Programmed Cell Death Ligand 1 Expression in Canine Cancer

KAZUHA SHOSU[1], MASASHI SAKURAI[2], KUMI INOUE[1], TAKAYUKI NAKAGAWA[3], HIROKI SAKAI[4], MASAHIRO MORIMOTO[2], MASARU OKUDA[5,6], SHUNSUKE NOGUCHI[1,6] and TAKUYA MIZUNO[1,6]

[1]Laboratory of Molecular Diagnostics and Therapeutics, [2]Laboratory of Veterinary Pathology,
[5]Laboratory of Veterinary Internal Medicine, Joint Faculty of Veterinary Medicine,
Yamaguchi University, Yoshida, Yamaguchi, Japan;
[3]Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences,
University of Tokyo, Bunkyo-ku, Tokyo, Japan;
[4]Laboratory of Veterinary Pathology, Department of Veterinary Medicine,
Faculty of Applied Biological Sciences, Gifu University, Gifu, Japan;
[6]Biomedical Science Center for Translational Research, The United Graduate School of Veterinary Science,
Yamaguchi University, Yoshida, Yamaguchi, Japan

Abstract. *Background: Antibody therapy targeting programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) is a promising therapy in human cancer, but only limited information on PD-L1 expression in canine tumors is available. Materials and Methods: PD-L1 expression was examined in 31 canine tumor cell lines of various origins by flow cytometry and western blotting, and in canine tumor and normal tissue specimens by immunohistochemistry. Results: PD-L1 was only expressed on the cell surface of a small number of cell lines but was found expressed within the cells of almost all cell lines. Immunohistochemistry revealed that PD-L1 is frequently expressed in malignant melanoma, mammary gland tumor, mast cell tumor and lymphoma, but less frequently in soft-tissue sarcoma and hemangiosarcoma. PD-L1 was also expressed in some of the cells of normal canine tissue specimens. Conclusion: Canine tumors with PD-L1 expression that were identified in this study are potential candidates for antiPD-1 and antiPD-L1 therapy.*

Incidence of canine cancer has been gradually increasing over the past two decades due to the prolonged lifespan of dogs (1). Compared to human patients, early detection of canine cancer is challenging and cancer is often diagnosed at an advanced stage. Various types of cancer, such as solid tumors, hematological tumors, adenocarcinoma and soft-tissue sarcoma, can be seen in dogs, and cancer therapy in dogs generally follows the same principles as in human medicine. The three main therapies for cancer, namely surgery, radiation therapy and chemotherapy, have since long been practiced in veterinary oncology. However, novel therapies are still required due to unfavorable outcomes with the currently available treatments.

To date, a number of emerging cancer therapies have been reported in human medicine. Among them, immunotherapy has been regarded as one of the most promising. In particular, antibody therapy targeting immune checkpoint molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1), have shown favorable results in many clinical studies of human cancer (2, 3). CTLA4 and PD-1 are expressed in T-cells and work as immunosuppressive molecules to prevent excessive T-cell reaction. As a part of the normal immunosuppressive mechanism, PD-L1 on dendritic cells binds to PD-1 expressed on T-cells and this suppresses T-cell function (4). On the other hand, tumor cells also express PD-L1 on their cell surface and this has been reported to be involved in the evasion from host immunity through binding of PD-1 expressed on T-cells (5). Thus, antibodies against PD-1 and PD-L1 can be used as therapy to restore T-cell function, leading to enhancement of the host antitumor immunity. However, the role of immune checkpoint molecules in mammals other than humans and the mouse have not yet been elucidated. Even though canine cancers are good comparative models for spontaneously occurring cancer, only limited information on PD-1 and PD-L1 expression in canine cancer is available (6).

Correspondence to: Takuya Mizuno, Laboratory of Molecular Diagnostics and Therapeutics, Joint Faculty of Veterinary Medicine, Yamaguchi University, 1677-1 Yoshida, Yamaguchi 753-8515, Japan. Tel./Fax: +81 839335894, e-mail: mizutaku@yamaguchi-u.ac.jp

Key Words: Canine cancer, immunohistochemistry, programmed cell death ligand 1, PD-L1.

During the preparation of this article, a separate article reported on the molecular cloning of canine PD-1 and PD-L1 (6). The authors showed that antibody to bovine PD-L1 cross-reacts with the canine PD-L1 molecule. They also demonstrated PD-L1 expression in several canine tumors by immunohistochemical analysis using the same antibody. However, the number of tumor types used in the study was inadequate to provide sufficient information on PD-L1 expression in canine tumors. This information is essential in indicating tumor potential for antibody therapy against canine PD-1 and PD-L1.

In this study, we performed flow cytometry, western blotting and immunohistochemical analysis of PD-L1 expression in an extensive variety of canine tumors in the hope of elucidating potential canine tumors suitable as candidates for antibody therapy using antibodies to PD-1 and PD-L1 in the future.

Materials and Methods

Cell lines. Human embryonic kidney HEK293T cell line was maintained in D10 complete medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). Cell lines from canine malignant melanoma (CMeC1, CMeC2, KMeC, LMeC, CMGD2 and CMGD5), canine osteosarcoma (Abrams, D17, Gracie, MacKinley and Moresco), canine mammary gland tumor (CIPp, CIPm, CTBp, CTBm, CHMp, CHMm and CNMm), canine mast cell tumor (VIMC, CMMC, CoMS and HRMC) and canine lymphoma (GL-1, Ema, Nody-1, UL-1, CLGL-90, 17-71, CLBL-1, CLC and CLK) have been described in previous studies (7-9) and were cultured in R10 complete medium (RPMI-1640 supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol). All these cell lines were cultured at 37°C in a humidified incubator with 5% CO_2. HEK293T cells were provided by Dr. Kazuo Nishigaki (Yamaguchi University). Four canine malignant melanoma cell lines (CMeC1, CMeC2, KMeC and LMeC), all canine mammary gland tumor cell lines and three canine mast cell tumor cell lines (VIMC, CMMC and CoMS) were kindly provided by Dr. Takayuki Nakagawa (The University of Tokyo). Two canine malignant melanoma (CMGD2 and CMGD5) and all canine osteosarcoma cell lines were kindly provided by Dr. Jaime Modiano (University of Minnesota) and Dr. Douglas Thamm (Colorado State University), respectively. Ema, CLC and CLK cell lines were established in our laboratory. HRMC, CL-1 and UL-1 were kindly provided by Dr. Hajime Tsujimoto (The University of Tokyo). GL-1, Nody-1, CLGL-90, 17-71 and CLBL-1 were kindly provided by Dr. Munekazu Nakaichi (Yamaguchi University), Dr. Yasuhiko Okamura, Dr. Maxey Wellman (The Ohio State University), Dr. Steven Suter (North Carolina University) and Dr. Barbara Rutgen (University of Veterinary Medicine, Vienna), respectively.

Specimens. The normal tissue specimens used in this study were obtained from healthy beagle dogs kept for experimental purposes according to the Yamaguchi University Animal Care and Use Committee Regulations. Tumor specimens previously used for diagnostic purposes were obtained from several pathology laboratories in Japan (Laboratories of Veterinary Pathology of Yamaguchi University, Gifu University and University of Tokyo).

Flow cytometry. Cell-surface expression of PD-1 and PD-L1 in the canine tumor cell lines was detected by incubation with antibodies to canine PD-1 (4F12-E6) and PD-L1 (G11-6) (prepared in our laboratory, article in preparation), followed by phycoerythrin (PE)-conjugated anti-rat IgG antibody (Southern Biotech, Birmingham, AL, USA). Cells were incubated with propidum iodide immediately before flow cytometric analysis in order to allow gating out of dead cells. The samples were analyzed using BD Accuri C6 (BD Biosciences, San Jose, CA, USA), and the obtained results were analyzed using FlowJo software (Treestar, San Carlos, CA, USA). The canine tumor cell lines were also incubated with 10 ng/ml interferon-gamma (IFN-γ) (R&D Systems, Minneapolis, MN, USA) for 24 h and collected for staining with antibody to canine PD-L1 before cell-surface expression of PD-L1 was detected using the same method described above.

Transfection. To confirm the crossreactivity of commercially available anti-human PD-L1 antibody to canine PD-L1 exogenously overexpressed in HEK293T cells, the plasmid vector coding canine PD-L1 (pMx-IP-cPDL1, constructed in our laboratory) or empty vector, pMx-IP (kindly provided by Dr. Toshio Kitamura (The University of Tokyo), was transiently transfected into HEK293T cells. Briefly, HEK293T cells were seeded at 7.5×10^6 cells/well in 6-well plates one day prior to transfection. On the day of transfection, 1.25 μg of plasmid was mixed with PEI Max (Polysciences, Warrington, PA, USA) in the OPTI-MEM buffer, and was added onto culture after 5 min incubation. The transfected cells were collected for further analysis 48 h after transfection.

Western blot analysis. Plasmid-transfected HEK293T cells or canine tumor cell lines were collected and washed once with cold PBS. The cells were lyzed for 15 mins at 4°C in lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP40, 1 mM phenylmethylsulfonyl fluoride and complete Mini EDTA-free protease inhibitor mixture (Roche Diagnostics, Tokyo, Japan)], centrifuged at $20,600 \times g$ for 15 min at 4°C, and the supernatant (cell lysate) was collected. The amount of protein in the cell lysate was measured with Micro BCA Protein Assay Reagent Kit (Thermo Scientific, Yokohama, Japan) according to the manufacturer's recommendations. Equal amounts of samples (20-30 μg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 9% acrylamide gel before proteins were blotted onto Hybond ECL membranes (GE Healthcare, Tokyo, Japan). Immediately after blotting, the membranes were blocked with blocking buffer (Tris-buffered saline with 0.05% Tween 20% and 5% skimmed milk; TBS-T) for 1 h at RT. The membranes were then incubated overnight at 4°C with primary antibody, rabbit monoclonal antibody to human PD-L1 (EPR1161; Abcam, Tokyo, Japan) or anti-FLAG M2 (Sigma-Aldrich, Tokyo, Japan) in TBS-T with 0.5% skimmed milk. The membranes were washed for three times with TBS-T for 10 min before incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (1:4,000 dilution; goat anti-mouse IgG polyclonal antibody; Merck Millipore, Tokyo, Japan) for 1 h at RT. The membranes were washed three times with TBS-T for 10 min, and visualized by immersion in Western Lightning Chemiluminescence Reagent (Perkin–Elmer, Yokohama,

Table I. *Cell surface expression of caine programmed cell death-1 (PD-1) and programmed cell death-ligand (PD-L1) in canine tumor cell lines.*

	PD-1 No. of positive samples/total samples (Name of cell lines with positive expression)	PD-L1 No. of positive samples/total samples (Name of cell lines with positive expression)	PD-L1 (Cells treated with IFN-γ) (Name of cell lines showing positive expression)
Malignant melanoma	0/6	1/6 (KMeC)	5/6 (All except CMGD5)
Osteosarcoma	0/5	2/5 (D17 and MacKinley)	5/5
Mammary gland tumor	0/7	0/7	7/7
Mast cell tumor	2/4 (VIMC and CMMC)	3/4 (VIMC, CMMC and CoMS)	4/4
Lymphoma	2/9 (Ema and CLK)	2/9 (17-71 and CLBL-1)	2/9 (17-71 and CLBL-1)

IFN-γ: Interferon-γ.

Japan). Immunoreactive bands were quantified with Luminescent Image Analyzer Las 3000 mini (Fujifilm, Tokyo, Japan), and analyzed with Science Lab 2005 (Fujifilm). The membranes were stripped and reprobed with antibody to actin (Sigma-Aldrich) to verify equal loading of protein in each lane.

Immunohistochemical staining. Sections with 4 μm thickness were cut from archival paraffin-embedded tissues described above and placed on hydrophilic glass slides (MAS-GP type A; Matsunami, Osaka, Japan). After dewaxing in three xylene baths and rehydration in alcohol, antigen retrieval was performed in citrate buffer by heating for 30 s at 125°C. Next, the sections were washed 10 times in distilled water, and rinsed with PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 30 min at RT. After washing 10 times in distilled water, the sections were rinsed with PBS. In order to block non-specific protein activity, the sections were incubated with 5% skimmed milk and 5% bovine serum albumin (BSA) in PBS for 30 min at RT. The sections were then rinsed with PBS before they were incubated with antibody to human PD-L1 in 2% BSA in PBS overnight at 4°C. The sections were rinsed with PBS three times, followed by treatment with HRP-conjugated secondary antibody (goat anti-rabbit IgG antibody; Histofine Simple Stain MAX PO: Nichirei Corporation, Tokyo, Japan) for 30 min at RT. The sections were rinsed three times with PBS. Color was developed with Peroxidase Stain 3',3'-diaminobenzidine (DAB) Kit & Enhancer (Nacalai Tesque, Kyoto, Japan) for 5 min at RT. After washing in distilled water 20 times, the sections were counterstained for 5 min with Mayer's hematoxylin (Merck KGaA, Darmstadt, Germany), washed in distilled water, dehydrated in graded baths of ethanol, cleared in xylene, and mounted with xylene-based mounting solution (Matsunami). Melanoma specimens were stained using immunofluorescent staining in order to avoid overlapping of the brown color induced by DAB with the brown melanin granules. The steps involved in the immunofluorescent staining were the same as those for immunohistochemical staining, except that blocking of endogenous peroxidase activity with peroxide was omitted. Alexa488-conjugated secondary antibody was used instead of the HRP-conjugated secondary antibody, and was followed by counterstaining using ProLong Diamond Antifade Mountant with 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA, USA).

Evaluation of immunoreactivity. Immunoreactivity of PD-L1 in each tissue specimen was evaluated as previously described (10), where specimens in which more than 10% of tumor cells exhibited positive staining, were classified as positive. The immunoreactivity was independently evaluated by three observers (KS, MS and TM).

Results

Detection of canine PD-1 and PD-L1 expression on the cell surface of canine tumor cell lines by flow cytometry. In order to elucidate canine PD-1 and PD-L1 expression in canine tumors, an extensive number of canine tumor cell lines were stained with antibodies against canine PD1 (4F12-E6) and PD-L1 (G11-6) for flow cytometric analysis (Table I). As expected, PD-1 was not detected in all of the cell lines, only in two mast cell tumor (VIMC and CMMC) and two lymphoma (Ema and CLK) cell lines. PD-L1 expression was observed in one out of six melanoma (KMeC), two out of five osteosarcoma (D17 and Mackinley), three out of four mast cell tumor (VIMC, CMMC and CoMS), two out of nine lymphoma (17-71 and CLBL-1) and none of the seven mammary gland tumor cell lines. Only the flow cytometric results of the canine tumor cell lines that express canine PD-1 and PD-L1 are shown in Figure 1. Interestingly, IFN-γ treatment of the cell lines for 24 h was able to induce cell-surface expression of PD-L1 in all except the lymphoma cell lines (Table I).

Confirmation of the cross-reactivity of antibody to human PD-L1 to canine PD-L1 protein by western blotting. As the results of the flow cytometric analysis showed that cell-surface expression of PD-L1 in canine tumor cell lines was far less than expected, we decided to analyze the expression of PD-L1 in various types of canine tumor using formalin-fixed paraffin-embedded specimens. Unfortunately, the anti-canine PD-1 and anti-PD-L1 used in the flow cytometric analysis were not able to detect canine PD-1 and PD-L1
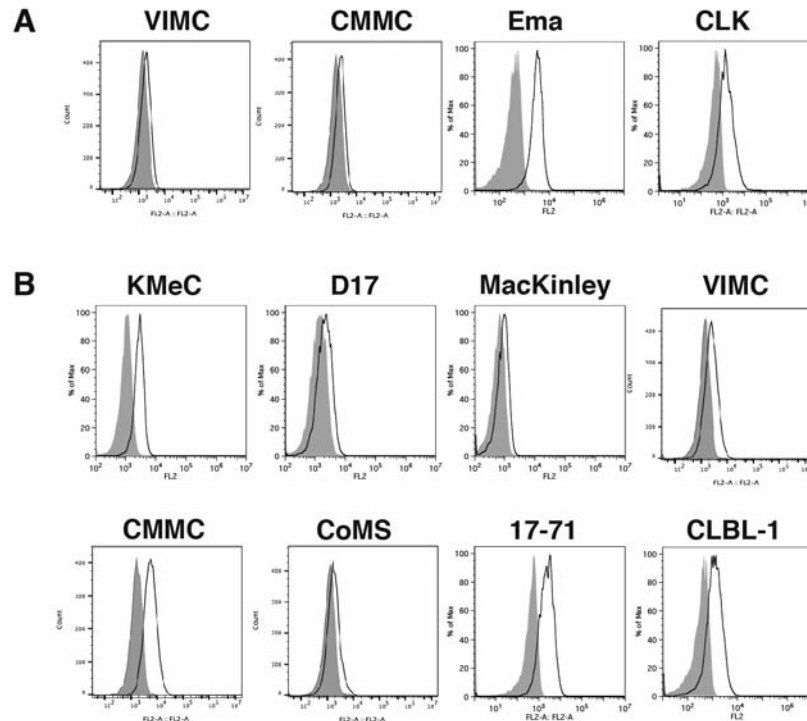
Figure 1. *Flow cytometric analysis of canine programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) expression in canine tumor cell lines. Canine tumor cell lines were collected and stained with antibodies to canine PD-1 (4F12-E6) and canine PD-L1 (G11-6), followed by staining with secondary antibody. Shaded histogram and open histogram indicate isotype control staining and anti-PD-1 (A) or anti-PD-L1 (B) staining, respectively.*

molecules in these specimens. Therefore, in search of a suitable antibody for our purpose, we examined the cross-reactivity of the monoclonal antibody against human PD-L1 to the canine PD-L1 molecule. Western blotting using the anti-human PD-L1 antibody showed a strong band in the lane where lysate of HEK293T cells transfected with canine PD-L1 was loaded (Figure 2A). We also confirmed that the band correlated with transfected canine PD-L1 as the same band was detected by the anti-FLAG antibody on the same membrane. These results verified that the monoclonal antibody to human PD-L1 cross-reacts with canine PD-L1.

Detection of PD-L1 expression in canine tumor cell lines by western blotting. PD-L1 expression in the canine tumor cell lines was examined by western blotting using the same anti-human PD-L1 antibody. As shown in Figure 2B-F, one clear band at the expected molecular weight was detected in all cell lines, despite the varied expression in flow cytometric analysis (Table I). These results indicate that the antibody to human PD-L1 cross-reacts with canine PD-L1 molecules and all canine cell lines used in this study expressed PD-L1, even though most of the them did not exhibit any PD-L1 expression on the cell surface.

Screening of PD-L1 expression in canine normal tissue specimens by immunohistochemical analysis. Next, we determined the expression of PD-L1 in canine formalin-fixed paraffin-embedded tissue specimens obtained from two healthy beagles (Figure 3 and Table II). PD-L1 expression was not found in the pituitary gland, thyroid gland, lung, heart, esophagus, pancreas, liver, adrenal gland and kidney. In the cerebrum and cerebellum, PD-L1 expression was detected in the glial cells, Purkinje cell axon and dendrites. The epithelial cells of the stomach and urinary bladder were positive in PD-L1 staining, but not the epithelial cells of the duodenum. In the thymus, spleen and lymph nodes, some of the lymphocytes were observed to be expressing PD-L1.

Detection of PD-L1 expression in canine tumor specimens by immunohistochemical analysis. The expression of PD-L1 was also examined in several types of canine tumor specimens. Specimens from malignant melanoma (n=15), osteosarcoma (n=3), mammary gland tumor (adenocarcinoma, n=6 and adenoma, n=9), mast cell tumor (n=13), malignant lymphoma (n=15), hemangiosarcoma (n=11), malignant fibrous histiocytoma (n=8), malignant nerve sheath tumor (n=11), hemangiopericytoma (n=10) and fibrosarcoma (n=6) were stained immunohistochemically. The results are shown in
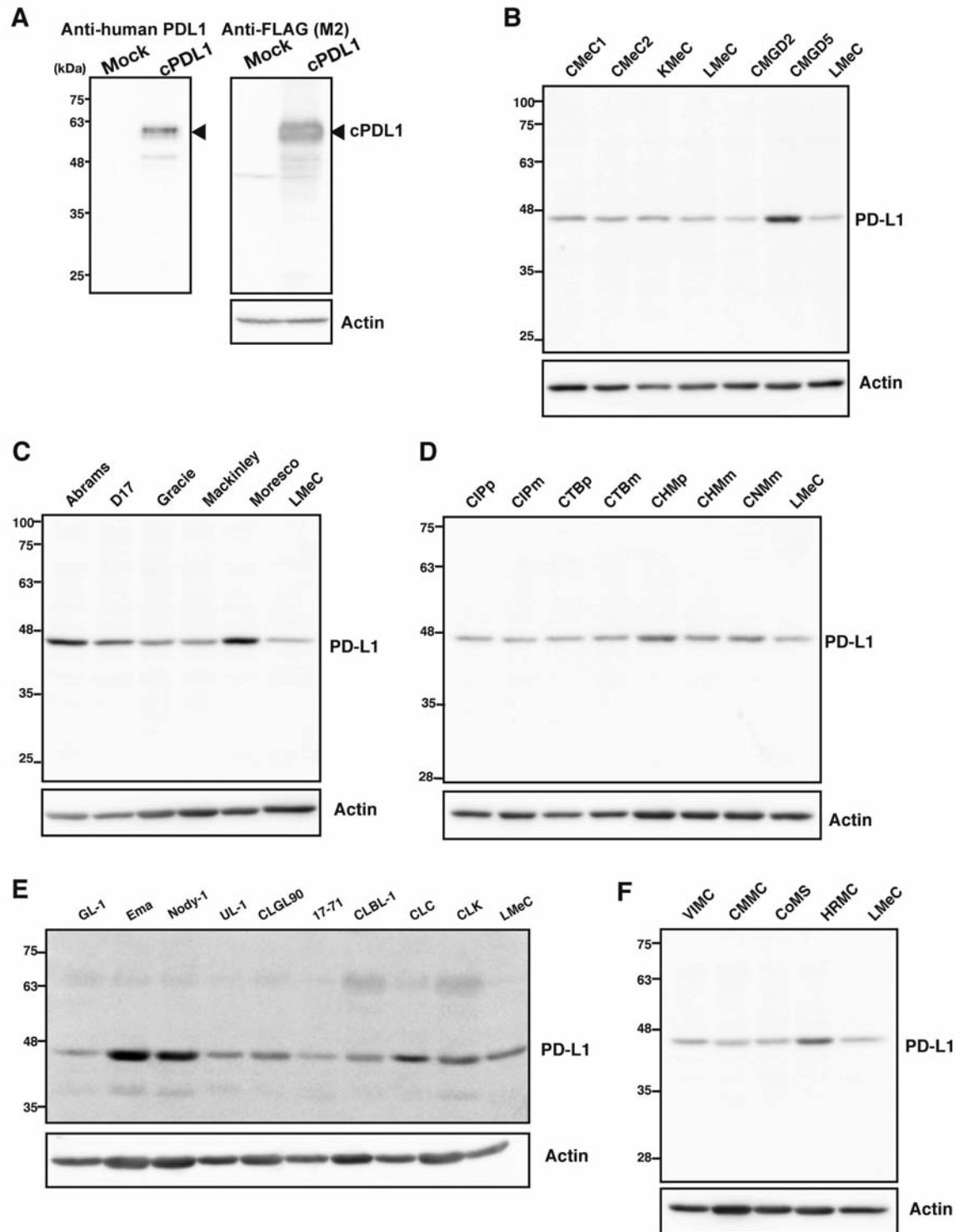
Figure 2. *Demonstration of cross-reactivity of antibody to human PD-L1 and western blotting of programmed cell death-1 (PD-1) and programmed cell death-ligand 1(PD-L1) in canine tumor cell lines. HEK293T cells were transfected with canine PD-L1-expressing plasmid. Cellular protein was then collected and western blotting was performed using antibody to human PD-L1 and anti-FLAG (M2) (A). Cellular protein of canine tumor cell lines, including melanoma (B), osteosarcoma (C), mammary gland tumor (D), lymphoma (E) and mast cell tumor (F) was collected before western blotting was performed using anti-human PD-L1 antibody. LMeC cell line was included in each membrane as a control. The results indicate that the antibody to human PD-L1 cross-reacts with canine PD-L1 molecules and all of the canine cell lines used in this study expressed PD-L1, although not necessarily on the cell surface (cf Figure 1).*

Table II. *Programmed death-ligand 1 (PD-L1) expression in normal canine tissue specimens.*

Canine tissue specimen	Expression of PD-L1
Cerebrum	+ (Glial cells)
Cerebellum	+ (Glial cells, Purkinje cell axon and dendrites)
Pituitary gland	–
Thyroid gland	–
Lung	–
Heart	–
Esophagus	–
Stomach	+ (Gastric mucosa epithelial cells)
Duodenum	+ (Lymphocytes)
Pancreas	–
Liver	–
Adrenal Gland	–
Kidney	–
Urinary bladder	+ (Transitional epithelial cells)
Ovary	+ (Interstitial endocrine cells)
Thymus	+ (Lymphocytes)
Spleen	+ (Lymphocytes)
Lymph node	+ (Lymphocytes and macrophages)

+: Positive expression; –: No expression.

Table III. *Immunohistochemical analysis of programmed death-ligand 1 (PD-L1) expression in canine tumor specimens.*

Canine tumor specimen	No. of positive samples/total samples
Malignant melanoma	11/15
Osteosarcoma	0/3
Mammary gland tumor	10/15
Adenocarcinoma	4/6
Adenoma	6/9
Mast cell tumor	11/13
Lymphoma	15/15
High-intermediate, T-cell	5/5
High-intermediate, B-cell	4/4
Low, T-cell	3/3
Low, B-cell	3/3
Hemangiosarcoma	2/11
Malignant fibrous histiocytoma	6/8
Malignant nerve sheath tumor	4/11
Hemagiopericytoma	4/10
Fibrosarcoma	4/6

Figure 4 and Table III. All lymphoma specimens were positively stained for PD-L1, while more than half of the tumor specimens from malignant melanoma, mammary gland tumor, mast cell tumor, malignant fibrous histiocytoma and fibrosarcoma exhibited positive PD-L1 staining. On the other hand, all three osteosarcoma specimens were negative for PD-L1, and less than half of the specimens from hemangiosarcoma, malignant nerve sheath tumor and hemangiopericytoma positively stained for PD-L1.

Discussion

In this study, we elucidated the expression of canine PD-L1 in canine tumor cell lines and tumor specimens. All tumor cell lines, irrespective of tumor type, expressed PD-L1 protein as shown by western blotting. On the contrary, PD-L1 expression of most cell lines was not detected by flow cytometric analysis, indicating that PD-L1 was not expressed on the cell surface but was found in the cells of most cell lines. However, PD-L1 expression was detected by flow cytometry after induction by IFNγ, a potent PD-L1 inducer (11), in all except the lymphoma cell lines. These results are consistent with those of Maekawa *et al*. (6) who showed that out of eight cell lines (four melanoma, two mast cell tumor, and two osteosarcoma), only two mast cell tumor cell lines expressed PD-L1; PD-L1 expression was successfully induced by IFNγ in six of the cell lines, while the two remaining osteosarcoma cell lines did not respond to IFNγ

treatment. The results in our study showed that PD-L1 expression on all of the five osteosarcoma cell lines was successfully induced by IFNγ. The discrepancy between these two results is most likely a reflection of the characteristics of the different cell lines. Maekawa *et al*. also showed that primary tumor cells obtained from a cancer-bearing dog exhibited PD-L1 expression on the cell surface (6). This suggests that tumor cell lines may have lost cell surface expression of PD-L1 during the cell culture process due to the absence of microenvironmental factors.

To the best of our knowledge, this study is the first to show PD-L1 expression in normal canine tissue specimens. Transcriptional expression of PD-L1 has been detected in the placenta, heart, lung, liver, spleen, lymph nodes and thymus of the mouse (12, 13). In a different study, mouse PD-L1 protein expression was detected in the spleen, thymus, heart, pancreas, small intestines and placenta (14). The results of previous reports differed regarding the expression pattern of PD-L1 from that of the normal canine non-hematopoietic tissue specimens found in this study. Perhaps this reflects the species-specific function of PD-L1 in dogs.

PD-L1 expression in canine tumor specimens has only been reported by Maekawa *et al*. (6). Their article presented very preliminary data on canine tumors as analysis was performed in only a limited number of tumor types. The results of their immunohistochemical analysis showed that all oral malignant melanoma (8/8), 4/6 mast cell tumors and 7/10 renal cell carcinomas expressed PD-L1. One of the objectives of our study was to expand the current pool of data on PD-L1 expression in various tumor types so that potential canine
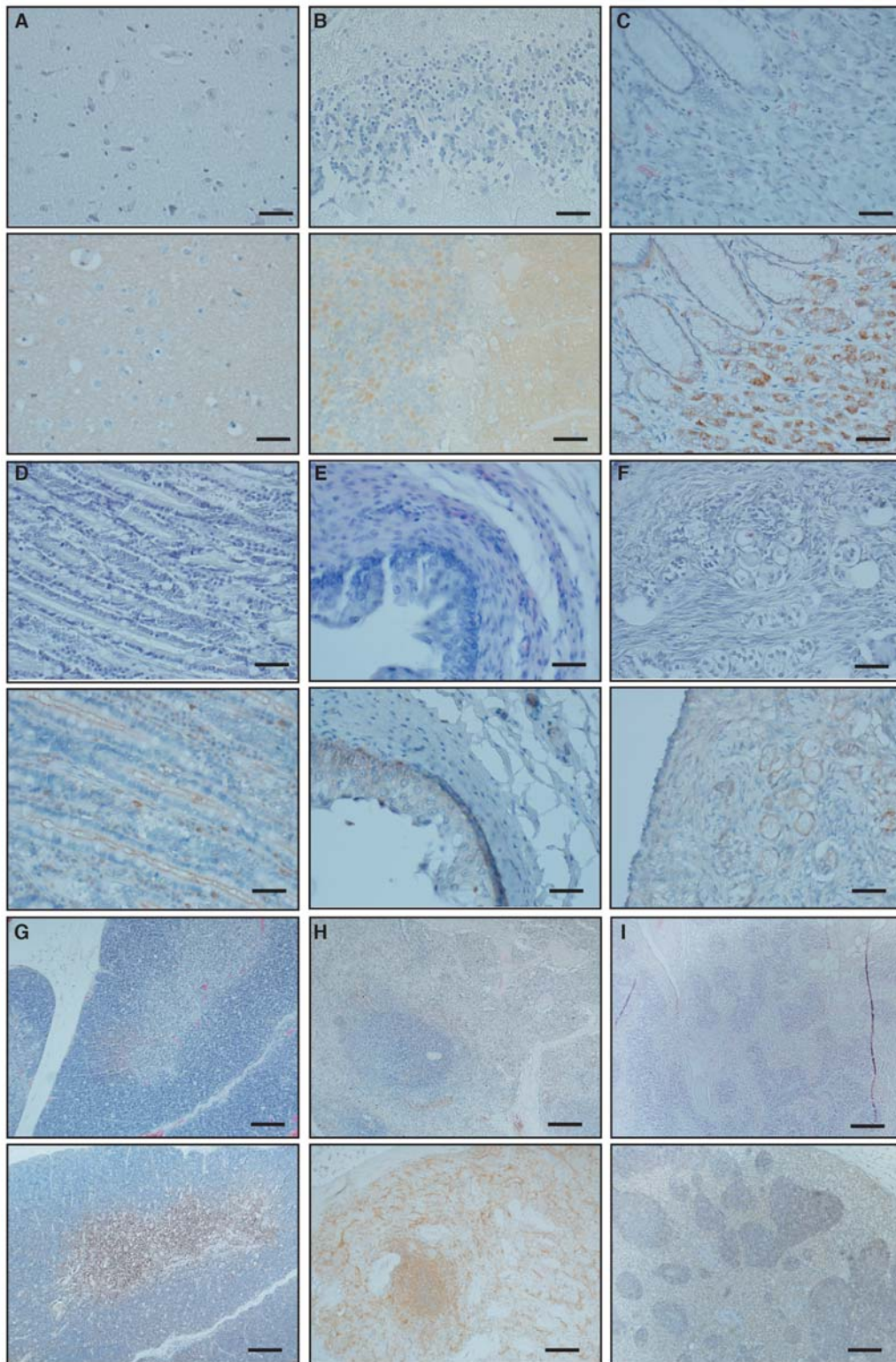
Figure 3. *Immunohistochemical analysis of programmed cell death-ligand 1 (PD-L1) expression in normal canine tissue specimens. Tissue specimens from two healthy beagles were stained immunohistochemically with anti-human PD-L1. Representative immunostaining of cerebrum (A), cerebellum (B), pituitary gland (C), stomach (D), duodenum (E), urinary bladder (F), ovary (G), thymus (H), spleen (I) and lymph node (J) are shown. The upper and lower panels represent hematoxylin and eosin staining and immunohistochemical staining of each tissue specimen, respectively. The scale bars of A-F and G-I are 40 µm and 160 µm, respectively.*
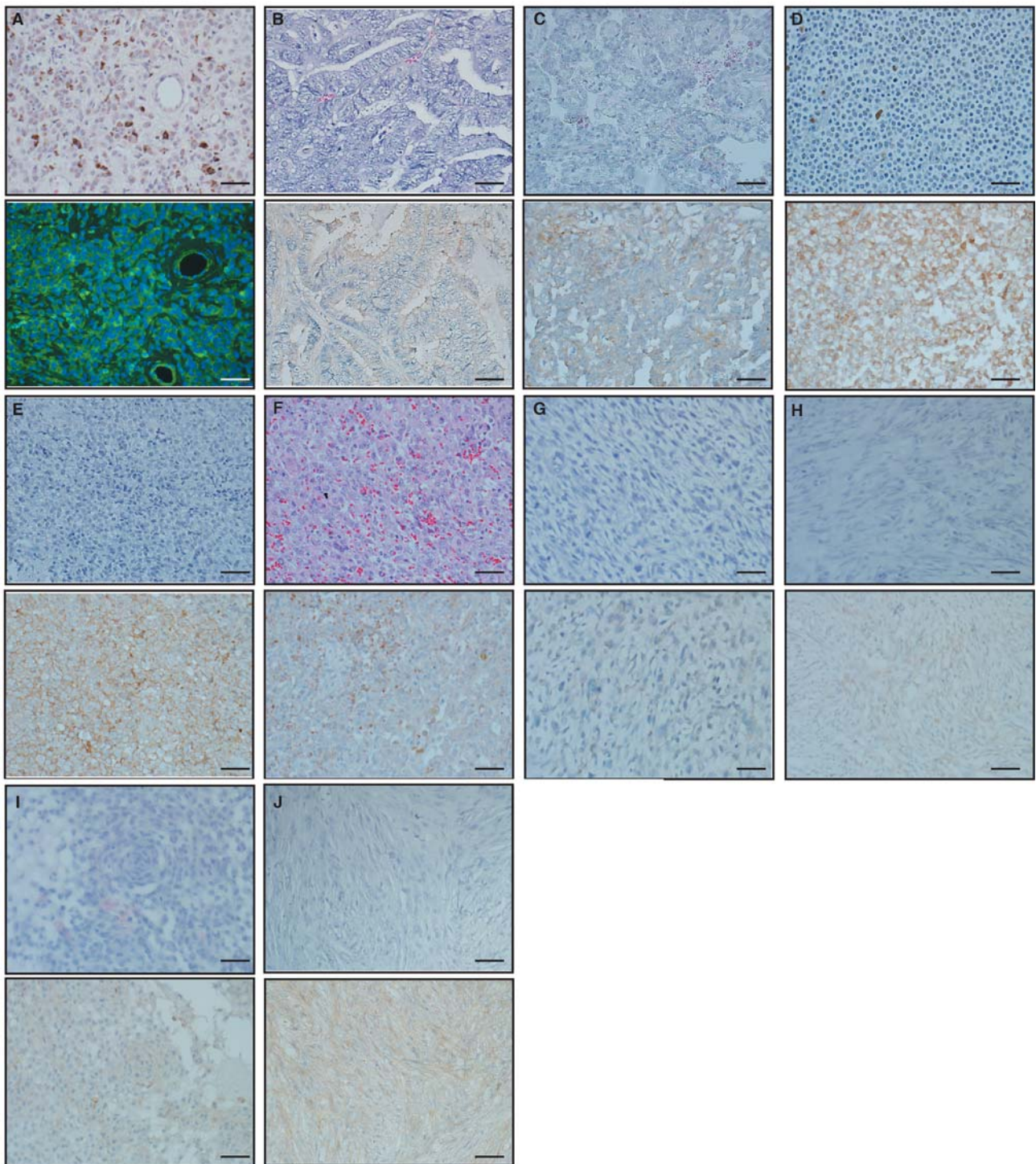
Figure 4. *Immunohistochemical analysis of PD-L1 expression in canine tumor specimens. Canine tumor specimens from cancer-bearing dogs were stained with antibody against human PD-L1. All specimens were immunohistochemically stained, except for malignant melanoma (A), that was stained using immunofluorescence. Representative immunostaining of malignant melanoma (A), mammary gland tumor (adenocarcinoma) (B), mammary gland tumor (adenocarcinoma) (C), mast cell tumor (D), lymphoma (E), hemangiosarcoma (F), malignant fibrous histiocytoma (G), malignant nerve sheath tumor (H), hemangiopericytoma (I) and fibrosarcoma (J) are shown. The upper and lower panels represent hematoxylin and eosin staining and immunofluorescent/immunohistochemical staining of each tumor specimen, respectively. The scale bar is 40 μm.*

tumor candidates can be narrowed down for therapy using antibodies PD-1 and PD-L1. Consistent with Maekawa *et al.*, our study showed that PD-L1 expression can be detected in high proportions in oral malignant melanoma (11/15) and mast cell tumor (11/13) (6). Although treatment options are available for canine mast cell tumor (7, 15), there is still a lack of effective treatment for oral malignant melanoma (16). That we found PD-L1 expression in a high number of specimens of canine malignant melanoma indicates that this type of cancer is a good candidate for antibody therapy against PD-1 and PD-L1, and the beneficed effects of such antibody therapy in actual clinical cases are highly anticipated.

In our study, PD-L1 expression was observed in all of the canine lymphoma specimens, including low-grade lymphoma and intermediate- to high-grade lymphoma, as well as both T-cell and B-cell lymphoma. We also confirmed that PD-L1 is expressed in some lymphocytes in the spleen and lymph nodes of healthy beagles. The results for normal canine specimens indicate that PD-L1 expression does not seem to be specific to tumor cells. However, in preliminary studies conducted in our laboratory, PD-L1 expression was not detected in canine peripheral blood mononuclear cell by flow cytometry (article in preparation). Since we did not examine the specific types of cells in the spleen and lymph nodes that expressed PD-L1 in this study, further studies are warranted to clarify this matter. Interestingly, PD-L1 expression was observed only in certain types of human lymphoma (17-20), and the expression of PD-L1 in human diffuse large B-cell lymphoma cells has been correlated to a worse prognosis (20). Therefore, future studies should include in-depth analysis of PD-L1 expression in a larger number of canine lymphoma specimens in order to gain a comprehensive understanding of the pathogenesis involving PD-L1 in dogs.

This study is also the first to report on the results of the analysis of PD-L1 expression in tumor specimens that included osteosarcoma, mammary gland tumor, hemangiosarcoma and soft-tissue sarcomas (malignant fibrous histiocytoma, malignant nerve sheath tumor, hemangiopericytoma and fibrosarcoma). These results contribute to a very important part of canine oncology because the tumors that were examined are common in dogs and are highly malignant. Osteosarcoma and hemangiosarcoma are common in larger-breed dogs and are known to have a grave prognosis (21). None of the osteosarcoma specimen (0/3) and only a few of the hemangiosarcoma specimens (2/11) were found to have PD-L1 expression. Since only three canine osteosarcoma specimens from this study and a small number of human osteosarcoma specimens (22) have been examined for the expression of PD-L1, more clinical data on osteosarcoma are needed in order to come to an acceptable conclusion on the PD-L1 expression of this tumor type. On the other hand, more than half of the canine mammary gland tumor specimens, including both adenoma and adenocarcinoma, exhibited PD-L1 expression,

which was consistent with the results for human breast cancer (23). Soft-tissue sarcoma is also common in dogs, and 18 out of 35 specimens exhibited PD-L1 expression. Among the four types of soft-tissue sarcoma, malignant fibrous histiocytoma and fibrosarcoma had a higher percentage of specimens that expressed PD-L1 as compared to malignant nerve sheath tumor and hemangiopericytoma. As antibodies to immune checkpoint proteins for human soft-tissue sarcoma are currently being tested in various clinical trials (24), similarly, canine soft-tissue sarcoma might be a good candidate for such therapy.

In this study, we were unable to analyze the correlation of PD-L1 expression and the prognosis of the canine cancer patients because there was a lack of patient data due to the retrospective nature of the study. In human medicine, many studies have reported on the positive correlation of the expression of PD-L1 in tumor cells and worse prognosis of patients with cancer (10, 23, 25-29). We also did not examine the rate of CD8^{+} T-cell infiltration in the tumor specimens, the functional analysis of PD-1 on CD8^{+} T-cells and the functional analysis of PD-L1 on tumor cells. These studies are essential to providing basic data for the evaluation of the potential of immune therapy using antibodies to PD-1 and PD-L1 in canine oncology.

In conclusion, canine mast cell tumor, malignant melanoma, mammary gland tumor, and soft-tissue sarcoma appear to be good tumor candidates for therapy using antibodies to PD-1 and PD-L1. Similarly to human medicine, there are high expectations for immune therapy in veterinary oncology and we hope to narrow the gap between the two as part of our efforts to provide better veterinary care.

Acknowledgements

References

1 Merlo DF, Rossi L, Pellegrino C, Ceppi M, Cardellino U, Capurro C, Ratto A, Sambucco PL, Sestito V, Tanara G and Bocchini V: Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. J Vet Intern Med *22*: 976-984, 2008.

2 Postow MA, Callahan MK and Wolchok JD: Immune checkpoint blockade in cancer therapy. J Clin Oncol *33*: 1974-1982, 2015.

3 Littman DR: Releasing the brakes on cancer immunotherapy. Cell *162*: 1186-1190, 2015.

4   Brown JA, Dorfman DM, Ma F-R, Sullivan EL, Munoz O, Wood CR, Greenfield EA and Freeman GJ: Blockade of programmed death-1 ligands on dendritic cells enhances T-cell activation and cytokine production. J Immunol 170: 1257-1266, 2003.

5   Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T and Minato N: Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. Proc Natl Acad Sci USA 99: 12293-12297, 2002.

6   Maekawa N, Konnai S, Ikebuchi R, Okagawa T, Adachi M, Takagi S, Kagawa Y, Nakajima C, Suzuki Y, Murata S and Ohashi K: Expression of PD-L1 on Canine Tumor Cells and Enhancement of IFN-γ Production from Tumor-Infiltrating Cells by PD-L1 Blockade. PLoS ONE 9: e98415, 2014.

7   Hwang CC, Umeki S, Kubo M, Hayashi T, Shimoda H, Mochizuki M, Maeda K, Baba K, Hiraoka H, Coffey M, Okuda M and Mizuno T: Oncolytic reovirus in canine mast cell tumor. PLoS ONE 8: e73555, 2013.

8   Hwang CC, Umeki S, Igase M, Coffey M, Noguchi S, Okuda M and Mizuno T: The effects of oncolytic reovirus in canine lymphoma cell lines. Vet Comp Oncol (in press). http://dx.doi.org/10.1111/vco.12124

9   Igase M, Hwang CC, Coffey M, Okuda M, Noguchi S and Mizuno T: The oncolytic effects of reovirus in canine solid tumor cell lines. J Vet Med Sci 77: 541-548, 2015.

10 Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, Nakamura S, Enomoto K, Yagita H, Azuma M and Nakajima Y: Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res 13: 2151-2157, 2007.

11 Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, Shin T, Tsuchiya H, Pardoll DM, Okumura K, Azuma M and Yagita H: Expression of programmed death 1 ligands by murine T cells and APC. J Immunol 169: 5538-5545, 2002.

12 Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, Malenkovich N, Okazaki T, Byrne MC, Horton HF, Fouser L, Carter L, Ling V, Bowman MR, Carreno BM, Collins M, Wood CR and Honjo T: Engagement of the PD-1 immuno-inhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 192: 1027-1034, 2000.

13 Dong H, Zhu G, Tamada K and Chen L: B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. Nat Med 5: 1365-1369, 1999.

14 Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ and Sharpe AH: Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. Eur J Immunol 33: 2706-2716, 2003.

15 London CA, Malpas PB, Wood-Follis SL, Boucher JF, Rusk AW, Rosenberg MP, Henry CJ, Mitchener KL, Klein MK, Hintermeister JG, Bergman PJ, Couto GC, Mauldin GN and Michels GM: Multi-center, placebo-controlled, double-blind, randomized study of oral toceranib phosphate (SU11654), a receptor tyrosine kinase inhibitor, for the treatment of dogs with recurrent (either local or distant) mast cell tumor following surgical excision. Clin Cancer Res 15: 3856-3865, 2009.

16 Smedley RC, Spangler WL, Esplin DG, Kitchell BE, Bergman PJ, Ho HY, Bergin IL and Kiupel M: Prognostic Markers markers for canine melanocytic neoplasms: A comparative review of the literature and goals for future investigation. Vet Pathol 48: 54-72, 2011.

17 Chen BJ, Chapuy B, Ouyang J, Sun HH, Roemer MGM, Xu ML, Yu H, Fletcher CDM, Freeman GJ, Shipp MA and Rodig SJ: PD-L1 expression is characteristic of a subset of aggressive B-cell lymphomas and virus-associated malignancies. Clin Cancer Res 19: 3462-3473, 2013.

18 Andorsky DJ, Yamada RE, Said J, Pinkus GS, Betting DJ and Timmerman JM: Programmed death ligand 1 is expressed by non-Hodgkin lymphomas and Inhibits the activity of tumor-associated T-Cells. Clin Cancer Res 17: 4232-4244, 2011.

19 Wilcox RA, Feldman AL, Wada DA, Yang Z-Z, Comfere NI, Dong H, Kwon ED, Novak AJ, Markovic SN, Pittelkow MR, Witzig TE and Ansell SM: B7-H1 (PD-L1, CD274) suppresses host immunity in T-cell lymphoproliferative disorders. Blood 114: 2149-2158, 2009.

20 Kiyasu J, Miyoshi H, Hirata A, Arakawa F, Ichikawa A, Niino D, Sugita Y, Yufu Y, Choi I, Abe Y, Uike N, Nagafuji K, Okamura T, Akashi K, Takayanagi R, Shiratsuchi M and Oshima K: Expression of programmed cell death ligand 1 is associated with poor overall survival in patients with diffuse large B-cell lymphoma. Blood: blood-2015-02-629600, 2015.

21 Clifford CA, Mackin AJ and Henry CJ: Treatment of canine hemangiosarcoma: 2000 and beyond. J Vet Intern Med 14: 479-485, 2000.

22 Shen JK, Cote GM, Choy E, Yang P, Harmon D, Schwab J, Nielsen GP, Chebib I, Ferrone S, Wang X, Wang Y, Mankin H, Hornicek FJ and Duan Z: Programmed cell death ligand 1 expression in osteosarcoma. Cancer Immunol Res 2: 690-698, 2014.

23 Ghebeh H, Mohammed S, Al-Omair A, Qattant A, Lehe C, Al-Qudaihi G, Elkum N, Alshabanah M, Bin Amer S, Tulbah A, Ajarim D, AL-Tweigeri T and Dermime S: The B7-H1 (PD-L1) T Lymphocyte-Inhibitory Molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. Neoplasia 8: 190-198, 2006.

24 Burgess M, Gorantla V, Weiss K and Tawbi H: Immunotherapy in sarcoma: future horizons. Curr Oncol Rep 17: 52, 2015.

25 Ohigashi Y, Sho M, Yamada Y, Tsurui Y, Hamada K, Ikeda N, Mizuno T, Yoriki R, Kashizuka H, Yane K, Tsushima F, Otsuki N, Yagita H, Azuma M and Nakajima Y: Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. Clin Cancer Res 11: 2947-2953, 2005.

26 Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, Sengupta S, Frank I, Parker AS, Zincke H, Blute ML, Sebo TJ, Cheville JC and Kwon ED: Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 66: 3381-3385, 2006.

27 Nakanishi J, Wada Y, Matsumoto K, Azuma M, Kikuchi K and Ueda S: Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. Cancer Immunol Immunother 56: 1173-1182, 2007.

28 Gao Q, Wang X-Y, Qiu S-J, Yamato I, Sho M, Nakajima Y, Zhou J, Li B-Z, Shi Y-H, Xiao Y-S, Xu Y and Fan J: Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res 15: 971-979, 2009.

29 Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM, Melief CJM and van der Burg SH: Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. Clin Cancer Res 15: 6341-6347, 2009.