J.; Roos, M.; Rohrer Bley, C. A Complication Probability Study for a Definitive-Intent, Moderately Hypofractionated Image-Guided Intensity-Modulated Radiotherapy Protocol for Anal Sac Adenocarcinoma in Dogs. *Vet Comp Oncol* **2019**, *17*, 21–31, doi:10.1111/vco.12441.
7. McQuown, B.; Keyerleber, M.A.; Rosen, K.; McEntee, M.C.; Burgess, K.E. Treatment of Advanced Canine Anal Sac Adenocarcinoma with Hypofractionated Radiation Therapy: 77 Cases (1999-2013). *Vet Comp Oncol* **2017**, *15*, 840–851, doi:10.1111/vco.12226.
8. Williams, C.; Parys, M.; Handel, I.; Serra, J.C.; Lawrence, J. Minimal Late Radiation Toxicity and Transient Early Toxicity Following Postoperative Definitive Intent Conformal Radiation Therapy (20 × 2.5 Gy) for Canine Apocrine Gland Anal Sac Adenocarcinoma. *Vet Radiol Ultrasound* **2022**, *63*, 224–233, doi:10.1111/vru.13042.
9. Swan, M.; Morrow, D.; Grace, M.; Adby, N.; Lurie, D. Pilot Study Evaluating the Feasibility of Stereotactic Body Radiation Therapy for Canine Anal Sac Adenocarcinomas. *Vet Radiol Ultrasound* **2021**, *62*, 621–629, doi:10.1111/vru.12998.
10. Wouda, R.M.; Hocker, S.E.; Higginbotham, M.L. Safety Evaluation of Combination Carboplatin and Toceranib Phosphate (Palladia) in Tumour-Bearing Dogs: A Phase I Dose Finding Study. *Vet Comp Oncol* **2018**, *16*, E52–E60, doi:10.1111/vco.12332.
11. London, C.; Mathie, T.; Stingle, N.; Clifford, C.; Haney, S.; Klein, M.K.; Beaver, L.; Vickery, K.; Vail, D.M.; Hershey, B.; et al. Preliminary Evidence for Biologic Activity of Toceranib Phosphate (Palladia(®)) in Solid Tumours. *Vet Comp Oncol* **2012**, *10*, 194–205, doi:10.1111/j.1476-5829.2011.00275.x.
12. Elliott, J.W. Response and Outcome Following Toceranib Phosphate Treatment for Stage Four Anal Sac Apocrine Gland Adenocarcinoma in Dogs: 15 Cases (2013–2017). *Journal of the American Veterinary Medical Association* **2019**, *254*, 960–966, doi:10.2460/javma.254.8.960.
13. Heaton, C.M.; Fernandes, A.F.A.; Jark, P.C.; Pan, X. Evaluation of Toceranib for Treatment of Apocrine Gland Anal Sac Adenocarcinoma in Dogs. *J Vet Intern Med* **2020**, *34*, 873–881, doi:10.1111/jvim.15706.
14. Pradel, J.; Berlato, D.; Dobromylskyj, M.; Rasotto, R. Prognostic Significance of Histopathology in Canine Anal Sac Gland Adenocarcinomas: Preliminary Results in a Retrospective Study of 39 Cases. *Vet Comp Oncol* **2018**, *16*, 518–528, doi:10.1111/vco.12410.
15. Simeonov, R.; Simeonova, G. Quantitative Analysis in Spontaneous Canine Anal Sac Gland Adenomas and Carcinomas. *Res Vet Sci* **2008**, *85*, 559–562, doi:10.1016/j.rvsc.2008.03.009.

16. Mosca, A.; Restif, O.; Dobson, J.; Hughes, K. Expression of Phosphorylated Signal Transducer and Activator of Transcription 3 and Its Prognostic Significance in Canine Anal Sac Adenocarcinoma. *J Comp Pathol* **2021**, *182*, 15–21, doi:10.1016/j.jcpa.2020.11.002.
17. Skorupski, K.A.; Alarcón, C.N.; de Lorimier, L.-P.; LaDouceur, E.E.B.; Rodriguez, C.O.; Rebhun, R.B. Outcome and Clinical, Pathological, and Immunohistochemical Factors Associated with Prognosis for Dogs with Early-Stage Anal Sac Adenocarcinoma Treated with Surgery Alone: 34 Cases (2002-2013). *J Am Vet Med Assoc* **2018**, *253*, 84–91, doi:10.2460/javma.253.1.84.
18. Wong, H.; Byrne, S.; Rasotto, R.; Drees, R.; Taylor, A.; Priestnall, S.L.; Leo, C. A Retrospective Study of Clinical and Histopathological Features of 81 Cases of Canine Apocrine Gland Adenocarcinoma of the Anal Sac: Independent Clinical and Histopathological Risk Factors Associated with Outcome. *Animals (Basel)* **2021**, *11*, 3327, doi:10.3390/ani11113327.
19. Morello, E.M.; Cino, M.; Giacobino, D.; Nicoletti, A.; Iussich, S.; Buracco, P.; Martano, M. Prognostic Value of Ki67 and Other Clinical and Histopathological Factors in Canine Apocrine Gland Anal Sac Adenocarcinoma. *Animals (Basel)* **2021**, *11*, 1649, doi:10.3390/ani11061649.
20. Yamazaki, H.; Tanaka, T.; Mie, K.; Nishida, H.; Miura, N.; Akiyoshi, H. Assessment of Postoperative Adjuvant Treatment Using Toceranib Phosphate against Adenocarcinoma in Dogs. *J Vet Intern Med* **2020**, *34*, 1272–1281, doi:10.1111/jvim.15768.
21. Ariyarathna, H.; Thomson, N.A.; Aberdein, D.; Perrott, M.R.; Munday, J.S. Increased Programmed Death Ligand (PD-L1) and Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4) Expression Is Associated with Metastasis and Poor Prognosis in Malignant Canine Mammary Gland Tumours. *Veterinary Immunology and Immunopathology* **2020**, *230*, 110142, doi:10.1016/j.vetimm.2020.110142.
22. Aresu, L.; Ferraresso, S.; Marconato, L.; Cascione, L.; Napoli, S.; Gaudio, E.; Kwee, I.; Tarantelli, C.; Testa, A.; Maniaci, C.; et al. New Molecular and Therapeutic Insights into Canine Diffuse Large B-Cell Lymphoma Elucidates the Role of the Dog as a Model for Human Disease. *Haematologica* **2019**, *104*, e256–e259, doi:10.3324/haematol.2018.207027.
23. Aresu, L.; Marconato, L.; Martini, V.; Fanelli, A.; Licenziato, L.; Foiani, G.; Melchiotti, E.; Nicoletti, A.; Vascellari, M. Prognostic Value of PD-L1, PD-1 and CD8A in Canine Diffuse Large B-Cell Lymphoma Detected by RNAscope. *Veterinary Sciences* **2021**, *8*, 120, doi:10.3390/vetsci8070120.
24. Igase, M.; Nemoto, Y.; Itamoto, K.; Tani, K.; Nakaichi, M.; Sakurai, M.; Sakai, Y.; Noguchi, S.; Kato, M.; Tsukui, T.; et al. A Pilot Clinical Study of the Therapeutic Antibody against Canine PD-1 for Advanced Spontaneous Cancers in Dogs. *Sci Rep* **2020**, *10*, 18311, doi:10.1038/s41598-020-75533-4.
25. Maekawa, N.; Konnai, S.; Takagi, S.; Kagawa, Y.; Okagawa, T.; Nishimori, A.; Ikebuchi, R.; Izumi, Y.; Deguchi, T.; Nakajima, C.; et al. A Canine Chimeric Monoclonal Antibody Targeting PD-L1 and Its Clinical Efficacy in Canine Oral Malignant Melanoma or Undifferentiated Sarcoma. *Sci Rep* **2017**, *7*, 8951, doi:10.1038/s41598-017-09444-2.
26. Maekawa, N.; Konnai, S.; Nishimura, M.; Kagawa, Y.; Takagi, S.; Hosoya, K.; Ohta, H.; Kim, S.; Okagawa, T.; Izumi, Y.; et al. PD-L1 Immunohistochemistry for Canine Cancers and Clinical Benefit of Anti-PD-L1 Antibody in Dogs with Pulmonary Metastatic Oral Malignant Melanoma. *npj Precis. Onc.* **2021**, *5*, 1–9, doi:10.1038/s41698-021-00147-6.
27. Choi, J.W.; Withers, S.S.; Chang, H.; Spanier, J.A.; Trinidad, V.L.D.L.; Panesar, H.; Fife, B.T.; Sciammas, R.; Sparger, E.E.; Moore, P.F.; et al. Development of Canine PD-1/PD-L1 Specific Monoclonal Antibodies and Amplification of Canine T Cell Function. *PLOS ONE* **2020**, *15*, e0235518, doi:10.1371/journal.pone.0235518.
28. Jiang, X.; Wang, J.; Deng, X.; Xiong, F.; Ge, J.; Xiang, B.; Wu, X.; Ma, J.; Zhou, M.; Li, X.; et al. Role of the Tumor Microenvironment in PD-L1/PD-1-Mediated Tumor Immune Escape. *Mol Cancer* **2019**, *18*, 10, doi:10.1186/s12943-018-0928-4.
29. Vojtěsek, B.; Bártek, J.; Midgley, C.A.; Lane, D.P. An Immunochemical Analysis of the Human Nuclear Phosphoprotein P53. New Monoclonal Antibodies and Epitope Mapping Using Recombinant P53. *J Immunol Methods* **1992**, *151*, 237–244, doi:10.1016/0022-1759(92)90122-a.

30. Pinard, C.J.; Hocker, S.E.; Poon, A.C.; Inkol, J.M.; Matsuyama, A.; Wood, R.D.; Wood, G.A.; Woods, J.P.; Mutsaers, A.J. Evaluation of PD-1 and PD-L1 Expression in Canine Urothelial Carcinoma Cell Lines. *Vet Immunol Immunopathol* **2022**, *243*, 110367, doi:10.1016/j.vetimm.2021.110367.
31. Hartley, G.; Faulhaber, E.; Caldwell, A.; Coy, J.; Kurihara, J.; Guth, A.; Regan, D.; Dow, S. Immune Regulation of Canine Tumour and Macrophage PD-L1 Expression. *Vet Comp Oncol* **2017**, *15*, 534–549, doi:10.1111/vco.12197.
32. Nagaya, T.; Okuyama, S.; Ogata, F.; Maruoka, Y.; Knapp, D.W.; Karagiannis, S.N.; Fazekas-Singer, J.; Choyke, P.L.; LeBlanc, A.K.; Jensen-Jarolim, E.; et al. Near Infrared Photoimmunotherapy Targeting Bladder Cancer with a Canine Anti-Epidermal Growth Factor Receptor (EGFR) Antibody. *Oncotarget* **2018**, *9*, 19026–19038, doi:10.18632/oncotarget.24876.
33. Li, C.-W.; Lim, S.-O.; Xia, W.; Lee, H.-H.; Chan, L.-C.; Kuo, C.-W.; Khoo, K.-H.; Chang, S.-S.; Cha, J.-H.; Kim, T.; et al. Glycosylation and Stabilization of Programmed Death Ligand-1 Suppresses T-Cell Activity. *Nat Commun* **2016**, *7*, 12632, doi:10.1038/ncomms12632.
34. Li, S.-M.; Zhou, J.; Wang, Y.; Nie, R.-C.; Chen, J.-W.; Xie, D. Recent Findings in the Posttranslational Modifications of PD-L1. *J Oncol* **2020**, *2020*, 5497015, doi:10.1155/2020/5497015.
35. Chen, D.S.; Mellman, I. Oncology Meets Immunology: The Cancer-Immunity Cycle. *Immunity* **2013**, *39*, 1–10, doi:10.1016/j.immuni.2013.07.012.
36. Chen, D.S.; Mellman, I. Elements of Cancer Immunity and the Cancer–Immune Set Point. *Nature* **2017**, *541*, 321–330, doi:10.1038/nature21349.
37. Von Rueden, S.K.; Fan, T.M. Cancer-Immunity Cycle and Therapeutic Interventions- Opportunities for Including Pet Dogs With Cancer. *Frontiers in Oncology* **2021**, *11*.
38. Haake, A.F.H.; Langenhagen, A.K.; Jovanovic, V.M.; Andreotti, S.; Gruber, A.D. 'Hot Versus Cold' – Can Transcriptome Analysis of Canine Perianal Tumours Help Illustrate Their Distinct Immunophenotypic Landscapes? *Journal of Comparative Pathology* **2022**, *191*, 14, doi:10.1016/j.jcpa.2021.11.032.
39. Marconato, L.; Frayssinet, P.; Rouquet, N.; Comazzi, S.; Leone, V.F.; Laganga, P.; Rossi, F.; Vignoli, M.; Pezzoli, L.; Aresu, L. Randomized, Placebo-Controlled, Double-Blinded Chemoimmunotherapy Clinical Trial in a Pet Dog Model of Diffuse Large B-Cell Lymphoma. *Clin Cancer Res* **2014**, *20*, 668–677, doi:10.1158/1078-0432.CCR-13-2283.
40. Riccardo, F.; Tarone, L.; Camerino, M.; Giacobino, D.; Iussich, S.; Barutello, G.; Arigoni, M.; Conti, L.; Bolli, E.; Quaglino, E.; et al. Antigen Mimicry as an Effective Strategy to Induce CSPG4-Targeted Immunity in Dogs with Oral Melanoma: A Veterinary Trial. *J Immunother Cancer* **2022**, *10*, e004007, doi:10.1136/jitc-2021-004007.
41. Alonso-Miguel, D.; Valdivia, G.; Guerrera, D.; Perez-Alenza, M.D.; Pantelyushin, S.; Alonso-Diez, A.; Beiss, V.; Fiering, S.; Steinmetz, N.F.; Suarez-Redondo, M.; et al. Neoadjuvant in Situ Vaccination with Cowpea Mosaic Virus as a Novel Therapy against Canine Inflammatory Mammary Cancer. *J Immunother Cancer* **2022**, *10*, e004044, doi:10.1136/jitc-2021-004044.
42. Cascio, M.J.; Whitley, E.M.; Sahay, B.; Cortes-Hinojosa, G.; Chang, L.-J.; Cowart, J.; Salute, M.; Sayour, E.; Dark, M.; Sandoval, Z.; et al. Canine Osteosarcoma Checkpoint Expression Correlates with Metastasis and T-Cell Infiltrate. *Vet Immunol Immunopathol* **2021**, *232*, 110169, doi:10.1016/j.vetimm.2020.110169.
43. Shosu, R. Programmed Cell Death Ligand 1 Expression in Canine Cancer. *in vivo* **2016**, 10.
44. Doroshow, D.B.; Bhalla, S.; Beasley, M.B.; Sholl, L.M.; Kerr, K.M.; Gnjatic, S.; Wistuba, I.I.; Rimm, D.L.; Tsao, M.S.; Hirsch, F.R. PD-L1 as a Biomarker of Response to Immune-Checkpoint Inhibitors. *Nat Rev Clin Oncol* **2021**, *18*, 345–362, doi:10.1038/s41571-021-00473-5.
45. Li, H.; Kuang, X.; Liang, L.; Ye, Y.; Zhang, Y.; Li, J.; Ma, F.; Tao, J.; Lei, G.; Zhao, S.; et al. The Beneficial Role of Sunitinib in Tumor Immune Surveillance by Regulating Tumor PD-L1. *Adv Sci (Weinh)* **2021**, *8*, 2001596, doi:10.1002/advs.202001596.