Studies on immune checkpoint molecules in canine cancers and development of a novel immunotherapy targeting these molecules

イヌ腫瘍における免疫チェックポイント分子の解析と
それらを標的とした新規免疫療法の検討

Naoya Maekawa

CONTENTS

ABBREVIATIONS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ABC	avidin-biotin peroxidase complex
ADCC	antibody-dependent cell-mediated cytotoxicity
APC	allophycocyanin
c4G12	canine chimeric 4G12
c6G7	canine chimeric 6G7
CBB	coomassie brilliant blue
cCD80	canine CD80
CDC	complement-dependent cytotoxicity
CHO	Chinese hamster ovary
ConA	concanavalin A
cPD-1	canine PD-1
cPD-L1	canine PD-L1
CR	complete response
CRPC	castration-resistant prostate cancer
CT	computed tomography
CTCAE	common terminology criteria for adverse events
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
dhfr	dihydrofolate reductase
EdU	thymidine analogue 5-ethynyl-2'-deoxyuridine
EGFP	enhanced green fluorescent protein
FCS	fetal calf serum
FSC	forward scatter
HC	healthy control
HSA	hemangiosarcoma
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motif
ITSM	immunoreceptor tyrosine-based switch motif
K_D	equilibrium dissociation constant
mAb	monoclonal antibody

MCT	mast cell tumor
MST	median survival time
MTX	methotrexate
NILs	normal tissue-infiltrating lymphocytes
NSCLC	non-small-cell lung cancer
OD	optical density
OMM	oral malignant melanoma
ORF	open reading frame
ORR	objective response rate
OS	osteosarcoma
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	progressive disease
PD-1	programmed cell death 1
PD-L1	programmed death ligand 1
PE	phycoerythrin
PMA	phorbol 12-myristate acetate
PR	partial response
PTEN	phosphatase and tensin homolog
RCC	renal-cell cancer
rpm	revolutions per minute
RT	room temperature
RU	response unit
SD	stable disease
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
SEB	staphylococcal enterotoxin B
SPR	surface plasmon resonance
SSC	side scatter
TCR	T cell receptor
TILs	tumor-infiltrating lymphocytes
TIMCs	tumor-infiltrating mononuclear cells
VCOG	veterinary cooperative oncology group
WHO	World Health Organization

NOTES

The contents of chapters I and II have been published in *PLoS ONE* as listed below.

Maekawa N, Konnai S, Ikebuchi R, Okagawa T, Adachi M, Takagi S, Kagawa Y, Nakajima C, Suzuki Y, Murata S, 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* 2014, **9(6)**:e98415.

Maekawa N, Konnai S, Okagawa T, Nishimori A, Ikebuchi R, Izumi Y, Takagi S, Kagawa Y, Nakajima C, Suzuki Y, Kato Y, Murata S, Ohashi K. Immunohistochemical analysis of PD-L1 expression in canine malignant cancers and PD-1 expression on lymphocytes in canine oral melanoma. *PLoS ONE* 2016, **11(6)**:e0157176.

The contents of chapters III and IV have been published in *Scientific Reports*.

Maekawa N, Konnai S, Takagi S, Kagawa Y, Okagawa T, Nishimori A, Ikebuchi R, Izumi Y, Deguchi T, Nakajima C, Kato Y, Yamamoto K, Uemura H, Suzuki Y, Murata S, Ohashi K. A canine chimeric monoclonal antibody targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma. *Sci Rep* 2017, **7(1)**:8951.

PREFACE

Immune system distinguishes self from non-self, and provides the maintenance of homeostasis by eliminating non-self, especially pathogens, from the host. Immunity is comprised of humoral and cell-mediated immunity, in both of which many different cell types and soluble mediators are coordinately and intricately involved. In general, cell-mediated immunity plays a pivotal role in the protection from viral pathogens and cancers.

Despite the elaborate coordination of immune responses, some pathogens and tumor cells can escape from the immune responses due to several mechanisms, which allow them to persist in the body. Of these, immune checkpoint molecules represent important mechanisms for immune suppression in cell-mediated immunity. T cells are activated through signaling from T cell receptor (TCR), and additional signals from costimulatory receptors, including CD28, are required to elicit sufficient effector functions of T cells [Chen and Flies, 2013]. Such costimulatory signals act as “accelerators” for T cell activation. On the other hand, the activation of T cells should be attenuated after the clearance of pathogens to minimize immunopathogenic aspects of the responses. Immune checkpoint molecules, including programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), serve as “brakes” in T cell responses. These coinhibitory receptors suppress the activation signal mediated by TCR, and thus providing an opportunity to inhibit effector functions of T cells, such as cytokine production and cell proliferation [Buchbinder and Desai, 2016]. They are indispensable for regulating excess immune responses; however, in chronic infections and cancers, the suppression of T cells by immune checkpoint molecules is often utilized by the pathogens and tumor cells to escape from the immune responses. In cancers, for example, PD-1 expression is upregulated on $CD4^+$ and $CD8^+$ tumor-infiltrating lymphocytes (TILs) in melanoma patients [Chapon *et al*., 2011], and patients with PD-1-expressing immune cells were at higher risk of cancer-specific death in renal cell carcinoma [Thompson *et al*, 2007]. Of note, a ligand for PD-1, PD-ligand 1 (PD-L1) is highly expressed in various cancer cells including melanoma, lung cancer, and ovarian cancer [Dong 2002], and this PD-L1 overexpression is associated with poor prognosis in several types of cancers such as renal cell carcinoma and gastric carcinoma [Thompson *et al*., 2006; Wu *et al*, 2006; Sznol and Chen, 2013]. These studies demonstrate the clinical importance of the PD-1/PD-L1 pathway as an immune evasion mechanism in various cancers (Figure 1A).

Interestingly, early studies on these molecules also demonstrated that the blockade of immune checkpoint molecules restores effector functions of T cells [Iwai *et al*., 2002; Blank *et al*., 2006; Wong *et al*., 2007], and thus immune checkpoint inhibitors, such as

anti-PD-1 antibody and anti-PD-L1 antibody, are recognized as candidates for anti-cancer biologics (Figure 1B). A proof-of-concept clinical study (Phase 1) using a monoclonal antibody (mAb) against PD-1 showed evidence of antitumor activity with the occurrence of tolerable adverse events [Brahmer *et al*., 2010]. A number of following clinical studies have confirmed the efficacy of anti-PD-1 or PD-L1 mAb in patient with various malignant cancers, including melanoma, non-small-cell lung cancer (NSCLC), and renal-cell cancer (RCC) [Brahmer *et al*., 2012; Topalian *et al*., 2012]. The objective response rates (ORR) were generally 10-30%, but were dependent on cancer types, blocking agent used, or tumor microenvironment of individuals including PD-L1 expression [Iwai *et al*., 2017]. Antibody drugs that block PD-1 or PD-L1 were approved for melanoma, NSCLC, RCC, classical Hodgkin's lymphoma, head and neck cancer, and bladder cancer in the last 3 years and now immunotherapy, including these antibody drugs, is considered the forth pillar of cancer treatment in addition to surgery, radiation, and chemotherapy.

In the field of veterinary medicine, there are many chronic infections and cancers for which effective treatment and prophylaxis are not available. The absence of countermeasures for them is partially due to the immune escape mechanisms that the pathogens and tumor cells have, but little is known about the association of the PD-1/PD-L1 pathway with animal diseases. Recent studies in our laboratory have revealed that the upregulation of PD-1 and PD-L1 expression is found in bovine leukemia virus infection, Johne's disease, amaplasmosis, and mycoplasmosis in cattle as well as in Marek's disease in chickens, and the expressions of these molecules could contribute to the disease progression and/or persistence of the pathogens [Ikebuchi *et al*., 2011; Matsuyama-Kato *et al.*, 2012; Ikebuchi *et al*., 2013; Okagawa *et al*., 2016a, b; Goto *et al.*, 2017]. In cats, PD-1 and PD-L1 are overexpressed in feline immunodeficiency virus infection and increase in the PD-1/PD-L1 levels may be related to immune dysfunction [Folkl *et al*., 2010]. However, in dogs, there was no report on the PD-1/PD-L1 pathway and its association with diseases was unclear.

In dogs, cancer is a major cause of death while the incidence of infectious diseases has declined because of the improved rearing environment and effective vaccinations. According to a recent report, cancer accounts for 27% of overall deaths [Adams *et al*., 2010]. Among malignant cancers in dogs, lymphoma, mast cell tumor (MCT), and mammary carcinoma are considered frequent [Dobson *et al*., 2002; Brønden *et al*., 2010]. Other malignant cancers such as malignant melanoma, osteosarcoma (OS), hemangiosarcoma (HSA), and undifferentiated sarcoma are also common and often arise as clinical problems because dogs with these malignancies usually have poor prognosis. Current treatment for canine cancers includes surgery, radiation, and chemotherapy as in

humans. However, in some dogs with malignant cancers, these treatments, either alone or in combination, are not curative for some reasons. For example, surgery and radiation are not optimal for metastatic cancers and sensitivities to radiation and chemotherapy can vary dependent on the cancer types. In addition, surgery is generally invasive, and adverse effects of radiation and chemotherapy often limit the treatment dosage and duration. Growing demand for better medical treatment in dogs encourages researchers to develop novel treatment modalities, including immunotherapy, as additional or alternative options for malignant cancers. In theory, the effect of immunotherapy is systemic and specific to cancers, covering the shortcomings of other treatment modalities. Some attempts have been made to develop immunotherapy against canine cancers including cancer vaccines; however, no immunotherapy has been widely used in veterinary clinics to date. It is because of the low efficacy of tested therapies, in which immunosuppressive microenvironment formed/induced by cancers may contribute to the poor responsiveness. Therefore, it is worth investigating the role of the PD-1/PD-L1 pathway in canine cancers, to obtain an insight into immune evasion mechanisms of canine cancers and to develop a novel immunotherapy for them targeting these molecules.

The development of biological drugs, including antibody drugs, for veterinary medicine is a growing field of research. There are several reports on the establishment of antibody drug in cattle and dogs [Singer *et al*., 2014; Rue *et al*., 2015; Okagawa *et al.*, 2017; Nishimori *et al.*, 2017], although none of those has yet been available in the clinics to date. For the preparation of therapeutic antibody, some strategies are required for reducing immunogenicity of the antibody to avoid allergic reactions and to prolong the biological half-life in the host. Among these, chimerization of antibody, in which constant regions of the antibody are exchanged for those of a given species, is a well-known, simple and effective strategy to prepare therapeutic antibody in humans. It is applicable to the development of therapeutic antibodies for animals because the only information needed for chimerization is the nucleotide sequences of constant regions of antibody derived from each animal species. Therefore, to develop therapeutic antibody for dogs, chimerization of antibody should be the first choice considering that limited information is available for canine antibody in the literature [Tang *et al*., 2001; Bergeron *et al*., 2014].

In this study, therapeutic potential of the PD-L1 blockade in canine cancers was evaluated *in vitro* and *in vivo*. In chapter I, canine *PD-1* and *PD-L1* genes were identified, and the expressions of PD-1 and PD-L1 were detected in canine cancers. Using tumor-infiltrating mononuclear cells (TIMCs), therapeutic effect of blocking anti-PD-L1 mAb was tested *in vitro*. In Chapter II, PD-L1 expression in various canine cancers was assessed by immunohistochemistry (IHC) to clarify the cancer types that could respond

to the PD-L1 blockade in future clinical studies. To prepare a therapeutic antibody, blocking anti-PD-L1 mAb was canine-chimerized, and the chimeric mAb was produced in a mammalian cell-based expression system which had been optimized for a large-scaled production in Chapter III. In Chapter IV, the clinical efficacy of the chimeric mAb was evaluated in dogs with oral malignant melanoma (OMM) or undifferentiated sarcoma in the Veterinary Teaching Hospital of Hokkaido University. Here, this study demonstrates the antitumor activity of an anti-PD-L1 mAb in canine malignant cancers, providing an opportunity to develop a novel treatment for them.

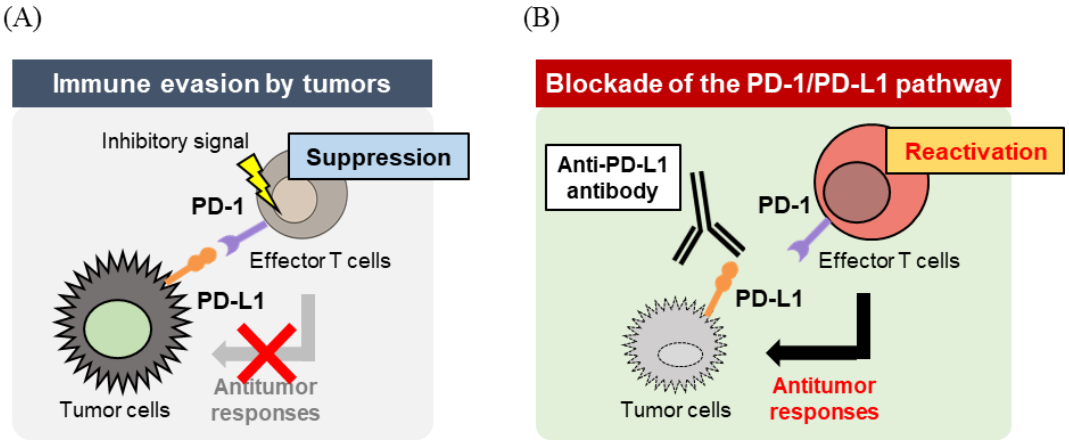

Figure 1. The PD-1/PD-L1 pathway in tumors. (A) The PD-1/PD-L1 pathway as a mechanism of immune evasion by tumors. PD-1 is an inhibitory receptor expressed on T cells, and the interaction of PD-1 with PD-L1, a ligand for PD-1 which is often expressed on tumor cells, suppresses effector functions of T cells. (B) Reinvigoration of antitumor responses by the PD-L1 blockade. The blockade of the PD-1/PD-L1 pathway by anti-PD-L1 antibody restores the effector functions of T cells, resulting in the clearance of tumor cells.

CHAPTER I

Identification and expression analysis of PD-1/PD-L1 and therapeutic potential of the PD-L1 blockade in canine cancers

INTRODUCTION

An immune checkpoint molecule, PD-1, is a B7:CD28 family receptor which is expressed mainly on T cells after activation. PD-1 negatively regulates the TCR signaling and inhibits effector functions of T cells, allowing the maintenance of peripheral tolerance or the persistence of certain pathogens in the host [Keir *et al*., 2008]. Two ligands for PD-1 have been identified, that is, PD-L1 and PD-L2 [Freeman *et al*., 2000; Latchman *et al*., 2001]. PD-L2 expression is restricted to certain cell types such as macrophages and dendritic cells, while PD-L1 expression can be induced in a wide variety of cell types, including hematopoietic cells and non-hematopoietic cells [Keir *et al*., 2008].

In humans, aberrant PD-L1 expression is found on many types of cancers [Dong *et al*., 2002; Sznol and Chen, 2013] and considered an immune evasion mechanism. In renal cell carcinoma, gastric cancer, and other cancer patients, PD-L1 expression is known to be associated with poor prognosis [Thompson *et al*., 2006; Wu *et al*., 2006; Sznol and Chen, 2013], indicating that PD-L1 is an important regulator of the immune system when it fights against cancer. Antibody drugs that target this pathway interfere with binding of PD-L1 to PD-1 and can enhance specific immune responses to tumor cells [Blank *et al*., 2006; Wong *et al*., 2007], subsequently resulting in the regression of cancer [Brahmer *et al*., 2010]. A number of clinical trials have revealed that objective responses can be obtained by anti-PD-1 or anti-PD-L1 antibodies in patients with malignant cancers, including advanced melanoma, NSCLC, and RCC [Brahmer *et al*., 2012; Topalian *et al*., 2012], and accumulating evidence demonstrates the potential of immune checkpoint inhibitors in cancer treatment.

Recent studies in our laboratory have shown that immune checkpoint molecules, including PD-1 and PD-L1, are upregulated in bovine chronic infections such as bovine leukemia virus infection, Johne's disease and bovine anaplasmosis, and the blockade of these molecules by specific mAbs is associated with enhanced immune responses [Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2013; Okagawa *et al*., 2016a, b]. In dogs, however, there was no report on the PD-1/PD-L1 pathway, and the molecular characteristics and contribution to immune evasion of canine cancers remain to be investigated. In this chapter, canine *PD-1* and *PD-L1* genes were identified and their molecular characterization was performed using recombinant proteins. Then, PD-1 and PD-L1 expressions were assessed in canine cancers, and the therapeutic potential of PD-L1 blockade by anti-PD-L1 antibody was evaluated *in vitro* using TIMCs.

MATERIALS AND METHODS

Canine samples

Animal use throughout the study was approved by the Institutional Animal Care and Use Committee (the serial number of approval was #1039), Faculty of Veterinary Medicine, Hokkaido University, which has been fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Peripheral blood samples were obtained from healthy 5- or 8-year-old beagles kept at the Experimental Animal Facility, Faculty of Veterinary Medicine, Hokkaido University. Clinical samples of canine cancer tissues were surgically excised and collected at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University in 2012-2013. Written informed consent was obtained from the owners of the dogs before the sample collection. For immunohistochemical analysis, tumor specimens submitted for histological diagnosis and kept in a commercial pathology Laboratory, North Lab (Sapporo, Japan), were used.

Cell culture

All cell cultures were performed at 37°C in a humid atmosphere of 5% CO_2. Cos-7 cells (SV40-transformed African green monkey kidney fibroblast cell line) [Gluzman, 1981] were cultured in complete RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA), which is supplemented with 10% fetal calf serum (FCS) (Valley Biomedical, Winchester, VA, USA), 2 mM L-glutamine (Life Technologies, Carlsbad, CA, USA), 200 μg/mL streptomycin (Life Technologies), and 200 U/mL penicillin (Life Technologies). Chinese hamster ovary-DG44 (CHO-DG44) cells were cultured in CD-DG44 medium (Life Technologies) containing GlutaMAX supplement (20 mL/L, Life Technologies) and 10% Pluronic F-68 (18 mL/L, Life Technologies). The canine melanoma cell lines CMeC, LMeC [Ohashi *et al*., 2002], CMM-1, and CMM-2 [Ohashi *et al*., 2001] were cultured in complete RPMI 1640 medium additionally supplemented with 2×10^{-5} M 2-mercaptoethanol. The canine MCT cell lines CM-MC [Takahashi *et al*., 2001] and CoMS [Ishiguro *et al*., 2001] were cultured in complete RPMI 1640 medium additionally supplemented with 12 mM HEPES, 2 mg/mL $NaHCO_3$. The canine OS cell lines POS [Kadosawa *et al*., 1994] and HMPOS [Barroga *et al*., 1999] were cultured in D-MEM (Life Technologies) containing 10% FCS, 2 mM L-glutamine, 200 μg/mL streptomycin, and 200 U/mL penicillin. To induce PD-L1 expression, the canine tumor cell lines were treated with 100 ng/mL canine recombinant interferon (IFN)-γ (Kingfisher Biotech, St. Paul, MN, USA) and cultured for 24 h. Canine peripheral blood mononuclear cells

(PBMCs) were isolated from heparinized blood samples by density gradient centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK) and cultured in complete RPMI 1640 medium. Concanavalin A (ConA) (5 μg/mL, Sigma-Aldrich), or phorbol 12-myristate acetate (PMA) (20 ng/mL, Sigma-Aldrich) and ionomycin (1 μg/mL, Sigma-Aldrich) were added to the medium to activate lymphocytes.

Identification of canine *PD-1* and *PD-L1* genes

Total RNA was isolated from the beagle and Samoyed PBMCs which had been stimulated with ConA for 4 h, white blood cells of the Labrador retriever, testis tissue of the Japanese Akita, and lung tissue of the Bernese mountain dog, using the TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Residual genomic DNA was removed from the total RNA by the treatment with DNase I (Life Technologies) treatment. cDNA was synthesized from 1 μg of the total RNA using Moloney murine leukemia virus reverse transcriptase (Takara, Shiga, Japan) and oligo-dT primer, as recommended by the manufacturer. To amplify the inner sequences of canine *PD-1* or *PD-L1*, canine *PD-1*- and *PD-L1*-specific primers were designed based on the putative canine *PD-1* and *PD-L1* mRNA sequence reported in the GenBank database (XM_543338 and XM_541302). Canine *PD-1* and *PD-L1* cDNA were amplified from beagle cDNA by polymerase chain reaction (PCR) using primers 5′-AGG ATG GCT CCT AGA CTC CC-3′ (*PD-1* inner forward), 5′-AGA CGA TGG TGG CAT ACT CG-3′ (*PD-1* inner reverse), 5′-ATG AGA ATG TTT AGT GTC TT-3′ (*PD-L1* inner forward), and 5′-TTA TGT CTC TTC AAA TTG TAT ATC-3′ (*PD-L1* inner reverse). The PCR cycling conditions were as follows: (1) initial denaturation at 94°C for 5 min, (2) 40 cycles of denaturation at 94°C for 1 min, annealing at 58°C (*PD-1*) or 50°C (*PD-L1*) for 1 min, and extension at 72°C for 1 min 30 s, and (3) final extension at 72°C for 7 min. PCR amplicons were purified using the FastGene gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan), cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and sequenced using the CEQ8000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA). 5′-RACE and 3′-RACE were then performed using the 5′-RACE system for rapid amplification of cDNA ends and 3′-RACE system for rapid amplification of cDNA ends (Life Technologies), respectively. The gene-specific primers for canine *PD-1/PD-L1* used for 5′-RACE were 5′-GTT GAT CTG TGT GTT G-3′ (*PD-1*) and 5′-TTT TAG ACA GAA AGT GA-3′ (*PD-L1*). The gene-specific primers for canine *PD-1/PD-L1* used for 3′-RACE were 5′-CGG GAC TTC CAC ATG AGC AT-3′ (*PD-1*) and 5′-GAC CAG CTC TTC TTG GGG AA-3′ (*PD-L1*). Based on the sequences obtained, new primer sets were designed to amplify the open reading frame (ORF) of the canine *PD-1* and *PD-L1* genes.

PCR was performed using primers 5′-GGG GGA GGC GAG CAG G-3′ (*PD-1* ORF F), 5′-GAG TCG AGA GAG GAG AGC CAT GAG-3′ (*PD-1* ORF R), 5′-GCC AGC AGG TCA CTT CAG AAC-3′ (*PD-L1* ORF F), and 5′-GCT GAA CTC AAA CCA CAG GCC-3′ (*PD-L1* ORF R) as described above, except that the annealing temperature used was 60°C. The resulting amplicons were sequenced as described above. To confirm the polymorphisms of *PD-1* and *PD-L1* genes among canine breeds, cDNA samples from other breeds were used and the ORF sequences were determined as described above. The established sequences were aligned, and unrooted neighbor-joining trees were constructed using the Mega version 5 software [Saitou and Nei, 1987; Tamura *et al*., 2011].

Preparations of canine PD-1- and PD-L1-expressing cells

To construct expression vectors for enhanced green fluorescent protein (EGFP) fusion proteins, the ORF region of canine *PD-1* (cPD-1) and *PD-L1* (cPD-L1) cDNA that did not have the stop codons was amplified by PCR using gene-specific primers 5′-CCG CTC GAG ATG GGG AGC CGG CGG GGG CC-3′ (*PD-1* F, containing an *Xho*I restriction site), 5′-CGC GGA TCC TGA GGG GCC ACA GGC CGG GTC-3′ (*PD-1* R, containing a *Bam*HI restriction site), 5′-GAA GAT CTA TGA GAA TGT TTA GTG TC-3′ (*PD-L1* F, containing a *Bgl*II restriction site), and 5′-GGA ATT CTG TCT CTT CAA ATT GTA TAT C-3′ (*PD-L1* R, containing an *Eco*RI restriction site). The amplicons were then cloned into the multicloning site of pEGFP-N2 vector (Clontech, Palo Alto, CA, USA). These vectors were named pEGFP-N2-cPD-1 and pEGFP-N2-cPD-L1, respectively. For transient expression, Cos-7 cells (5×10^4/cm^2) were transfected with 0.4 μg/cm^2 of either pEGFP-N2-cPD-1, pEGFP-N2-cPD-L1, or empty pEGFP-N2 vector (mock), using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. The cells were cultured for 48 h and harvested using a cell scraper. To confirm the expression of fusion proteins, the cells were observed under a confocal microscope LSM700 (Carl Zeiss Microscopy, Jena, Germany), and the subcellular distributions of EGFP were determined.

Preparations of recombinant canine PD-1- and PD-L1-rabbit IgG Fc fusion proteins (cPD-1-Ig, cPD-L1-Ig)

For the construction of cPD-1-Ig and cPD-L1-Ig expression vectors, cDNA sequences of canine *PD-1* and *PD-L1* encoding the putative extracellular regions were amplified by PCR using gene-specific primers 5′-CGC GGC TAG CAT GGG GAG CCG GCG GGG GCC-3′ (*PD-1* F, containing an *Nhe*I restriction site), 5′-CGC GGA TAT CCA GCC CCT GCA ACT GGC CGC-3′ (*PD-1* R, containing an *Eco*RV restriction site), 5′-CGC GGC

TAG CAT GAG AAT GTT TAG TGT CTT-3′ (*PD-L1* F, containing an *Nhe*I restriction site), and 5′-CGC GGA TAT CAG TCC TCT CAC TTG CTG GAA-3′ (*PD-L1* R, containing an *Eco*RV restriction site). The amplicons were then cloned into the multicloning site of pCXN-2.1-rabbit IgG Fc vector (kindly provided by Dr T. Yokomizo, Graduate School of Medicine, Juntendo University; modified) [Zettlmeissl *et al*., 1990; Niwa *et al*., 1991]. These vectors were named pCXN2.1-Rabbit IgG Fc-cPD-1 and pCXN-2.1-rabbit IgG Fc-cPD-L1, respectively. For stable expression, 4×10^6 CHO-DG44 cells were transfected with 2.5 μg either pCXN-2.1-rabbit IgG Fc-cPD-1 or pCXN-2.1-rabbit IgG Fc-cPD-L1 using Lipofectamine LTX (Life Technologies), as recommended by the manufacturer. Forty-eight hours later, the cells were collected and resuspended in the supplemented CD-DG44 medium containing 800 μg/mL G418 (Enzo Life Science, Farmingdale, NY, USA). Stably expressing cells were cloned by limiting dilution, and cell lines with high expression of recombinant proteins were established. The culture supernatant of these cell lines was collected after 7 days of shaking culture {37 °C, 5% CO_2, 125 revolutions per minute (rpm)}. The supernatant containing Ig fusion proteins was concentrated by ultrafiltration using Centricon Plus-70 (Merck Millipore, Billerica, MA, USA) and the Ig fusion proteins were purified by Ab-Capcher Extra (Protenova, Kagawa, Japan). Buffers were exchanged with phosphate-buffered saline (PBS) using PD MiniTrap G-25 (GE Healthcare). The concentrations of the Ig fusion proteins were evaluated using a rabbit IgG ELISA quantitation set (Bethyl Laboratories, Montgomery, TX, USA). To confirm the expression and purification of these Ig fusion proteins, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with Immobilon-P transfer membrane (Merck Millipore) were performed as described elsewhere [Ikebuchi *et al*., 2013]. The membrane was incubated with Immobilon Western chemiluminescent HRP substrate (Merck Millipore) to visualize the signals and analyzed by a Fluor-S MultiImager (Bio-Rad Laboratories, Hercules, CA, USA).

Flow cytometry

To analyze the binding of cPD-1 to cPD-L1, Cos-7 cells expressing cPD-1-EGFP or cPD-L1-EGFP were incubated with 10 μg/mL of either cPD-1-Ig, cPD-L1-Ig, or rabbit IgG isotype control antibody at room temperature (RT) for 30 min. Cells were washed twice and then incubated with Alexa Fluor 647-conjugated goat antirabbit IgG (H+L) F(ab')2 (Beckman Coulter) at RT for 30 min. After washing, cells were analyzed by FACS Verse (BD Biosciences, San Jose, CA, USA) and FACS Express 4 (De Novo Software, Glendale, CA, USA). PBS containing 10% goat serum (Sigma-Aldrich) was used in all

washing processes and dilutions of Ig fusion proteins or antibodies.

To detect canine PD-L1 expressed on the cell surfaces, flow cytometric analysis was performed using rat anti-bovine PD-L1 mAbs 6G7-E1; rat IgM (κ), 5A2-A1; rat IgG1 (κ), and 4G12-C1; rat IgG2a (κ), which were established in our laboratory [Ikebuchi *et al*., 2014]. In brief, cells were stained with 10 μg/mL mAbs as described above, using allophycocyanin (APC)-conjugated goat anti-rat Ig antibody (Beckman Coulter) as a secondary antibody. Rat IgM (κ), IgG1 (κ), IgG2a (κ) isotype control (BD Biosciences) were used as isotype-matched negative control antibodies.

To assess the blockade of canine PD-1/PD-L1 binding by anti-PD-L1 mAb, Cos-7 cells expressing cPD-L1-EGFP (2×10^5) were incubated with various concentrations (0.5, 1.0, 2.5, and 5.0 μg/mL) of 6G7-E1 at 37°C for 15 min prior to the incubation with 1 μg/mL (final concentration) of cPD-1-Ig at 37°C for 30 min. The cells were washed twice, and binding of cPD-1-Ig was detected by a flow cytometer as described above. Rat IgM (κ) (BD Biosciences) was used as a control antibody.

To assess whether PD-1 expression is upregulated in the context of canine cancers, flow cytometric analysis was performed with cross-reactive anti-human PD-1 polyclonal antibody [Esch *et al*., 2013]. To obtain TIMCs, freshly excised solid tumor tissues were cut into small pieces, and mechanically dispersed into single cells using 100 μm cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The cell suspensions were overlaid onto 1.055 g/mL Percoll separation solution. The tumor cells were separated by density gradient centrifugation at 800 ×*g* for 20 min, and the cell pellets were collected and resuspended in complete RPMI 1640 medium. Then, cells were again overlaid onto 1.077 g/mL Percoll separation solution and centrifuged. Cells in the gradient interfaces were collected and washed 3 times with PBS. The resulting cells containing TILs were stained with optimal concentrations of fluorescein isothiocyanate-conjugated anti-CD4/phycoerythrin (PE)-conjugated anti-CD8 antibody cocktail (AbD Serotec, Oxford, UK) and biotinylated anti-human PD-1 antibody (R&D Systems, Minneapolis, MN, USA) for 30 min at RT. After washing, cells were incubated with Streptavidin APC-eFluor 780 (eBioscience, San Diego, CA, USA) for 30 min at RT. Fluorescence was analyzed as described above. Biotinylated normal goat IgG (R&D systems) was used as isotype-matched control antibody. For the analysis, lymphocytes population was gated by forward scatter (FSC) and side scatter (SSC) and PD-1 expression on $CD4^+$ cells or $CD8^+$ cells was evaluated.

Immunohistochemical analysis of PD-L1

Formalin-fixed and paraffin-embedded tumor tissues were cut into 4-μm-thick sections

and dried on silane-coated slides. The dried sections were deparaffinized in xylene. Antigen retrieval was performed in citrate buffer (0.37 g/mL of citric acid and 2.4 g/mL of trisodium citrate dihydrate) by microwave heating for 10 min. Endogenous peroxidase activity was blocked by incubating the sections in methanol containing 0.3% hydrogen peroxide for 15 min. Primary antibody incubation was performed at RT for 30 min using anti-PD-L1 mAb 5A2-A1 (1.2 μg/mL) or rat IgG1 isotype-matched control antibody (Biolegend, San Diego, CA, USA). The sections were washed twice with PBS, and incubated with Histofine simple stain MAX PO (rat) (Nichirei, Tokyo, Japan) at RT for 30 min. Positive staining was visualized with 3-diaminobenzidine tetrahydrochloride. The sections were observed under an optical microscope.

Blocking assay using anti-PD-L1 mAb

To prepare TIMCs, single cell suspensions from solid tumor tissues were obtained as described above, or by enzymic digestion. In brief, small pieces of tumor tissues were treated with 2 mg/mL collagenase D (Roche Applied Science, Indianapolis, IN, USA) in complete RPMI 1640 medium at 37°C for up to 2h. Dispersed cells were collected using 100 μm cell strainer (Becton, Dickinson and Company). TIMCs were enriched from single cell suspensions by density gradient centrifugation as described above.

The TIMCs and PBMCs obtained from healthy adult beagles (2×10^6/mL) were cultured with 20 μg/mL of anti-PD-L1 mAb 6G7-E1 for 48 h, and the culture supernatant was collected. As a negative control, low-endotoxin, azide-free rat IgM isotype control antibody (Acris Antibodies, Herford, Germany) was used. The concentration of canine IFN-γ in the culture supernatant was evaluated by Duoset ELISA canine IFN-γ (R&D systems) according to the manufacturer's protocol.

Statistical analyses

In the blocking assay of protein binding with anti-PD-L1 mAb, Tukey's test was conducted among groups. In the flow cytometric analysis of PD-1 expression, Mann-Whitney U test was performed. The Wilcoxon signed rank-sum test was conducted to compare the data from the same individuals. For all tests, $p < 0.05$ was considered statistically significant.

Nucleotide sequence accession numbers

The sequences of canine *PD-1* and *PD-L1* genes have been submitted to the GenBank database under accession numbers, AB898677 (PD-1) and AB898678 (PD-L1).

RESULTS

Identification of canine *PD-1* and *PD-L1* genes

The complete nucleotide sequences and deduced amino acid sequences of canine *PD-1* and *PD-L1* are determined using RNA samples extracted from beagle PBMCs (Figures I-1A and I-2A). Canine *PD-1* and *PD-L1* mRNA sequences were found to be 1,781 and 1,561 bp in length, encoding ORFs for 288 and 289 amino acids, respectively. Canine PD-1 and PD-L1 were predicted to be type I transmembrane proteins, which consist of a putative signal sequence, an extracellular domain, a transmembrane domain, and an intracellular domain (Figures I-1B and I-2B). The intracellular domain of canine PD-1 contained an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) [Chemnitz *et al*., 2004], as reported in other mammalian species (Figures I-1B and 1D). Phylogenetic analyses revealed that canine PD-1 and PD-L1 are closely related to those of cat, cattle, and pig, and relatively less similar to those of rodents (Figures I-1C and I-2C). Canine PD-1 showed 87.8%, 77.1%, 75.7%, and 68.4% amino acid similarities with cat, cattle, human and mouse PD-1, respectively (Table I-1). Canine PD-L1 had 87.9%, 86.2%, and 82.4% amino acid similarities with cattle, human and mouse PD-L1, respectively (Table I-2). To assess the polymorphism of PD-1 and PD-L1 among canine breeds, *PD-1* gene sequences of Samoyed and Japanese Akita, and *PD-L1* gene sequences of Samoyed, Labrador retriever, and Bernese mountain dog were determined. The sequences of these canine breeds were 100% identical to those of beagle (Figures I-1B and I-2B).

Canine PD-1 binds to PD-L1, and the binding is disturbed by anti-bovine PD-L1 mAb

To confirm that canine PD-1 binds to PD-L1, canine PD-1 and PD-L1 recombinant proteins that are fused to EGFP (membrane bound form) or to rabbit IgG Fc (soluble form) were prepared. cPD-1-EGFP- and cPD-1-EGFP-expressing cells were established using Cos-7 cells, and the cell surface expression of fusion proteins were detected under a confocal microscope (Figure I-3A). On the other hand, Ig-fusion proteins, named cPD-1-Ig and cPD-L1-Ig, were expressed using CHO-DG44 cells, and their expression and purification were confirmed by SDS-PAGE and Western blot analysis (Figure I-3B). Subsequently, the bindings of cPD-1-Ig or cPD-L1-Ig to cPD-1-EGFP or cPD-L1-EGFP-expressing cells were assessed by a flow cytometer, confirming the specific binding of canine PD-1 to PD-L1 (Figure I-3C).

For expression and functional analysis of canine PD-1/PD-L1, cross-reactivities of

anti-bovine PD-L1 mAbs, which were previously established in our laboratory, were examined. The anti-bovine PD-L1 mAbs 4G12-C1, 5A2-A1, and 6G7-E1 bound to canine PD-L1-expressing cells and mitogen-stimulated canine lymphocytes (Figure I-4A). Furthermore, 6G7-E1 clearly blocked the binding of cPD-1-Ig to cPD-L1-EGFP-expressing cells, and the effect was observed in a dose-dependent manner. (Figure I-4B).

PD-1 and PD-L1 are expressed in canine cancers

Using cross-reactive anti-PD-L1 mAb, expression analysis of PD-L1 was performed in canine cancers. In a flow cytometric analysis, PD-L1 was detected on MCT cell lines CM-MC and CoMS, and the expression was enhanced by IFN-γ treatment. Interestingly, PD-L1 expression was induced by IFN-γ on all tested melanoma cell lines CMeC, LMeC, CMM-1, and CMM-2, although the expression was not found without stimulation. On the other hand, in OS cell lines POS and HMPOS, PD-L1 expression was not found even after the IFN-γ treatment (Figure I-5A and Table I-3). The expression of PD-L1 in canine cancers was further assessed by IHC, demonstrating that 69.2% of melanoma, 66.7% of grade III MCT, and 70.0% of renal cell carcinoma were PD-L1-positive. Although the number of tested samples were limited, PD-L1 was found in all oral melanoma cases (Figure I-5B and Table I-4).

Next, to assess the expression level of PD-1 on tumor-associated lymphocytes, TILs were obtained from surgically excised cancer tissues, and a flow cytometric analysis was performed using cross-reactive polyclonal antibody against human PD-1. Compared to peripheral blood lymphocytes from healthy dogs, PD-1 was highly expressed on both $CD8^+$ and $CD4^+$ TILs from OMM (Figure I-6A, $p < 0.05$). The positive rate of PD-1 expression on $CD8^+$ or $CD4^+$ TILs was 70.9-96.6% or 80.2-96.8%, respectively (Table I-5). In the case of hepatic tumors, more optimal control cells were available; normal tissue-infiltrating lymphocytes (NILs) from the same individuals were similarly prepared and used for the analysis. Both in hepatocellular adenoma and in hepatocellular carcinoma, TILs expressed the higher level of PD-1 than NILs (Figure I-6B).

The blockade of the PD-1/PD-L1 axis enhances IFN-γ production from canine mononuclear cells

To obtain an insight into the effect of the PD-L1 blockade on canine immune cells, PBMCs from healthy dogs were cultivated in the presence of blocking anti-PD-L1 mAb, 6G7-E1. IFN-γ production from PBMCs were significantly enhanced by 6G7-E1 treatment (Figure I-7A, $p < 0.05$). Moreover, 6G7-E1 also increased the IFN-γ production from TIMCs obtained from hepatocellular carcinoma, myelolipoma, and OMM, while the

effect was not found in TIMCs obtained from seminoma (Figure I-7B and Table I-6).

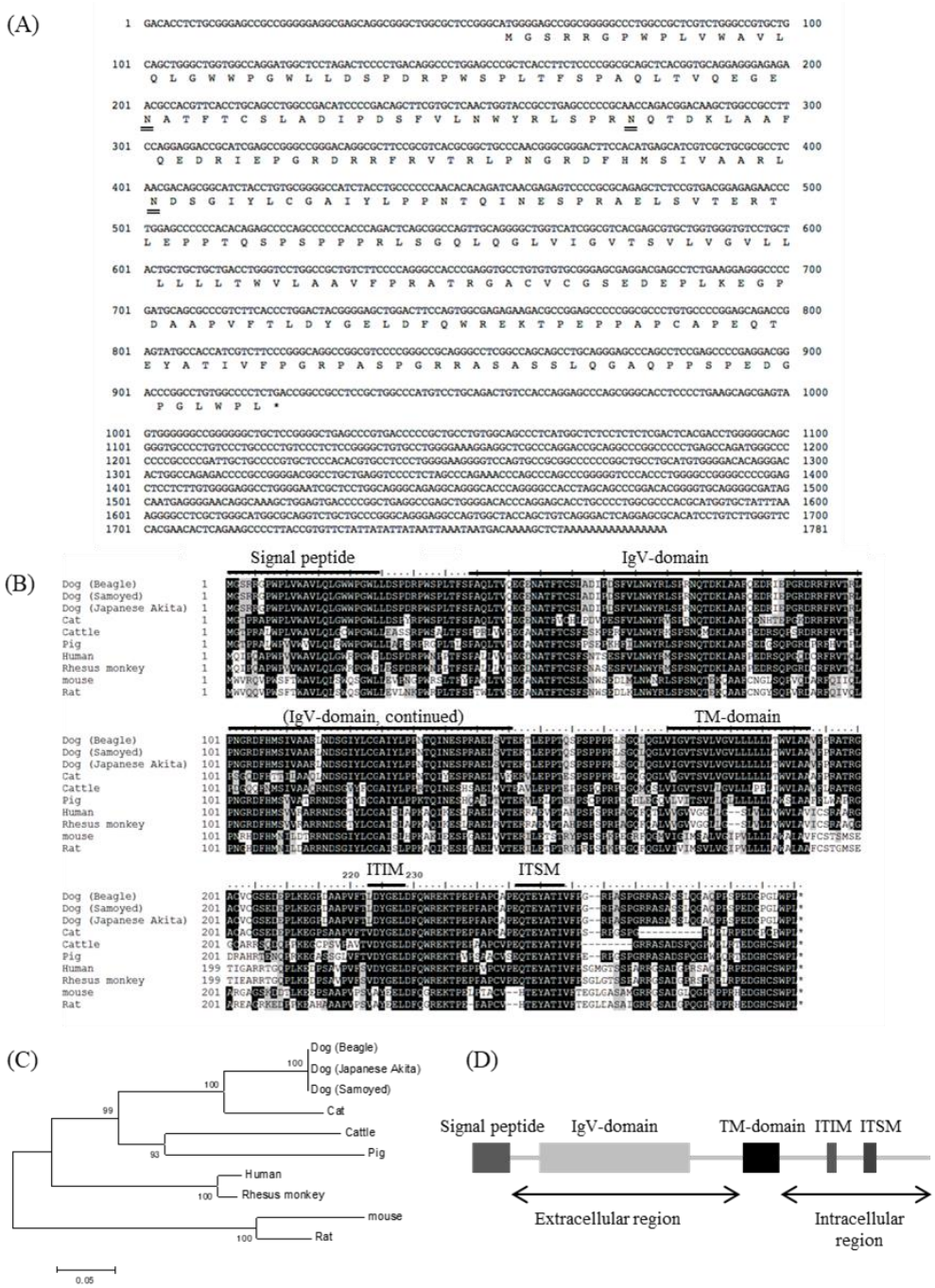

Figure I-1. Sequence analysis of canine PD-1. (A) Nucleic acid and deduced amino acid sequences of canine *PD-1*. Predicted N-glycosylation sites are double-underlined. (B) Multiple sequence alignment of vertebrate PD-1 amino acid sequences. Predicted domains and motifs of canine PD-1 are shown. Signal peptide, 1-24; IgV domain, 39-145; transmembrane domain, 170-192; ITIM, 223-228; ITSM, 246-253. (C) Phylogenetic tree of the canine PD-1 sequence in relation to those of other vertebrate species. The bootstrap consensus tree was inferred from 1,000 replicates (the numbers next to the branches indicate the bootstrap percentage). The scale indicates the divergence time. (D) Schematic image of predicted functional motifs in canine PD-1. Canine PD-1 consists of an extracellular region, a transmembrane region, and an intracellular region.

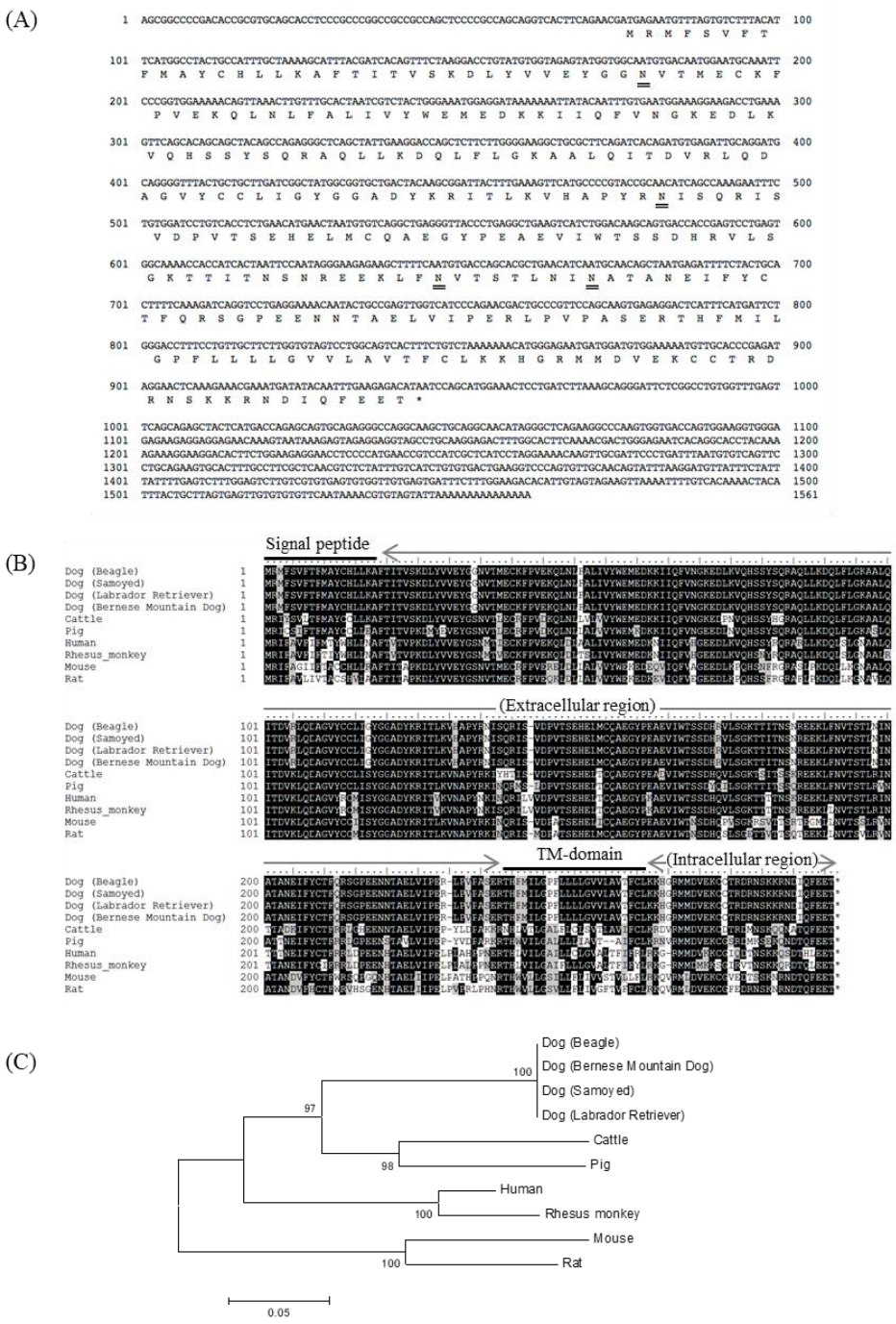

Figure I-2. Sequence analysis of canine PD-L1. (A) Nucleic acid and deduced amino acid sequences of canine *PD-L1*. Predicted N-glycosylation sites in the amino acid sequence of canine PD-L1 are double-underlined. (B) Multiple sequence alignment of vertebrate PD-L1 amino acid sequences. Predicted domains and regions of canine PD-L1 are shown. Signal peptide, 1-18; transmembrane domain, 237-259. Canine PD-L1 consists of an extracellular region, a transmembrane region, and an intracellular region. (C) Phylogenetic tree of the canine PD-L1 sequence in relation to those of other vertebrate species. The bootstrap consensus tree was inferred from 1,000 replicates (the numbers next to the branches indicate the bootstrap percentage). The scale indicates the divergence time.

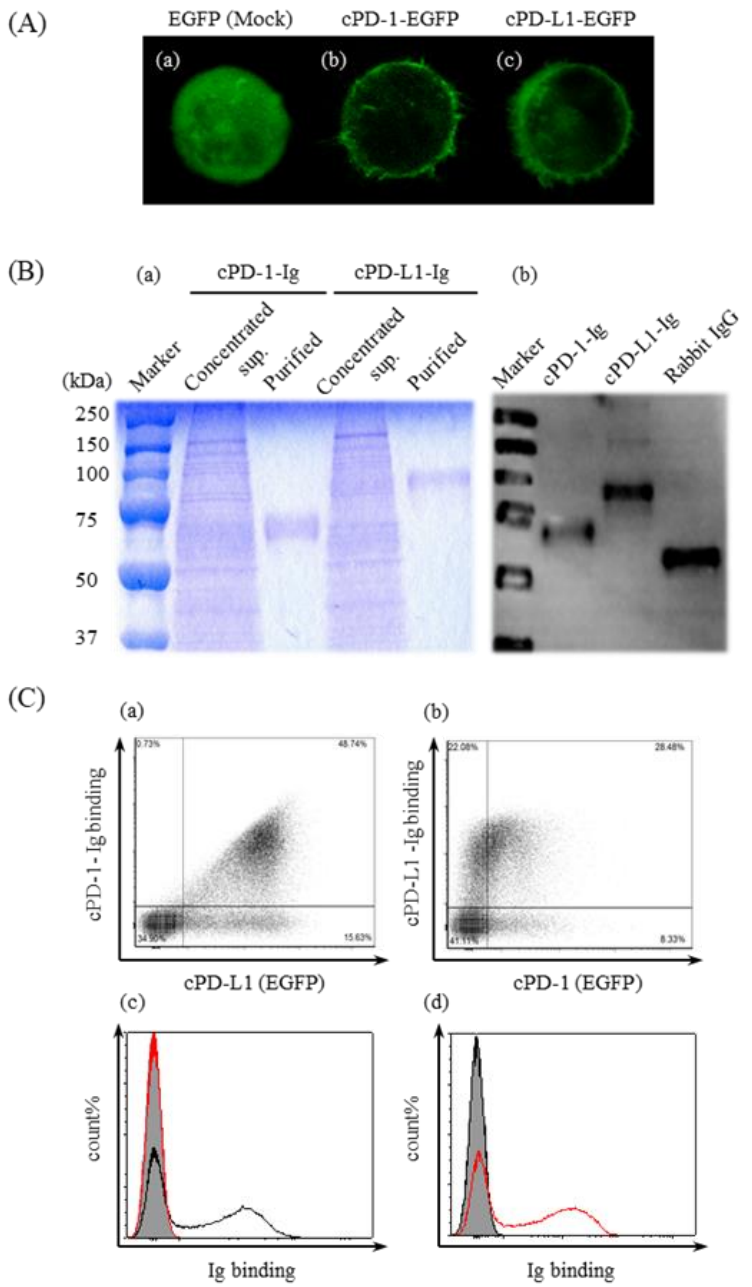

Figure I-3. Establishment of canine PD-1- or PD-L1-expressing cells and Ig fusion proteins. (A) Canine PD-1-EGFP- or PD-L1-EGFP-expressing cell. The subcellular distributions of (a) EGFP only (Mock), (b) cPD-1-EGFP, or (c) cPD-L1-EGFP were analyzed under a confocal microscope (400×). (B) Production and purification of Ig fusion proteins. (a) SDS-PAGE analysis of concentrated culture supernatant and purified proteins. (b) Western blot analysis of purified Ig fusion proteins. Rabbit IgG was used as a positive control. (C) Canine PD-L1 binds to canine PD-1. cPD-1-EGFP- or cPD-L1-EGFP-expressing cells were stained with either cPD-L1-Ig or cPD-1-Ig. (a, c) Binding of cPD-1-Ig to cPD-L1-expressing cells. (b, d) Binding of cPD-L1-Ig to cPD-1-expressing cells. Black line, cPD-1-Ig; red line, cPD-L1-Ig; shaded area, rabbit IgG.

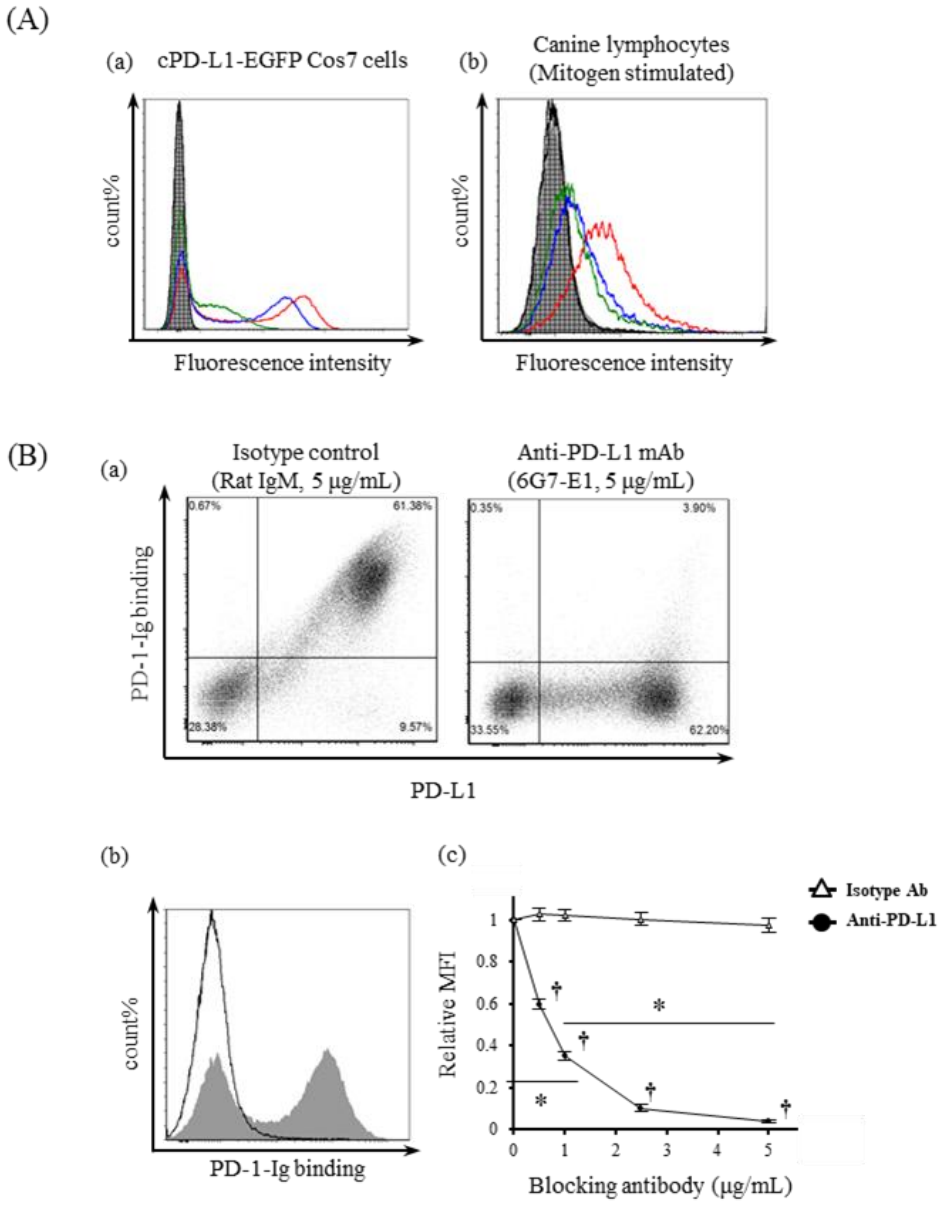

Figure I-4. MAbs which recognize canine PD-L1. (A) Cross-reactivities of anti-bovine PD-L1 mAbs. (a) cPD-L1-EGFP-expressing Cos-7 cells and (b) canine PBMCs stimulated with PMA/ionomycin were stained with anti-PD-L1 mAbs (10 μg/mL). Red line, 4G12-C1; blue line, 5A2-A1; green line, 6G7-E1; shaded area, rat IgG2a; vertical-striped area, rat IgG1; horizontal-striped area, rat IgM. (B) Blockade of cPD-1/cPD-L1 binding by anti-PD-L1 mAb 6G7-E1. cPD-L1-EGFP-expressing cells were preincubated with 6G7-E1 and then cPD-1-Ig bindings were evaluated. (a) Representative dot plot and (b) histogram from the blockade assay of protein binding. Shaded area, isotype control; solid line, 6G7-E1. (c) Dose-dependent blockade of 6G7-E1 on cPD-1/cPD-L1 binding. Each point indicates the average value of relative MFI obtained from three independent experiments {compared to no antibody control, error bar; standard error (SE)}. * $p < 0.05$, between the group treated with 0 μg/mL of 6G7-E1 and that with 1 μg/mL and between the group treated with 1 μg/mL and that with 5 μg/mL. † $p < 0.05$, between the group treated with each concentration of 6G7-E1 and that treated with the same concentration of isotype control antibody.

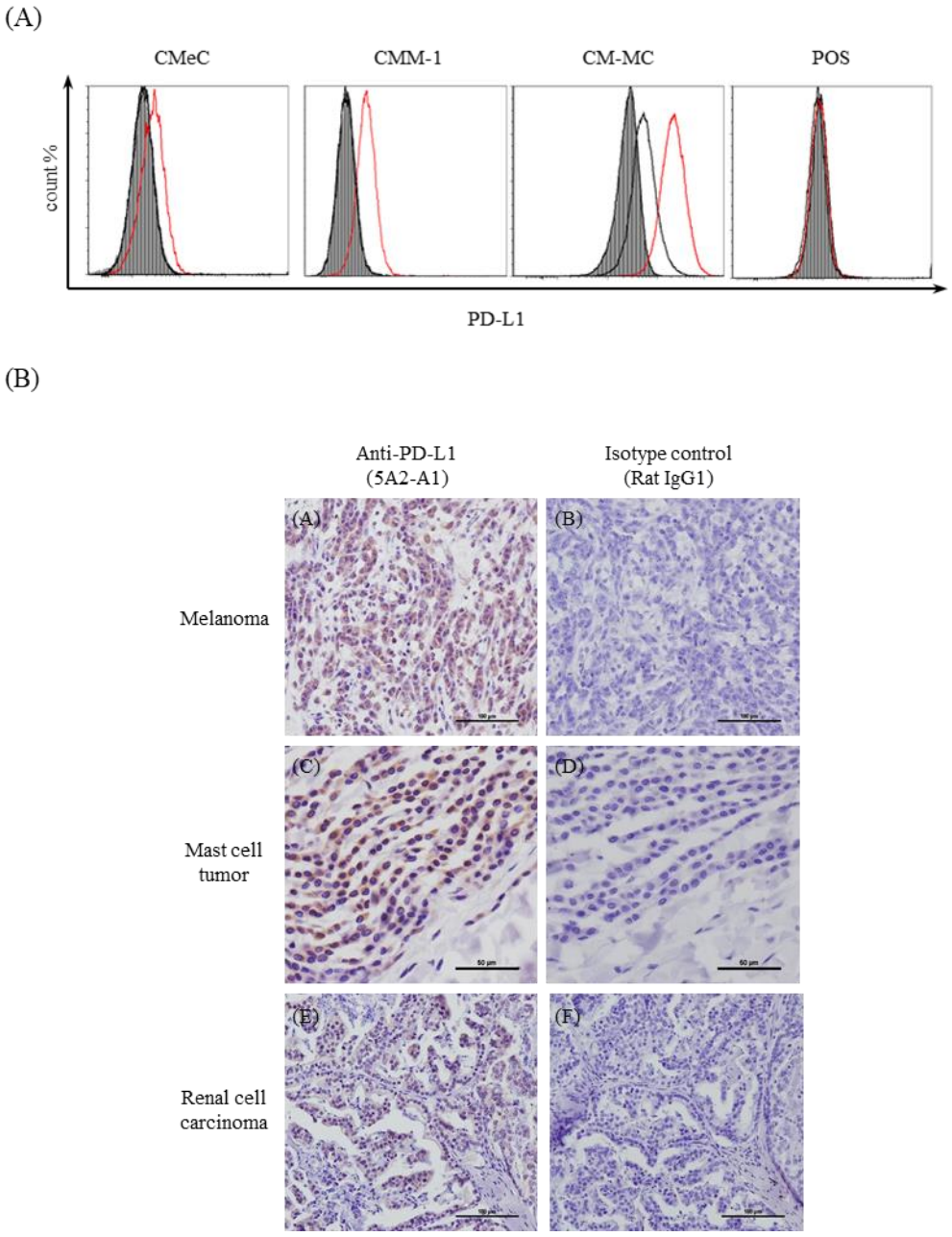

Figure I-5. Expression of PD-L1 in canine cancer cells. (A) Representative histograms for the analysis of PD-L1 expression on canine cancer cell lines. Cells maintained in the medium or those stimulated by IFN-γ (100 ng/mL) were stained with anti-PD-L1 mAb 4G12-C1 or isotype control antibody (rat IgG2a). Black line, medium/4G12-C1; red line, IFN-γ/4G12-C1; shaded area, medium/isotype control; vertical-striped area, IFN-γ/isotype control. (B) Immunohistochemical analysis of PD-L1 expression in canine cancer tissues. Tissue sections were stained with anti-PD-L1 mAb 5A2-A1 or isotype control antibody (rat IgG1). (a,b) Representative immunohistochemical staining of melanoma. (c,d) Representative immunohistochemical staining of mast cell tumor (grade III). (e,f) Representative immunohistochemical staining of renal cell carcinoma.

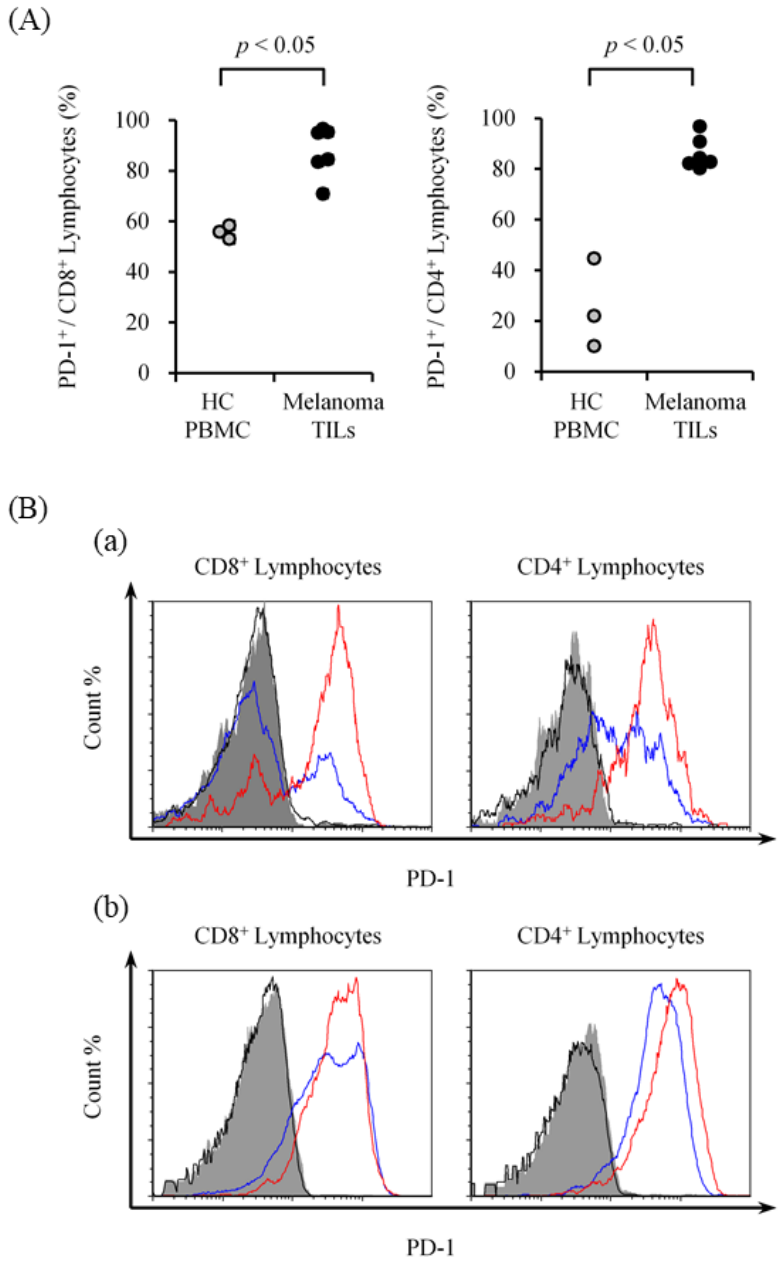

Fig I-6. PD-1 expression on TILs obtained from OMM and hepatic tumors. TILs were collected from surgically excised cancer tissues and the expression level of PD-1 was evaluated by a flow cytometer. (A) PD-1 expression on TILs in OMM. Left panel, PD-1 expression on $CD8^+$ lymphocytes. Right panel, PD-1 expression on $CD4^+$ lymphocytes. PBMCs obtained from healthy dogs were used as control (healthy control, HC). $p < 0.05$ was considered statistically significant (Mann-Whitney U test). (B) PD-1 expression on TILs in hepatic tumors. TILs in (a) hepatocellular adenoma and (b) hepatocellular carcinoma were analyzed for PD-1 expression (red line). NILs obtained from adjacent healthy liver tissue of the same individuals (blue line) were used as control. Black line, isotype control for TILs. Shaded area, isotype control for NILs.

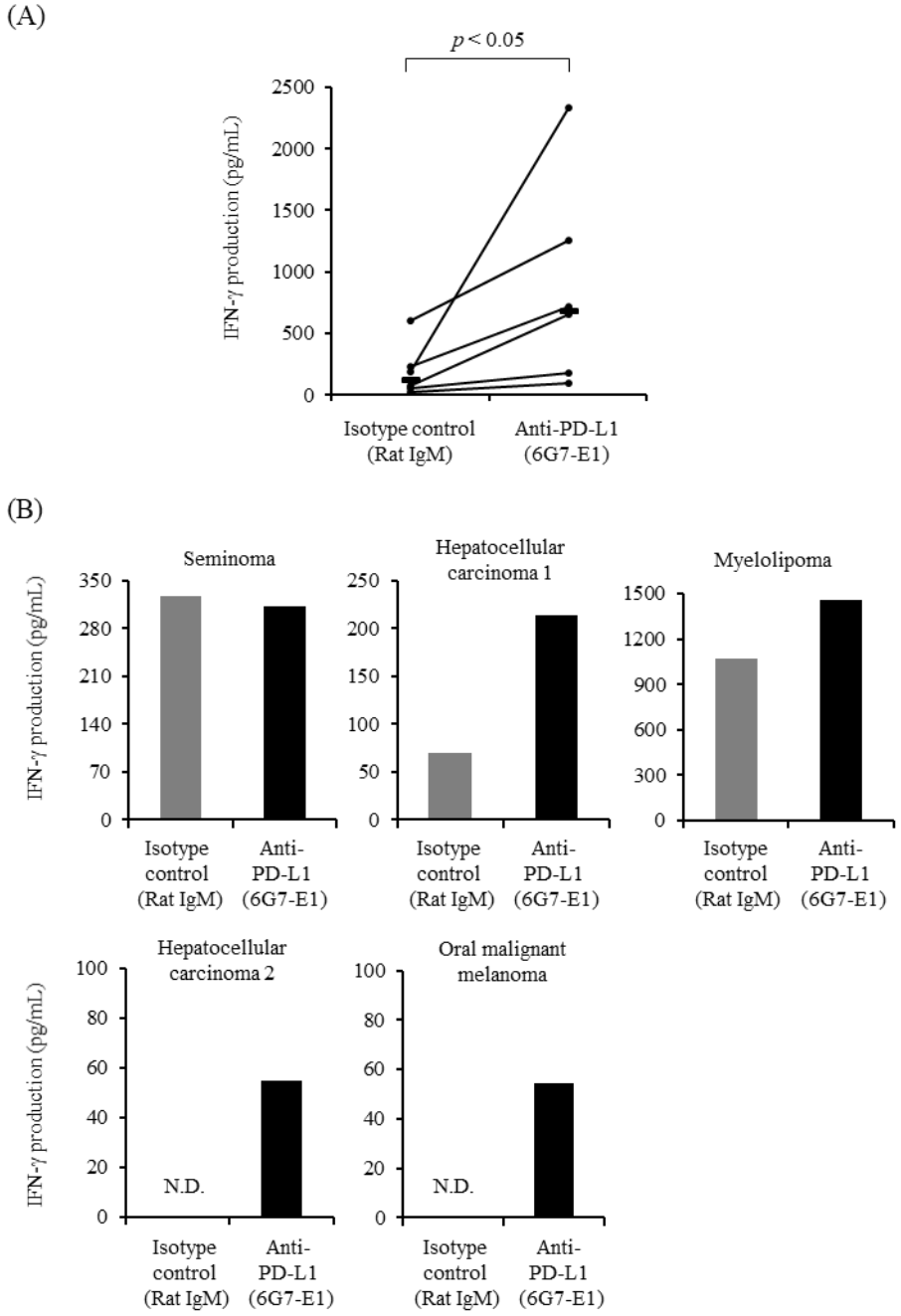

Figure I-7. Effects of PD-L1 blockade by anti-PD-L1 mAb. (A) Effect of anti-PD-L1 mAb 6G7-E1 on IFN-γ production from canine PBMCs. PBMCs obtained from healthy dogs were cultured with 6G7-E1 or isotype-matched control antibody (20 μg/mL) for 2 days and the concentration of IFN-γ in the culture supernatant was measured by ELISA. Statistical significance was evaluated by the Wilcoxon signed rank-sum test (n = 6). (B) Effect of PD-L1 blockade on IFN-γ production from TIMCs. TIMCs were cultured with 20 μg/mL of 6G7-E1 or isotype-matched control antibody for 2 days. Details of each tumor sample are shown in Table I-6. N.D., Not Detected.

Table I-1. Nucleotide and amino acid sequence similarities (%) of PD-1 among vertebrate species.

Species (GenBank accession number)	Dog	Cat	Cattle	Pig	Human	Rhesus monkey	Mouse	Rat
Dog	-	83.4	78.1	78.5	76.4	77.3	69.2	69.0
Cat (NM_001145510)	87.8	-	76.7	74.5	72.1	72.5	65.3	64.7
Cattle (AB510901)	77.1	76.6	-	80.0	74.9	75.1	66.6	67.6
Pig (NM_001204379)	76.4	75.7	79.9	-	76.8	77.0	69.8	71.2
Human (NM_005018)	75.7	73.3	72.9	71.9	-	95.8	71.4	72.0
Rhesus monkey (NM_001114358)	75.7	73.6	73.6	72.6	98.3	-	71.3	71.9
Mouse (NM_008798)	68.4	64.2	65.6	67.4	71.5	72.6	-	91.3
Rat (NM_001106927)	69.4	65.9	68.3	70.1	71.2	72.2	90.3	-

Upper section; similarities in nucleotide level, Lower section; similarities in amino acid level. Genbank accession numbers are shown in the table.

Table I-2. Nucleotide and amino acid sequence similarities (%) of PD-L1 among vertebrate species.

Species (GenBank accession number)	Dog	Cattle	Pig	Human	Rhesus monkey	Mouse	Rat
Dog	-	83.3	84.6	83.2	82.4	73.2	73.6
Cattle (NM_001163412)	87.9	-	87.4	83.0	81.7	73.3	74.4
Pig (NM_001025221)	89.3	92.0	-	84.4	83.2	74.2	75.2
Human (AK314567)	86.2	85.2	86.9	-	95.7	76.3	76.5
Rhesus monkey (EF444816)	85.2	83.4	85.2	96.2	-	75.2	75.1
Mouse (AF317088)	82.4	80.7	82.4	82.8	82.1	-	87.1
Rat (NM_001191954)	82.8	80.3	82.1	82.8	82.1	92.1	-

Upper section; similarities in nucleotide level, Lower section; similarities in amino acid level. Genbank accession numbers are shown in the table.

Table I-3. Expressions of PD-L1 on canine cancer cell lines.

Cell line	Pathology	PD-L1 expression	
		Medium	IFN-γ*
CMeC	Melanoma	-	+
LMeC	Melanoma	-	+
CMM-1	Melanoma	-	++
CMM-2	Melanoma	-	++
CM-MC	Mast cell tumor	+	+++
CoMS	Mast cell tumor	+	+++
POS	Osteosarcoma	-	-
HMPOS	Osteosarcoma	-	-

The expression of PD-L1 was evaluated by a flow cytometer using anti-PD-L1 mAb 4G12-C1. -; < 3% positive, +; 3-30% positive, ++; 30-60% positive, +++; > 60% positive. *Cells were incubated with recombinant canine IFN-γ (100 ng/ml) for 24 h before the analysis.

Table I-4. Immunohistochemical analysis of PD-L1 expression in canine cancer tissues.

Pathology		Positive case/Tested samples	(%positive)
Melanoma	Oral cavity	8/8	(100%)
	Skin	1/3	(33.3%)
	Eye	0/2	(0%)
	All	9/13	(69.2%)
Mast cell tumor		4/6	(66.7%)
Renal cell carcinoma		7/10	(70.0%)

Canine cancer tissues surgically excised from clinical cases were stained with anti-PD-L1 mAb 5A2-A1. Melanoma cases were divided into 3 groups dependent on the tumor site (oral cavity, skin, and eye).

Table I-5. OMM samples used in the flow cytometric analysis of PD-1 expression.

				PD-1 expression (%)	
Breed	Age	Sex	Site	$CD8^+$	$CD4^+$
American cocker spaniel	12	Male	Maxilla, left	84.4	84.2
Golden retriever	14	Female, spayed	Mandible, left	83.5	82.2
Miniature dachshund	13	Male	Mandible, right	70.9	80.2
Golden retriever	14	Male, castrated	Lip, upper left	95.2	82.7
Chihuahua	13	Female	Maxilla, left	95.1	96.8
Mix	9	Male	Mandible, right	96.6	90.7

Breed of dog, age, sex, tumor site, and positive rate of PD-1 expression (%) on $CD8^+$ or $CD4^+$ lymphocytes were shown in the table.

Table I-6. Canine cancer tissues used in the blockade test of the PD-1/PD-L1 pathway.

Pathology	N-fold	Site	Breed	Age
Seminoma	0.96	Testis	Beagle	11
Hepatocellular carcinoma 1	3.06	Liver	Boston terrior	11
Myelolipoma	1.36	Spleen	Beagle	7
Hepatocellular carcinoma 2	-	Liver	Shih-tzu	9
Malignant melanoma	-	Oral cavity	American cocker spaniel	12

Pathology, tumor site, breed of dog, and age of each tumor sample are shown. Relative value of IFN-γ production from TIMCs treated with anti-PD-L1 mAb compared to that treated with isotype control was also shown as N-fold in the table.

DISCUSSION

Immunotherapies are considered the forth modality of cancer treatment in humans, in addition to surgery, radiation, and chemotherapy. Of those, immune checkpoint inhibitors such as anti-PD-1 mAb gained attention for their clear antitumor activities in some portion of patients. However, in dogs, even the gene sequences of *PD-1* and *PD-L1* had not been reported in the literature.

In this study, to evaluate the potential of the PD-1/PD-L1 pathway as a therapeutic target for canine cancers, gene sequences of canine *PD-1* and *PD-L1* were firstly identified. Canine *PD-1* and *PD-L1* genes were highly similar to those in other mammalian species. In the cytoplasmic tail of canine PD-1, functional motifs known as ITIM and ITSM, which are required for immunoinhibitory signaling of PD-1 [Chemnitz *et al*., 2004], were found, indicating that canine PD-1 is likely to send an inhibitory signal into T cells. *PD-1* and *PD-L1* genes were not polymorphic in several canine samples tested in this study, suggesting that these genes are well conserved among canine breeds. Using recombinant proteins, binding of canine PD-1 to PD-L1 was detected and the blockade of the PD-1/PD-L1 pathway by an anti-PD-L1 mAb enhanced the cytokine production from canine PBMCs. This result suggested that the PD-1/PD-L1 pathway also acts as an inhibitory mechanism of immune response in dogs. Flow cytometric and immunohistochemical analyses demonstrated the expressions of PD-1 and PD-L1 in several types of canine cancers, confirming that the PD-1/PD-L1 axis could be a common mechanism for tumor cells to evade the immune responses. Finally, the effect of PD-L1 blockade on TIMCs was assessed as an indicator of therapeutic potential of anti-PD-L1 mAb. As expected, IFN-γ production from TIMCs was enhanced by a blocking anti-PD-L1 mAb, encouraging the development of antibody drugs which target the PD-1/PD-L1 pathway as a novel immunotherapy against canine cancers.

In the expression analysis of PD-L1 on canine cancer cell lines, PD-L1 expression on MCT and melanoma was enhanced by treating these cells with IFN-γ. In human cells, PD-L1 expression is induced by cytokines including IFNs [Eppihimer *et al*., 2002; Lee *et al*., 2006], and thus, that in cancer cells is considered a mechanism for adaptive resistance to cytokine-producing TILs. The cytokine environment within cancer tissue is also likely to be one of the mechanisms that induce PD-L1 expression in canine cancers, because all melanoma cell lines tested in this study did not show PD-L1 expression without IFN-γ stimulation while most of the melanoma tissues tested by IHC were PD-L1-positive. In the immunohistochemical analysis, all melanoma tissues from oral cavity expressed PD-L1, while most of those from eye and skin did not show PD-L1 expression. Although the

tested sample size is small and further investigations are needed, this is possibly because the tumor microenvironment of eye and skin melanoma differs from that of oral cavity. It is not surprising that MCT cell lines expressed PD-L1 without stimulation, because mast cells are known to express PD-L1 constitutively and modulates T cell responses by expressing various costimulatory molecules and producing cytokines [Nakae *et al*., 2006]. Similarly, renal tubular epithelial cells, from which renal cell carcinoma originates, are reported to express PD-L1 and inhibit T cell proliferation and cytokine synthesis [de Haij *et al*., 2005]. The PD-L1-expressing characteristics of these original cells may contribute to the tendency of PD-L1 expression in cancers. At least, because PD-L1 expression was found in most of oral melanoma, MCT, and renal cell carcinoma tissues, these cancer types could be candidates for immunotherapy targeting the PD-1/PD-L1 pathway.

In the blockade assay using TIMCs, IFN-γ production was enhanced in hepatocellular carcinoma, myelolipoma, and OMM samples, while seminoma TIMCs were not affected by the treatment with an anti-PD-L1 mAb. Seminoma is a common cancer in testis, which is an immune privileged site with immunosuppressive local environment. Although the mechanisms of immune suppression in testis is not fully understood, several characteristics are known to be involved, including cytokine production from male germ cells, presence of regulatory T cells, and structural separation by the blood-testis-barrier [Zhao *et al*., 2014]. Careful interpretation should be made when the therapeutic potential of immune checkpoint blockade is considered in cancers that are found in immune privileged sites, such as testis, brain, and eye ball, because the immunological local environment may largely differ from that in other organs.

In conclusion, results shown in this chapter suggested that PD-1 and PD-L1 are expressed in canine cancers and immune cell functions within cancer tissues can be enhanced by the PD-L1 blockade. Further studies are needed to clarify which cancer types could respond to this therapeutic strategy in more detail, and the antitumor activity of anti-PD-L1 mAb should be evaluated *in vivo*.

SUMMARY

An immune checkpoint molecule, PD-1, is a coinhibitory receptor expressed on activated T cells. When it binds to its ligand, PD-L1, effector functions of T cells are attenuated through the inhibition of TCR signaling. Aberrant PD-L1 expression is found on various human cancers and considered an immune evasion mechanism of cancers. The blockade of PD-1 or PD-L1 using mAbs restores antitumor responses in cancer patients. However, almost no information was available for canine PD-1 and PD-L1.

In this chapter, gene sequences of canine *PD-1* and *PD-L1* were determined, and the expression and function of those molecules were assessed using cross-reactive antibodies. In canine melanoma, MCT, and renal cell carcinoma, approximately 70% of tissue samples expressed PD-L1. In OMM and hepatic tumors, PD-1 was highly expressed on TILs, suggesting that the PD-1/PD-L1 pathway is an immune evasion mechanism for canine cancers. Because the PD-L1 blockade by an anti-PD-L1 mAb enhanced the IFN-γ production form TIMCs, the PD-1/PD-L1 pathway could be a promising therapeutic target for canine cancers.

CHAPTER II

Immunohistochemical analysis of PD-L1 expression in various canine malignant cancers

INTRODUCTION

Dogs often develop spontaneous cancers, which may either cause death [Adams *et al*., 2010] or reduce the quality of life of the patients. Among malignant cancers, lymphoma, MCT, OS, soft tissue sarcoma, and mammary carcinoma are relatively frequent [Dobson *et al*., 2002; Brønden *et al*., 2010] and often recognized as fatal diseases in clinical settings. OMM and HSA are also common malignancies in dogs, and patient dogs with these cancer types usually have poor prognosis [MacEwen *et al*., 1986; Wood *et al*., 1998]. The standards of care for each cancer type have been established and successfully improved the prognosis; however, the treatment outcomes are not always satisfying because of the low-to-moderate response rates or limited prolongation of survival times.

In chapter I, PD-L1 expression was found in canine OMM, MCT, and renal cell carcinoma and the PD-L1 blockade by an anti-PD-L1 mAb enhanced IFN-γ production from TIMCs. These results suggest that anti-PD-L1 mAbs may have therapeutic effects on cancers in dogs. In theory, PD-L1-expressing cancers are more likely to respond to immune checkpoint blockade targeting the PD-1/PD-L1 axis. Indeed, in a human clinical trial with an anti-PD-1 antibody, PD-L1 expression was reported to be associated with the treatment outcome; patients with PD-L1-positive tumors had an ORR of 36%, while none with PD-L1-negative tumors had an objective response [Topalian *et al*., 2012]. In this chapter, to assess which cancer types could respond to the PD-L1 blockade, PD-L1 expression was further investigated in various canine malignant cancers. Here, the immunohistochemical analysis showed that some canine cancers including OMM, OS, HSA, and MCT express PD-L1, suggesting that the PD-1/PD-L1 axis is an immune evasion mechanism in these cancers and thus these cancer types could be candidates for therapeutic blockade of PD-L1 in dogs.

MATERIALS AND METHODS

Specimens

Specimens were randomly selected from formalin-fixed and paraffin-embedded tissues of canine malignant tumors (110 samples in total), which had been submitted for histological diagnosis and kept in a commercial pathology laboratory (North Lab, Sapporo, Japan). All malignant melanoma samples (n = 40) used in this study originated from oral cavity. MCT samples (n = 5) were all classified into Patnaik grade III [Patnaik *et al*., 1984]. For mammary adenocarcinoma sample (n = 5), no inflammatory mammary carcinoma samples were included. In addition to above-mentioned specimens, OS (n = 10), HSA (n = 10), prostate adenocarcinoma (n = 5), skin squamous cell carcinoma (n = 5), diffuse large B-cell lymphoma (n = 5), nasal adenocarcinoma (n = 5), soft tissue sarcoma (n = 5), histiocytic sarcoma (n = 5), transitional cell carcinoma (n = 5), and anal sac gland carcinoma with metastatic disease (n = 5) specimens were studied by IHC.

Flow cytometry

To check the binding specificity of a recently established anti-PD-L1 mAb [Ikebuchi *et al*., 2014], flow cytometric analyses were performed as described in chapter I, with some modifications. In brief, canine melanoma cell lines, CMeC and CMM-1, which are PD-L1-negative under normal conditions, were transfected with pEGFP-N2 vector or pEGFP-N2-cPD-L1 vector using Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. One day after the transfection, cells were harvested and stained with anti-PD-L1 mAb 6G7-E1 or an isotype-matched control antibody (rat IgM) (BD Biosciences) at the final concentration of 10 μg/mL. After washing, the cells were incubated with an APC-conjugated anti-rat Ig antibody (Beckman Coulter) and analyzed by a FACSVerse flow cytometer (BD Biosciences). For transfected cells, EGFP-positive cells were gated and used for analysis.

Immunohistochemistry

Immunohistochemical staining was examined by the avidin-biotin peroxidase complex (ABC) procedure. Sections (4 μm) were dewaxed in xylene and hydrated through graded alcohols. To remove endogenous peroxidase, the sections were immersed in a 3% hydrogen peroxide solution at RT for 10 min. Following this, the sections were incubated with anti-PD-L1 mAb 6G7-E1 (10 μg/mL). After washing with PBS, the sections were incubated in a secondary antibody solution (biotin-labeled goat anti-rat IgM) (Jackson ImmnunoResearch Laboratories, West Grove, PA, USA) at RT for 30 min. Following the

incubation, the sections were reacted using the ABC in the VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) at RT for 30 min. Visualization was accomplished using a 0.05% 3,3′-diaminobenzidine solution. Mayer's hematoxylin stain was used as a counterstain. For negative controls, sections were subjected to the same procedures without the primary antibody or with rat IgM isotype control antibody (Acris Antibodies). The specimen was considered positive for PD-L1 if there was histological evidence of cell staining. To prepare the OS specimens, decalcification was performed using a standard protocol with Plank-Rychlo's solution. Berlin blue staining was performed to distinguish hemosiderin deposits from positive staining in HSA samples, when required.

RESULTS

Anti-PD-L1 mAb 6G7-E1 specifically binds to canine PD-L1

Among anti-PD-L1 mAbs established in our laboratory, 6G7-E1 showed sufficient staining in IHC of formalin-fixed and paraffin-embedded canine cancer sections. To test the specificity of 6G7-E1 for the detection of canine PD-L1, Western blot analysis was firstly performed using cPD-L1-Ig as a positive control protein. However, no binding was observed when 6G7-E1 was used as a primary antibody, while anti-rabbit IgG Fc antibody detected the specific band (data not shown). Because this result suggests that the binding of 6G7-E1 is conformation-specific, subsequent flow cytometric analysis was performed using canine melanoma cell lines CMeC and CMM-1. No binding was observed with non-transfected or mock (EGFP only)-transfected canine melanoma cells, while 6G7-E1 bound to cells transfected with canine PD-L1-EGFP (Figure II-1). These results indicated that 6G7-E1 specifically binds to canine PD-L1.

Canine malignant cancers express PD-L1

Immunohistochemical analysis was performed to detect PD-L1 expression in various malignant cancers. In total, 110 specimens were tested, among which 36 OMM, 7 OS, 6 HSA, 3 MCT (grade III), 4 mammary adenocarcinoma, and 3 prostate adenocarcinoma specimens were PD-L1 positive (Figures II-2 and II-3, and Table II-1). Both cytoplasmic and cell-surface staining were observed. No obvious staining was found with a negative control antibody (Figure II-4). The positive rates of PD-L1 expression were 90% (36/40) for OMM, 70% (7/10) for OS, 60% (6/10) for HSA, 60% (3/5) for grade III MCT, 80% (4/5) for mammary adenocarcinoma, and 60% (3/5) for prostate adenocarcinoma (Table II-1). The other 7 types of cancers, namely, squamous cell carcinoma, diffuse large B-cell lymphoma, nasal adenocarcinoma, soft tissue sarcoma, histiocytic sarcoma, transitional cell carcinoma, and anal sac gland carcinoma did not express PD-L1, although the tested sample size was substantially limited (5 cases for each tumor type, Table II-1).

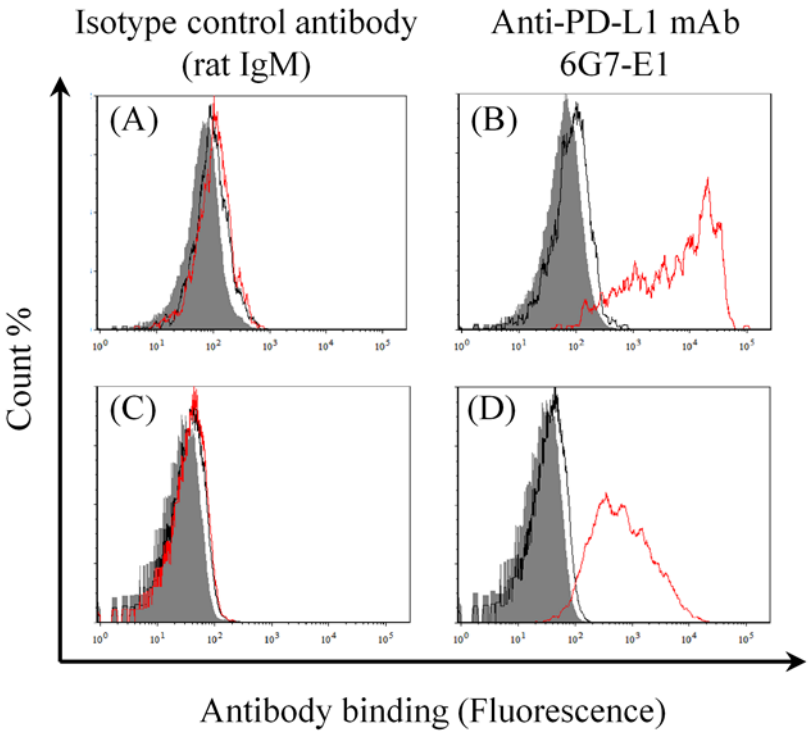

Figure II-1. Binding specificity of anti-PD-L1 mAb 6G7-E1. Canine melanoma cell lines, CMeC and CMM-1, were transfected with pEGFP-N2 vector (mock) or pEGFP-N2-cPD-L1, and antibody binding was analyzed by a flow cytometer. The shaded area, black line, and red line represent the results for the cells with no treatment, mock-transfected cells, and cPD-L1-EGFP-transfected cells, respectively. (A) Binding of the isotype control antibody to CMeC; (B) binding of 6G7-E1 to CMeC; (C) binding of the isotype control antibody to CMM-1; and (D) binding of 6G7-E1 to CMM-1 are shown in the histograms.

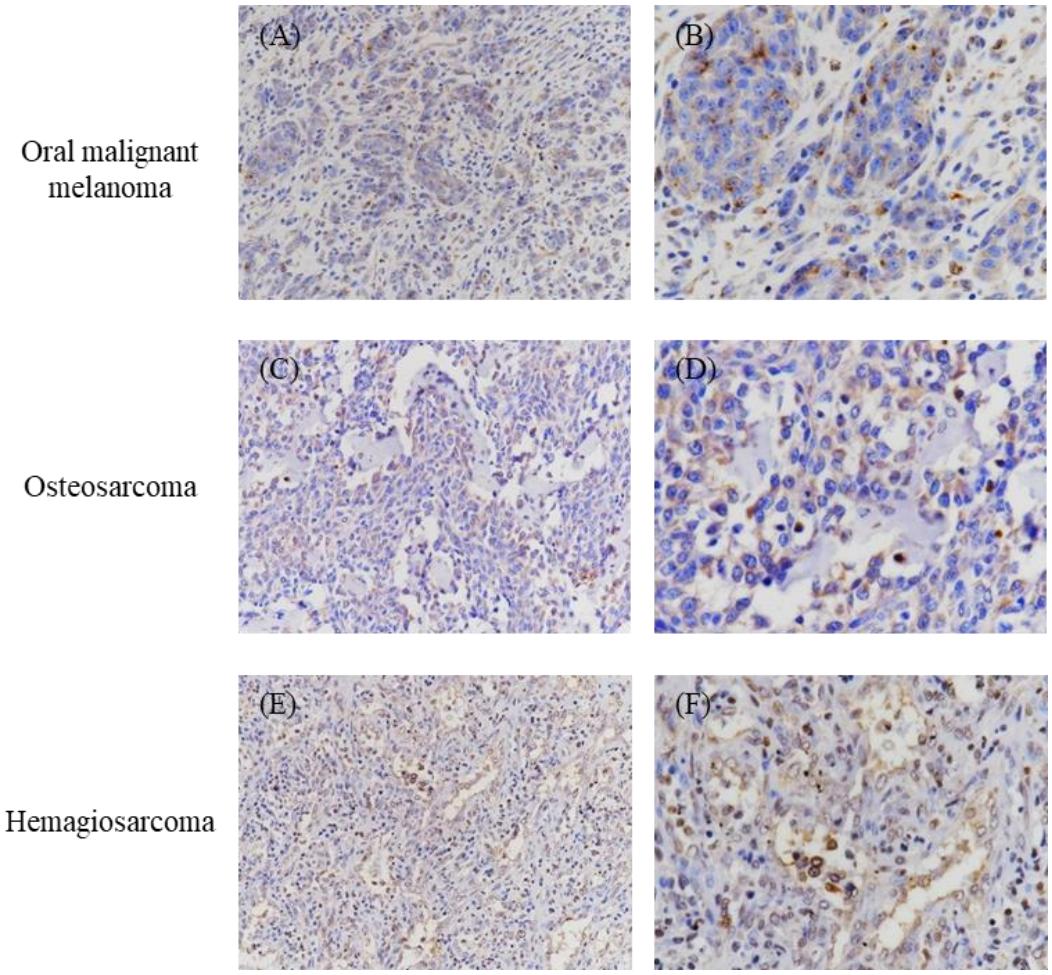

Figure II-2. Immunohistochemical analysis of PD-L1 in OMM, OS, and HSA. Formalin-fixed and paraffin-embedded tumor tissues were examined by IHC. The sections were stained with anti-PD-L1 mAb 6G7-E1. Representative positive staining of (A, B) OMM, (C, D) OS, and (E, F) HSA are shown. Original magnification: (A, C, E) 200×; (B, D, F) 400×.

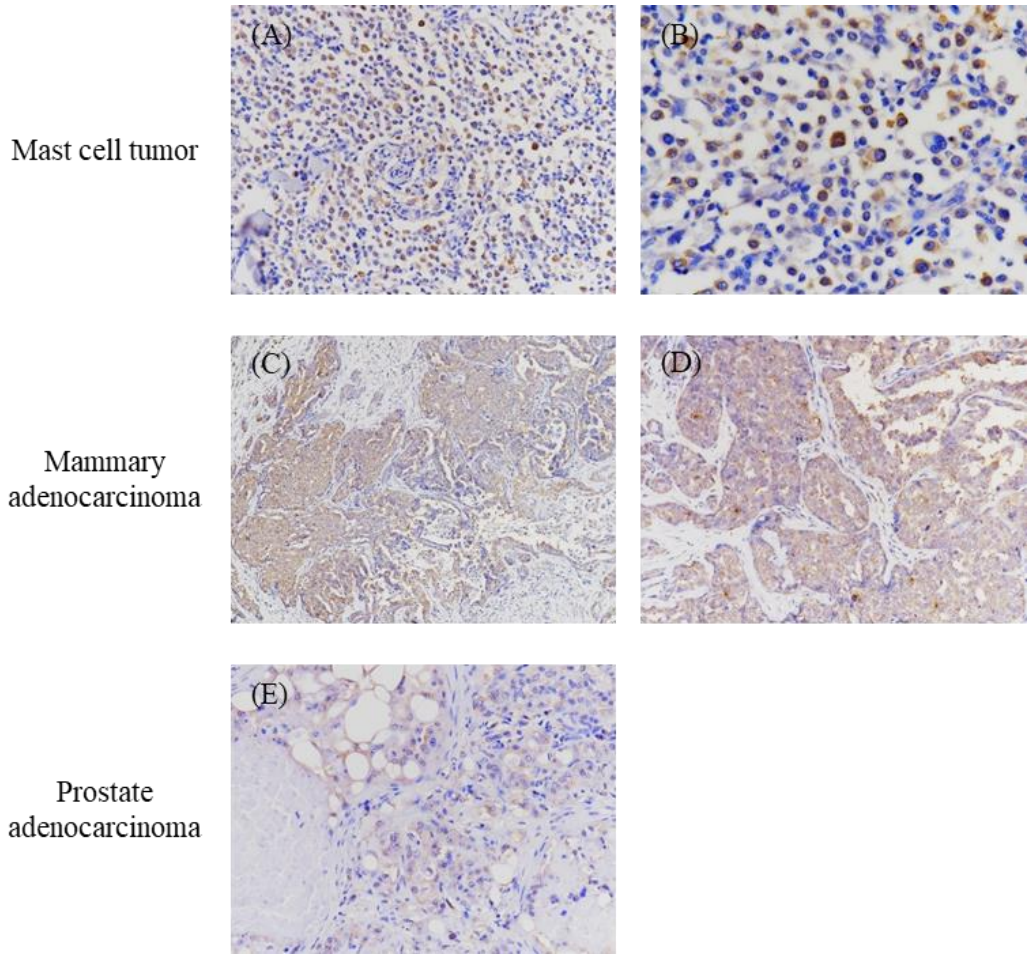

Figure II-3. Immunohistochemical analysis of PD-L1 in MCT, mammary adenocarcinoma, and prostate adenocarcinoma. Formalin-fixed and paraffin-embedded tumor tissues were examined by IHC. The sections were stained with anti-PD-L1 mAb 6G7-E1. Representative positive staining of (A, B) MCT, (C, D) mammary adenocarcinoma, and (E) prostate adenocarcinoma are shown. Original magnification: (A, D, E) 200×; (B) 400×; (C) 100×.

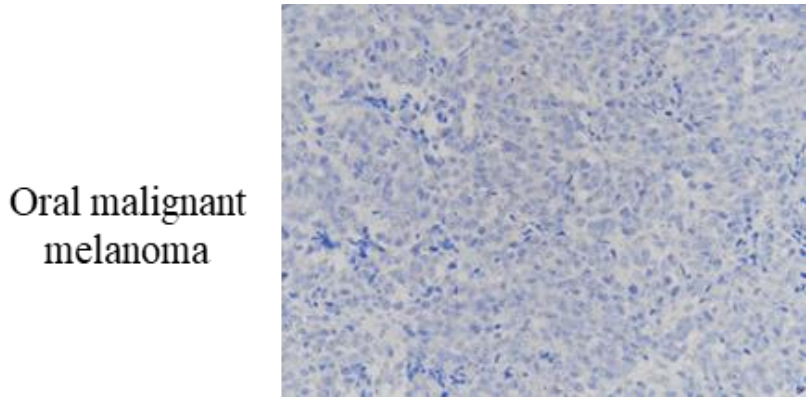

Figure II-4. Representative IHC with isotype-matched control antibody. OMM specimen was stained with rat IgM isotype control antibody. No staining was observed. Original magnification, 200×.

Table II-1. PD-L1 expression in various canine malignant cancers.

Pathology	Positive cases/Tested samples	% Positive
Malignant melanoma (oral)	36/40	90%
Osteosarcoma	7/10	70%
Hemangiosarcoma	6/10	60%
Mast cell tumor (grade III)*	3/5	60%
Mammary adenocarcinoma**	4/5	80%
Prostate adenocarcinoma	3/5	60%
Squamous cell carcinoma (skin)	0/5	0%
Diffuse large B-cell lymphoma	0/5	0%
Nasal adenocarcinoma	0/5	0%
Soft tissue sarcoma	0/5	0%
Histiocytic sarcoma	0/5	0%
Transitional cell carcinoma	0/5	0%
Anal sac gland carcinoma	0/5	0%

The results of immunohistochemical analysis were summarized. *Grading of mast cell tumor was performed in accordance with the Patnaik grading method [Patnaik *et al*., 1984]. **No inflammatory mammary carcinoma was included in this study.

DISCUSSION

Malignant melanoma is a relatively common type of tumor, which accounts for 7% of all malignant cancers and is the most common oral malignancy in dogs. Canine malignant melanoma can occur in haired skin, oral cavity, nail bed, foot pad, eye, or mucocutaneous junction [Smith *et al*., 2002]. Among these forms, OMM is considered highly intractable because it frequently causes severe local invasion and metastatic disease. Unfortunately, OMM is generally resistant to chemotherapy, making it difficult to treat metastatic lesions, which eventually kill diseased dogs [Murphy *et al*., 2005]. The absence of effective systemic therapy encourages the researchers to develop novel therapeutic modalities, including immunotherapies. In humans, immune checkpoint inhibitors such as anti-PD-1 and anti-PD-L1 antibodies have been used for the treatment of advanced melanoma, showing promising efficacies, with ORRs of 28% and 17%, respectively [Brahmer *et al*., 2012; Topalian *et al*., 2012]. In this study, as much as 90% (36/40) of OMM were found to express PD-L1, suggesting that the PD-1/PD-L1 axis is a major mechanism of immune evasion in OMM. This result is well consistent with the result described in chapter I, in which 100% (8/8) of OMM expressed PD-L1. Considering that malignant melanoma in dogs shares some similarities with human melanoma, inhibitors of the PD-1/PD-L1 axis could also provide therapeutic effects for canine OMM.

OS is the most common bone cancer in dogs, and is highly metastatic. With amputation alone, median survival time (MST) for dogs with OS is only 19 weeks, and approximately 90% of the dogs develop metastasis [Spodnick *et al*., 1992; Szewczyk *et al*., 2015]. Therefore, aggressive surgery in combination with systemic chemotherapy has been a standard treatment for canine OS, and new systemic therapies are needed to be investigated. In humans, anti-PD-L1 antibodies are expected to have therapeutic effects on OS because 75% (12/16) of metastatic OS showed PD-L1 expression. In a mouse model, an anti-PD-L1 antibody improved the function of cytotoxic T lymphocytes and increased the survival of tumor-bearing mice [Lussier *et al*., 2015]. Because 70% (7/10) of primary OS in dogs were found to express PD-L1, blocking antibodies may also have a therapeutic efficacy against canine OS. However, it may be important to note that, in contrast to its metastatic lesions, none of human primary OS expressed PD-L1 [Lussier *et al*., 2015], whereas canine primary OS expressed PD-L1 at a high rate. The difference in PD-L1 expression should be taken into careful consideration when extrapolating findings from human OS studies to canine OS, or vice versa.

Dogs have a relatively high risk for developing HSA [MacVean *et al*., 1978], which is a malignant cancer originating from vascular endothelial cells. Considering that canine

HSA is highly metastatic, adjuvant chemotherapy remains important although it can provide only a modest benefit in survival times [Gregory *et al*., 1996]. Because angiosarcoma in humans is a rare disease, reports on PD-L1 expression in angiosarcoma have been limited [Kim *et al*., 2013; D'Angelo *et al*., 2015] and its therapeutic implication has not been well studied. Because PD-L1 expression was found in 60% (6/10) of canine HSA samples, it is worth investigating the therapeutic potential of PD-1/PD-L1 inhibitors as novel systemic immunotherapies for canine HSA. As for PD-L1 expression, HSA may share the characteristics of its originating vascular endothelial cells, because vascular endothelial cells express PD-L1 in response to several cytokines such as IFNs [Eppihimer *et al*., 2002; Rodig *et al*., 2003]. It may be reasonable to suggest that HSA utilizes the PD-1/PD-L1 axis as an immune evasion mechanism because PD-L1 on vascular endothelial cells has been reported to negatively regulate $CD8^+$ T-cell responses, including IFN-γ production and cytolytic activity [Rodig *et al*., 2003].

MCT is the most common cutaneous cancer in dogs, accounting for up to 21% of all canine skin neoplasms [Welle *et al*., 2008]. Although the clinical behavior of MCT can vary, histologic grading [Patnaik *et al.*, 1984] is widely used and considered a reliable prognostic factor for dogs with MCT. According to a previous report, for high-grade or undifferentiated MCT, MST is only 13 weeks [Bostock *et al*., 1989] and the metastatic rate is as high as 56 to 96%. In this study, high-grade (Patnaik grade III) MCT was analyzed for PD-L1 expression, and 60% (3/5) of the samples were positively stained. This result is also well consistent with the result described in chapter I, in which 66.7% (4/6) of grade III MCTs showed PD-L1 expression. Although some cytotoxic chemotherapy drugs and tyrosine kinase inhibitors, such as toceranib, have been used to treat MCT systemically [London *et al*., 2003], immune checkpoint inhibitors targeting the PD-1/PD-L1 axis may also have the potential to be included in the treatment because such chemotherapies are sometimes insufficient because of the emergence of drug resistance.

Mammary gland tumors are the most common neoplasms in sexually intact female dogs, accounting for 70% of all cancer cases in this subpopulation [Merlo *et al*., 2008]. Among malignant mammary tumors, mammary adenocarcinoma represents a common histopathology, and dogs with mammary adenocarcinoma treated by surgery alone sometimes suffer from a recurrence and/or metastasis. Chemotherapy has some benefits for mammary malignancies; however, in many cases it is not curative, and alternative or additional systemic therapies are needed to be explored. In humans, PD-L1 expression in tumor cells was observed in 34% (15/44) of patients with breast cancer, and its expression was significantly correlated with some important prognostic factors such as the

expression of hormone receptors [Ghebeh *et al*., 2006]. In dogs, 80% (4/5) of the mammary adenocarcinoma samples expressed PD-L1, although its association with the prognosis remains unclear. However, it is worth to evaluate the therapeutic potential of PD-1/PD-L1 inhibitors as novel systemic therapies against this cancer.

Prostate adenocarcinoma is a relatively uncommon disease in male dogs. Although the incidence is low, prostate adenocarcinoma often arises as a clinical problem because local progression and metastases are the common sequelae. At the time of diagnosis, regional or distant metastasis are often found, and MSTs for dogs with no treatment are considered less than a month [Sorenmo *et al*., 2004]. To date, no consensus for the standard of care has been achieved, and conventional treatments such as surgery, radiation, and chemotherapy are palliative but not curative in most dogs. In humans, 50% (8/16) of castration-resistant prostate cancer (CRPC) cases were reported to express PD-L1 [Massari *et al*., 2015]; however, none of CRPC patients (n = 17) who received an anti-PD-1 mAb had objective response in a clinical trial [Topalian *et al*., 2012]. No response in the CRPC patients is possibly due to the absence of PD-L1 expression in the tumor tissues, although only a few samples from the enrolled patients (4/17) were examined and found to be PD-L1-negative [Topalian *et al*., 2012; Taube *et al*., 2014]. In this study, PD-L1 was expressed in 60% (3/5) of the canine prostate adenocarcinoma samples, but its therapeutic implications remain obscure. Further studies are needed to clarify the clinical benefit of PD-1/PD-L1 inhibitors for this tragic cancer.

The other cancer types tested in this study did not show any PD-L1 expression. The absence of PD-L1 expression in these tumors may be due to the sensitivity of anti-PD-L1 mAb, differences in the origin of tumor cells, and/or immunological properties of tumor microenvironment of each patient. Because the sample size tested in this study was very small, further analysis should be performed before making a conclusion on the expression status of PD-L1 in those cancers. Recently, mechanisms of PD-L1 upregulation in human cancers have been extensively investigated. One of the mechanisms for inducing PD-L1 expression in tumor cells is in response to cytokines including IFN-γ [Dong *et al*., 2002], which are likely produced by immune cells in the tumor microenvironment during antitumor immune responses. Another mechanism, for example, is associated with signaling from a well-defined tumor suppressor, phosphatase and tensin homolog (PTEN). The loss of the PTEN function has been reported to upregulate PD-L1 expression in glioma and triple-negative breast cancer cells [Parsa *et al*., 2007; Mittendorf *et al*., 2014]. In dogs, mutations or loss of PTEN were also reported in melanoma, OS, HSA, and malignant mammary gland tumors [Koenig *et al*., 2002; Levine *et al*., 2002; Dickerson *et al*., 2005; Kanae *et al*., 2006]. This may contribute, at least in part, to the aberrant PD-L1

expression in these cancers in dogs.

To the best of my knowledge, this is the first report of PD-L1 expression in various types of canine malignant cancers. Canine cancers with PD-L1 expression shown in this chapter may represent the candidate cancers that could respond to PD-1/PD-L1 inhibitors in future clinical studies. However, in humans, PD-L1 IHC alone is not considered a clear-cut predictive biomarker for clinical efficacy of PD-1/PD-L1 inhibiting-mAbs, because as much as 41% of patients with PD-L1-negative melanoma still did respond to anti-PD-1 mAb therapy in another clinical study [Larkin *et al*., 2015]. This is possibly due to the sensitivity of anti-PD-L1 antibody used in the IHC, timing of tissue sample collection, cutoff criteria for PD-L1 positivity, and/or induction of PD-L1 during the therapy as adaptive resistance [Patel and Kurzrock, 2015]. Further studies are needed to clarify precise biomarkers that can predict treatment outcome in dogs along with the clinical studies using PD-1/PD-L1 inhibitors.

SUMMARY

Spontaneous cancers are common cause of death in dogs. The standards of care for canine malignant cancers include surgery, radiation and chemotherapy; however, novel treatment options are needed to be established because effective systemic therapy is not available for most cancer types. In chapter I, therapeutic potential of PD-L1 blockade as a novel immunotherapy was discussed in limited cancer types. In this chapter, to clarify the cancer types which could respond to the PD-L1 blockade, immunohistochemical analysis of PD-L1 expression was extensively performed in various types of malignant cancers in dogs.

Among 13 cancer types, PD-L1 expression was found in OMM, OS, HSA, MCT, mammary adenocarcinoma, and prostate adenocarcinoma, with positive rates of 90% (36/40), 70% (7/10), 60% (6/10), 60% (3/5). 80% (4/5), and 60% (3/5), respectively. The other 7 types of cancers, including squamous cell carcinoma, diffuse large B-cell lymphoma, nasal adenocarcinoma, soft tissue sarcoma, histiocytic sarcoma, transitional cell carcinoma, and anal sac gland carcinoma did not express PD-L1, although the tested sample size was small (n = 5 for each cancer type). Nonetheless, it is worth investigating the therapeutic efficacy of PD-L1 blockade in these PD-L1-expressing cancer types in future clinical studies.

CHAPTER III

Establishment of a canine chimeric monoclonal antibody targeting PD-L1 as a novel biological drug for the treatment of canine malignant cancers

INTRODUCTION

Recently, many attempts have been made to find new options for canine cancer treatment. In addition to surgery, radiation, and chemotherapy, some molecular-targeted therapies including tyrosine kinase inhibitors are available for the treatment of a limited number of cancer types including MCT [London *et al*., 2003]. In addition to those, some candidate therapeutic antibodies have been developed and tested for their efficacy *in vitro* and *in vivo* in the last 3 years [Singer *et al*., 2014; Rue *et al*., 2015]. In chapters I and II, therapeutic potential of an anti-PD-L1 mAb, which was established in our laboratory, has been demonstrated in several types of canine malignant cancers. The next attempt should be an evaluation of antitumor effect of this mAb in dogs with spontaneous cancers.

When preparing therapeutic mAb for humans, four major types of antibody have been developed and tested for their efficacy and safety. The first type is murine mAb, which is xenogeneic protein for humans. As expected, murine mAb induces human anti-murine antibody response, leading to the decrease of the therapeutic effect [Kuus-Reichel *et al*., 1994]. Moreover, murine mAb does not have efficient ability to trigger human effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC). To reduce the immunogenicity of murine mAb and to provide it effector functions which depend on the constant region of mAb, murine-human chimeric mAb has been developed using genetic engineering techniques. Chimeric mAb consists of murine variable regions and human constant regions, and is approximately 75% human IgG. Because the immunogenicity of mAb largely depends on the constant regions [Brüggemann *et al*., 1989], this chimeric mAb is expected to be much less immunogenic. There are some chimeric mAbs that have been widely used as biologics in human medicine, including rituximab (anti-CD20 mAb), cetuximab (mAb against epidermal growth factor receptor), and infliximab (mAb against tumor necrosis factor receptor), with sufficient efficacy and tolerable side effect profiles [Maloney *et al*., 1997; Targan *et al*., 1997; Saltz *et al*., 2004]. The other two types include humanized mAb, in which only complementarity-determining regions of murine mAb are grafted onto human IgG framework, and fully humanized mAb, which can be generated through the expression of human antibody genes in genetically modified mice, phage, or yeast. The latter two types are technically difficult to apply to the preparation of therapeutic mAb for dogs, because the comprehensive conformational analysis of canine antibody, or specialized expression system for canine antibody genes are needed. Therefore, in this study, a canine chimeric mAb against PD-L1 was prepared using the variable regions of rat anti-PD-L1 mAb to perform a clinical study in dogs.

For therapeutic anti-PD-L1 mAb, ADCC and CDC activities are not required for the mode of action. Rather, these effector functions may cause depletion of immune cells because PD-L1 can also be expressed on activated T cells, B cells, macrophages and dendritic cells [Keir *et al*., 2008]. In dogs, four IgG subclasses are identified and characterized for their effector functions. Among them, canine IgGD is reported to be equivalent to human IgG4 with minimum ADCC activity and complement binding [Tang *et al*., 2001; Bergeron *et al*., 2014]. Therefore, IgGD was selected as the source of constant region for heavy chain of canine chimeric anti-PD-L1 mAb. As for the light chain, canine lambda chain was chosen as the source of constant region because, in dogs, lambda chains are considered more abundant than kappa chains [Sandusky *et al*., 1987; Arun *et al*., 1996].

In this chapter, the blocking effect of rat anti-PD-L1 mAbs were compared using recombinant canine PD-1 and PD-L1 proteins. Then chimeric mAbs were designed and produced in a transient expression system, followed by further comparison of resulting chimeric mAbs. To prepare a large amount of chimeric mAb for a clinical study, a stable producer cell line for the candidate chimeric mAb was established, and the product was tested for immunostimulatory effects on canine PBMCs *in vitro*.

MATERIALS AND METHODS

Animals

Animal use throughout this study was approved by the Institutional Animal Care and Use Committee, Hokkaido University (Approval number: 15-0149) and the Faculty of Veterinary Medicine, Hokkaido University (Approval number: 15028). All experiments were performed in accordance with relevant guidelines and regulations of the Faculty of Veterinary Medicine, Hokkaido University, which has been fully accredited by the AAALAC International. Peripheral blood samples of clinically healthy dogs were collected from 1- to 3-year-old beagles kept at the Experimental Animal Facility, Faculty of Veterinary Medicine, Hokkaido University.

Preparation of canine chimeric mAb by a mammalian transient expression system

The method for rapid amplification of cDNA ends was performed to determine the nucleotide sequence of rat anti-PD-L1 mAb 4G12 or the 6G7 [Ikebuchi *et al*., 2014] variable region using a 5′-RACE system (Life Technologies) following the manufacturer's instruction. Total RNA was extracted from the hybridoma 4G12 or 6G7 and the following rat immunoglobulin gene-specific primers were used in PCR: 5′-ACA AGG ATT GCA TTC CCT TGG-3′ and 5′-CTC AAT TTT CTT GTC CAC CTT GGT GC-3′ for 4G12 heavy chain; 5′-CAC ATT GGG GTT CAT CTC AAC-3′ and 5′-TGG AAT CGG CAC ATG CAG ATC T-3′ for 6G7 heavy chain; 5′-CTC ATT CCT GTT GAA GCT CTT GAC GAC-3′ and 5′-CTC ATT CCT GTT GAA GCT CTT GAC GAC GGG-3′ for 4G12 and 6G7 light chains. The amplicons were analyzed with a CEQ8000 DNA analysis system (Beckman Coulter) as described in chapter I. The nucleotide sequences of the light and heavy chain variable region were combined with those of canine lambda chain constant region (Genbank accession number E02824) and canine IgGD constant region (Genbank accession number AF354267), respectively, using Mega 5 software [Tamura *et al*., 2011]. The resulting sequences were modified according to the optimal codon usage for the expression in CHO cells (except for the signal peptide sequences, which were optimized for the expression in *Pichia pastoris*), synthesized (Medical & Biological Laboratories, Aichi, Japan), and cloned into pDC6 expression vector (kindly provided by Dr. Y. Suzuki, Research Center for Zoonosis Control, Hokkaido University). The expression vector was named pDC6-c4G12 (canine chimeric 4G12) or pDC6-c6G7 (canine chimeric 6G7).

The expression vector for c4G12 or c6G7 were transfected into Expi293F cells (Life

Technologies) and chimeric mAbs were produced according to the manufacturer's instructions. Culture supernatant was harvested on days 2 and 7. Chimeric mAbs were purified from culture supernatant by affinity chromatography using Ab-Capcher Extra (Protenova), and the buffers were exchanged with PBS using PD-MidiTrap G-25 (GE Healthcare). Concentrations of chimeric mAbs were measured by a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific). To confirm the expression and purification of chimeric mAbs, SDS-PAGE was performed in a non-reducing condition using 10% acrylamide gel and 2× Laemmli Sample Buffer (Bio-Rad). Precision Plus Protein All Blue Standards (Bio-Rad) was used as a molecular-weight size marker and proteins were visualized by coomassie brilliant blue (CBB) staining with Quick-CBB (Wako, Osaka, Japan). Purities of chimeric mAbs were evaluated by densitometry using CS Analyzer version 3.0 software (Atto, Tokyo, Japan). For further purification, gel filtration chromatography was performed using a Hiload 16/60 Superdex 200 pg (GE Healthcare) prepacked column and an ÄKTAexplorer FPLC system (Amersham Biosciences, Piscataway, NJ, USA). Rat mAb 4G12 and dog IgG (Jackson ImmunoResearch Laboratories) were used as control proteins.

Blocking assay of PD-1/PD-L1 binding and CD80/PD-L1 binding

To evaluate the ability of anti-PD-L1 mAb to block PD-1/PD-L1 binding, a blocking assay was conducted on a microwell plate using recombinant canine PD-1 and PD-L1 proteins (cPD-1-Ig and cPD-L1-Ig). The expression vector for cPD-1-Ig or cPD-L1-Ig (pCXN2.1-rabbit IgG Fc-cPD-1 or pCXN-2.1-rabbit IgG Fc-cPD-L1; described in chapter I) was introduced into Expi293F cells (Life technologies) and the recombinant proteins were expressed according to the manufacturer's instructions. cPD-1-Ig and cPD-L1-Ig were purified from culture supernatant harvested on days 2 and day 7 using Ab-Capcher Extra (Protenova), and the buffers were exchanged with PBS using PD-MidiTrap G-25 (GE Healthcare). Concentration of cPD-1-Ig or cPD-L1-Ig was measured by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). cPD-L1-Ig was biotinylated using Lightning-Link Biotin Conjugation Kit (Innova Biosciences, Cambridge, UK). A Maxisorp flat bottom microwell plate (Thermo Fisher Scientific) was coated with cPD-1-Ig and blocked with PBS containing 1% bovine serum albumin (Sigma-Aldrich) and 0.05% Tween20 (Kanto Chemical, Tokyo, Japan). Biotinylated cPD-L1-Ig was preincubated with anti-PD-L1 mAb 4G12, 5A2, 6G7, c4G12 or c6G7 at various concentrations (0, 2.5, 5, 10 μg/mL) for 30 min at 37 °C and added to the plate. cPD-L1-Ig binding was detected using Neutravidin-horseradish peroxidase (Thermo Fisher Scientific) and TMB one component substrate (Bethyl Laboratories). The reaction was

stopped by adding 0.18 M H_2SO_4, and the optical density (OD) at 450 nm was measured by a microplate reader MTP-900 (Corona Electric, Ibaraki, Japan). Relative OD (% OD) was calculated from the OD in comparison with that of control without antibody (0 μg/mL). Rat IgG (Sigma-Aldrich), rat IgM (BD Biosciences), or dog IgG (Jackson ImmunoResearch Laboratories) was used as negative control. Statistical analysis was performed by Tukey's test among groups treated with 10 μg/mL of antibody. A *p* value of less than 0.05 was considered statistically significant.

For the preparation of recombinant canine CD80 protein (cCD80) fused to rabbit IgG Fc (cCD80-Ig), the nucleotide sequence encoding the putative extracellular region of cCD80 was amplified by PCR using cDNA obtained from beagle PBMCs stimulated with 20 ng/mL PMA (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) and gene-specific primers (5′-CGC GGA TAT CAT GGA TTA CAC AGC GAA GTG-3′ and 5′-CGG GGT ACC CCA GAG CTG TTG CTG GTT AT-3′). The amplicon was then cloned into the multicloning site of pCXN-2.1-rabbit IgG Fc vector (kindly provided by Dr T. Yokomizo, Graduate School of Medicine, Juntendo University; modified) [Zettlmeissl *et al*., 1990; Niwa *et al*., 1991] using *Eco*RV and *Kpn*I restriction enzyme sites. The resulting expression vector, pCXN2.1-rabbit IgG Fc-cCD80, was introduced into Expi293F cells (Life Technologies) and the recombinant protein was expressed according to the manufacturer's instructions. cCD80-Ig was purified, and the ability of anti-PD-L1 mAb to block CD80/PD-L1 binding was evaluated similarly as described for the PD-1/PD-L1 binding.

Establishment of stable producer cell lines for c4G12

For stable expression of chimeric mAb, pDC6-c4G12 was introduced into dihydrofolate reductase (dhfr)-deficient CHO-DG44 cells (Life Technologies) using Lipofectamine LTX Reagent (Life Technologies) following the manufacturer's instructions. Stable producer cells were selected in CD OptiCHO medium (Life Technologies) supplemented with 2 mM GlutaMAX I (Life Technologies). Cloning of expressing cells was performed following a standard protocol of limiting dilution. Gene amplification was performed by the dhfr/methotrexate (MTX) method using CD OptiCHO medium supplemented with 2 mM GlutaMAX I and 60 nM MTX (Alexis Biochemicals, San Diego, CA, USA) in combination with further cell cloning. The culture supernatant of the expressing cells was harvested on day 14 of shaking culture (37°C, 5% or 8% CO_2, 125 rpm) in CD OptiCHO medium or in Dynamis medium (Life Technologies) supplemented with 2 mM GlutaMAX I. For the culture in Dynamis medium, 4 g/L, 4 g/L, and 6 g/L glucose was added on days 3, 5, and 7, respectively, and

3.3% v/v EfficientFeed B+ (Life Technologies) reconstituted at 3× concentration was added on days 3, 5, 7, and 10.

The concentration of c4G12 in the culture supernatant was measured by a sandwich ELISA. Goat anti-dog IgG1antibody (Bethyl Laboratories) was coated on a 96 well plate and the captured chimeric mAb was detected by goat anti-dog light chain antibody (Bethyl Laboratories) conjugated with HRP and TMB substrate as described above. HRP conjugation was performed by using Lightning Link HRP conjugation kit (Innova Biosciences).

Purification of chimeric mAb from the culture supernatant was performed by affinity chromatography using Ab-Capcher Extra (Protenova), and the buffer was exchanged with PBS by ultrafiltration using Amicon Ultra-15 Ultracel-50 (Merck Millipore). The concentration of c4G12 was measured by Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific). To confirm the expression and purification of c4G12, SDS-PAGE was performed in reducing or non-reducing conditions as described above. For SDS-PAGE analysis, rat anti-PD-L1 mAb 4G12 was used as the control protein.

Measurement of binding avidity to canine PD-L1

To assess binding avidity of 4G12, c4G12, cPD-1-Ig, and cCD80-Ig to canine PD-L1, surface plasmon resonance (SPR) analysis was performed using polyhistidine-tagged canine PD-L1 protein (cPD-L1-His). For the preparation of cPD-L1-His, the nucleotide sequence of the putative extracellular region of canine PD-L1 was amplified by PCR as described in chapter I, except for the use of gene-specific primers containing the C-terminal 6× polyhistidine-tag-encoding sequence (5′-CGC GGC TAG CAT GAG AAT GTT TAG TGT CTT-3′ and 5′-CGC GGA TAT CTT AAT GGT GAT GGT GAT GGT GAG TCC TCT CAC TTG CTG G-3′). The amplicon was then cloned into the multicloning site of pCXN-2.1 vector (kindly provided by Dr T. Yokomizo, Graduate School of Medicine, Juntendo University) [Niwa *et al*., 1991] using *Nhe*I and *Eco*RV restriction enzyme sites. The resulting expression vector, named pCXN2.1-cPD-L1-His, was introduced into Expi293F cells (Life Technologies) and the recombinant protein was expressed according to the manufacturer's instructions. cPD-L1-His was purified from culture supernatant harvested on days 2 and 7 using TALON Metal Affinity Resin (Clontech), and the buffer was exchanged with PBS by ultrafiltration using Amicon Ultra-4 Ultracel-3 (Merck Millipore). The concentration of cPD-L1-His was measured with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

SPR analysis was performed using Biacore X100 system with a CM5 sensor chip and His Capture Kit (GE Healthcare). Anti-histidine antibody was immobilized on the sensor

chip by amine coupling following the manufacturer's instructions. cPD-L1-His was captured on the sensor chip to obtain approximately 35 response units (RU) to analyze 4G12 or c4G12 binding, or approximately 400 RU to analyze cPD-1-Ig or cCD80-Ig binding. HBS-EP+ (GE Healthcare) was used for both the running and dilution buffers. Control run responses containing buffer only were subtracted to obtain specific binding responses. The kinetic constants of 4G12 or c4G12 were determined by fitting with the 1:1 kinetic binding model, and that of cPD-1-Ig or cCD80-Ig was determined by fitting with the two state reaction model because a conformational change of PD-L1 upon binding with PD-1 and CD80 has been suggested [Ghiotto *et al*., 2010].

***In vitro* functional assay of anti-PD-L1 chimeric mAb**

PBMCs were prepared from healthy beagles and cultured as described in chapter I, in the presence of 5 μg/mL staphylococcal enterotoxin B (SEB, Sigma-Aldrich) and 20 μg/mL c4G12. Dog IgG (Jackson ImmunoResearch Laboratories) was used as a control antibody. For cytokine assays, the culture supernatant was harvested on day 3 and the concentrations of interleukin (IL)-2 and IFN-γ were measured by Duoset ELISA canine IL-2 and IFN-γ (R&D systems), respectively, according to the manufacturer's instructions. For the proliferation assay, incorporation of thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) was evaluated using a Click-iT Plus EdU flow cytometry assay kit (Life Technologies). EdU was added to the culture medium at the final concentration of 10 μM on day 2, and cells were harvested after additional incubation for 2 h. Cells were stained with optimal concentrations of anti-canine CD4 antibody (R&D systems) conjugated with peridinin-chlorophyll-protein complex-cyanin 5.5 using a Lightning-Link Antibody Labeling Kit (Innova Biosciences) and anti-canine CD8 antibody (LifeSpan BioSciences, Seattle, WA, USA) labelled with R-PE using a Zenon Mouse IgG2a Labeling Kit (Thermo Fisher Scientific). After washing, incorporated EdU was labelled with Alexa Fluor 647 following the manufacturer's instructions. Fluorescence of the cells was analyzed with a FACS Verse flow cytometer (BD Biosciences). For the analysis, the lymphocyte population was gated using FSC and SSC, and the EdU incorporation in $CD4^+$ or $CD8^+$ cells was evaluated within the lymphocyte population. A Wilcoxon signed rank-sum test was performed to compare data obtained from the same individuals. The result was considered statistically significant if the p value was less than 0.05.

RESULTS

Selection of anti-PD-L1 mAb

To prepare therapeutic antibody for canine cancers, three different rat anti-PD-L1 mAbs, that were recently established in our laboratory, were compared for their therapeutic potential. In the blocking assay of canine PD-1/PD-L1 binding, 6G7 (rat IgM) and 4G12 (rat IgG2a) showed efficient blocking ability, while 5A2 (rat IgG1) did not have an apparent blocking effect (Figure III-1A). To reduce immunogenicity, rat mAb was canine-chimerized prior to clinical use (Figure III-1B). For this purpose, IgM antibody was converted to IgG in our chimeric mAb expression system, although the conversion between antibody classes may weaken the binding avidity and blocking ability. To assess this possibility, canine chimeric mAb, c4G12 or c6G7, was prepared using the variable regions of 4G12 or 6G7 and the constant regions of canine IgGD and the lambda chain. They were produced in a mammalian cell-based transient expression system and purified by affinity chromatography using protein A derivative. The purities of these proteins were evaluated by SDS-PAGE and found to be < 80%, thus they underwent further purification by gel filtration chromatography to obtain > 90% purities (Figure III-1C). In the blocking assay of PD-1/PD-L1 binding, the blocking ability of c6G7 was weakened, whereas that of c4G12 remained sufficient throughout the conversion to canine chimeric IgG form (Figure III-1D). Therefore, c4G12 was selected as the therapeutic candidate and used for the following experiments.

c4G12 had similar binding and blocking properties to its original rat mAb

For stable expression of c4G12, CHO-DG44 cells were transfected with the c4G12 expression vector and high-producer cell clones were established by the dhfr/MTX method. The best producer cell line selected in this study produced as much as approximately 700 mg/L of chimeric mAb in the culture supernatant in 14 days of shaking culture, along with preferable cell growth and survival (Figure III-2). The expression and purification of c4G12 was confirmed by SDS-PAGE analysis (Figure III-3A). The purity was routinely > 90%, thus no further purification was needed. Because CD80/PD-L1 interaction also inhibits T-cell responses [Butte *et al*., 2007], the blocking ability of c4G12 was tested in both PD-1/PD-L1 binding and CD80/PD-L1 binding assays. c4G12 significantly blocked the binding of recombinant cPD-L1 to both cPD-1 (Figure III-3B) and cCD80 (Figure III-3C), and possessed comparable blocking ability to its original rat mAb 4G12. In the SPR analysis, c4G12 was bound to cPD-L1 with a similar binding property {equilibrium dissociation constant (K_D) = 2.30 ± 0.07 nM, Table III-1} to 4G12.

The K_D value of c4G12 was 10-fold smaller than that of cPD-1-Ig and cCD80-Ig, indicating that the binding avidity of c4G12 was practically higher than those of cPD-1-Ig and cCD80-Ig (Table III-1). This result was consistent with the observation that c4G12 effectively blocked PD-1/PD-L1 and CD80/PD-L1 binding.

Cytokine production and lymphocyte proliferation were enhanced by c4G12

To assess the immunostimulatory effect of c4G12, canine PBMCs were stimulated with a superantigen, SEB, in the presence of c4G12. Cell proliferation and cytokine production were evaluated on days 2 and 3, respectively. Treatment with c4G12 significantly enhanced IL-2 and IFN-γ production from canine PBMCs (Figures III-4A and B). The proliferation of $CD4^+$ and $CD8^+$ lymphocytes, indicated by the incorporation of nucleotide analogue EdU, was also enhanced by the c4G12 treatment (Figures III-4C and D). Taken together, c4G12 appeared to restore the effector functions of canine lymphocytes which are suppressed via the PD-1/PD-L1 and/or CD80/PD-L1 axis.

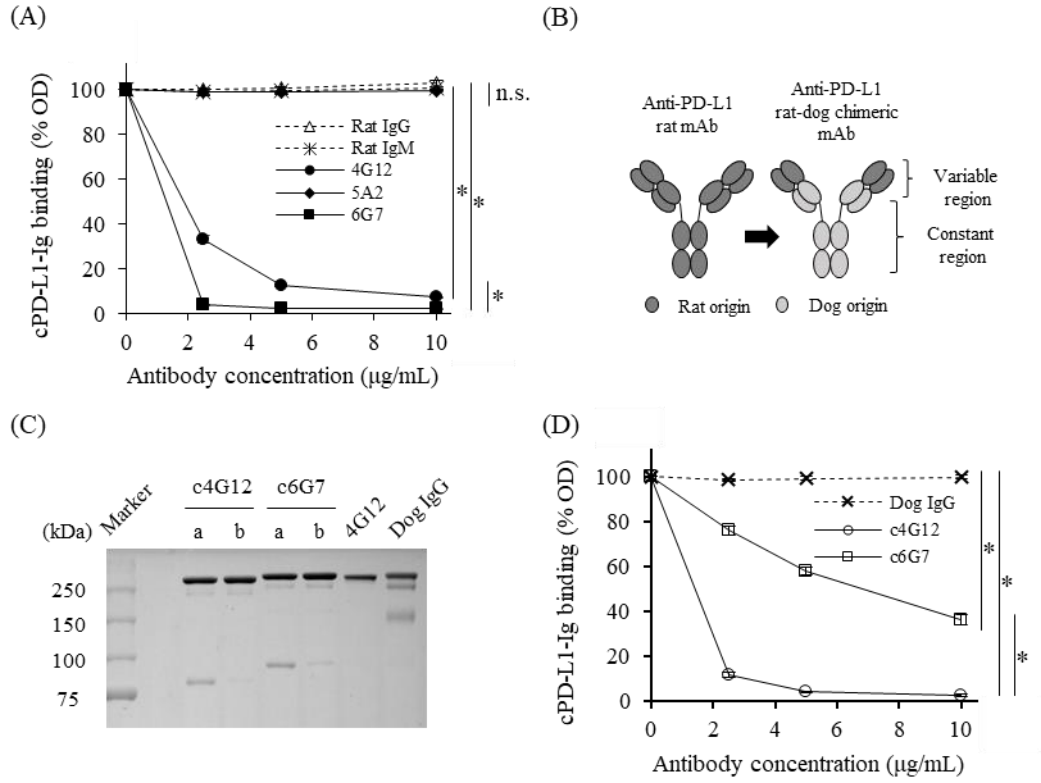

Figure III-1. Selection of anti-PD-L1 mAb for the preparation of canine chimeric mAb. (A) Blocking effect of anti-PD-L1 mAbs. cPD-1-Ig was coated on a microwell plate and the binding of cPD-L1-Ig, which had been preincubated with various concentrations of anti-PD-L1 mAbs 4G12, 5A2, and 6G7, was detected on the plate. Rat IgG and rat IgM were used as control antibodies. (B) Schematic image of rat mAb and canine chimeric mAb. (C) Expression and purification of canine chimeric 4G12 (c4G12) and c6G7. Chimeric mAbs were expressed using Expi293F cells and purified from culture supernatant by a) protein A derivative or b) protein A derivative plus gel filtration chromatography. SDS-PAGE and CBB staining were performed. Rat mAb 4G12 and dog IgG were used as control proteins. (D) Blocking effects of chimeric mAbs c4G12 and c6G7. cPD-L1 binding to cPD-1-Ig was assessed similarly after preincubation with chimeric mAbs. Dog IgG was used as a control antibody. Each point represents a mean value of relative OD (%) obtained from three independent experiments. Error bar; SE. Statistical analysis was performed by Tukey's test. $*p < 0.05$; n.s., not significant.

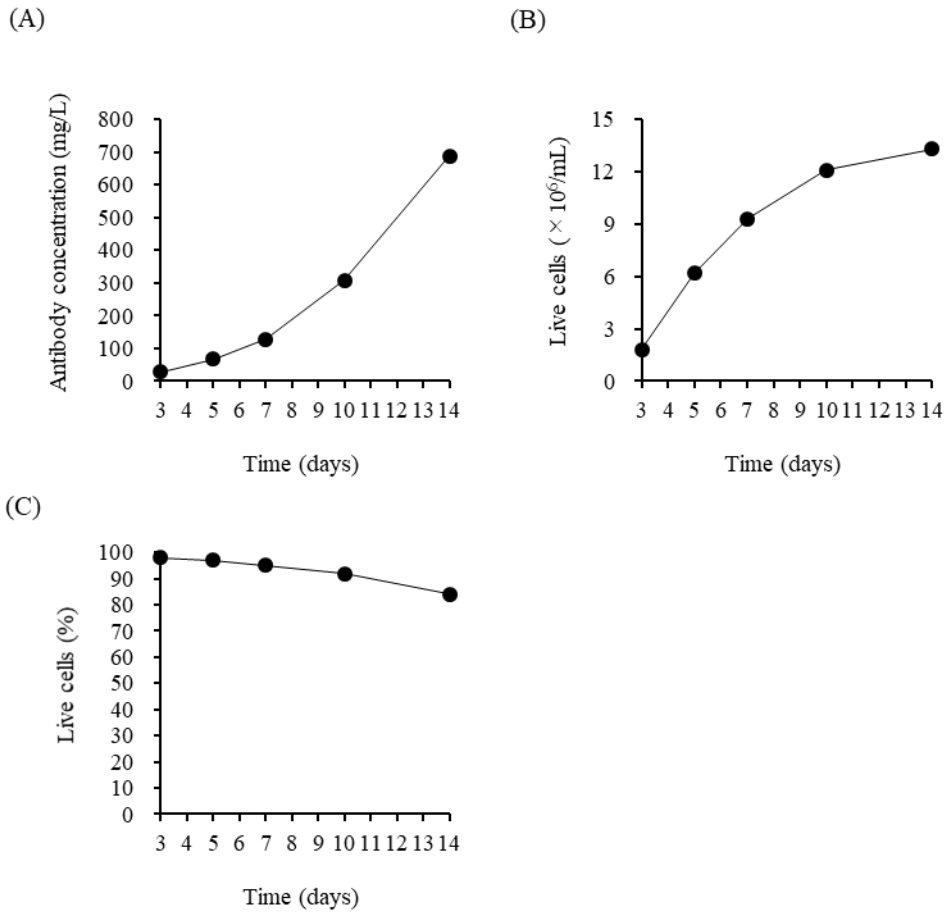

Figure III-2. Production of c4G12 by a stable producer cell line. A CHO-DG44 cell line which stably produces c4G12 was cultured in Dynamis medium with appropriate feeding on days 3, 5, 7, and 10. (A) Chimeric mAb production in the culture supernatant, (B) live cell count, and (C) percentage of live cells in 14 days of shaking culture.

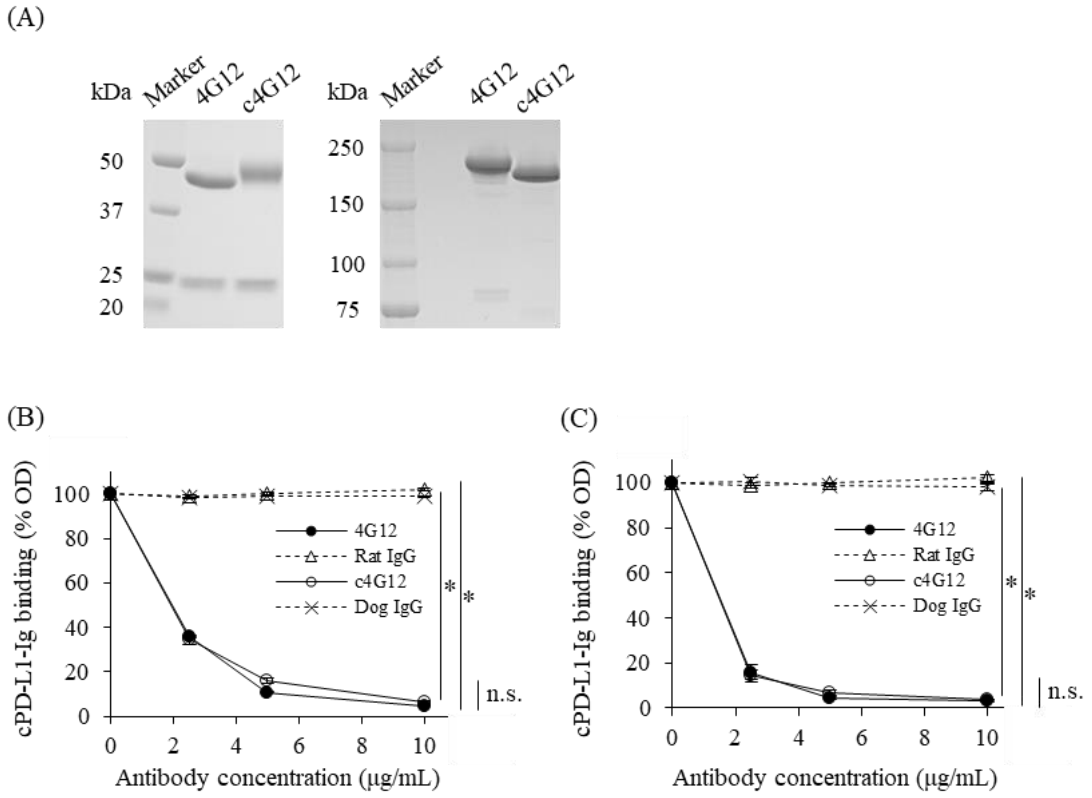

Figure III-3. Preparation and evaluation of canine chimeric anti-PD-L1 mAb c4G12. (A) Expression and purification of c4G12. A CHO-DG44 cell line which stably produce c4G12 were established and the chimeric mAb was purified from the culture supernatant by protein A derivative. SDS-PAGE and CBB staining were performed. Left panel, reduced condition; Right panel, non-reduced condition. Rat mAb 4G12 was used as a control protein. (B) Blocking of PD-1/PD-L1 binding by c4G12. cPD-1-Ig was coated on a microwell plate and binding of cPD-L1-Ig, which had been preincubated with various concentrations of anti-PD-L1 mAbs 4G12 or c4G12, was detected on the plate. Rat IgG and dog IgG were used as control antibodies. (C) Blocking of CD80/PD-L1 binding by c4G12. cCD80-Ig was coated on a plate and cPD-L1-Ig binding was evaluated similarly. Each point represents a mean value of relative OD (%) obtained from three independent experiments. Error bar; SE. Statistical analysis was performed by Tukey's test. *$p < 0.05$; n.s., not significant.

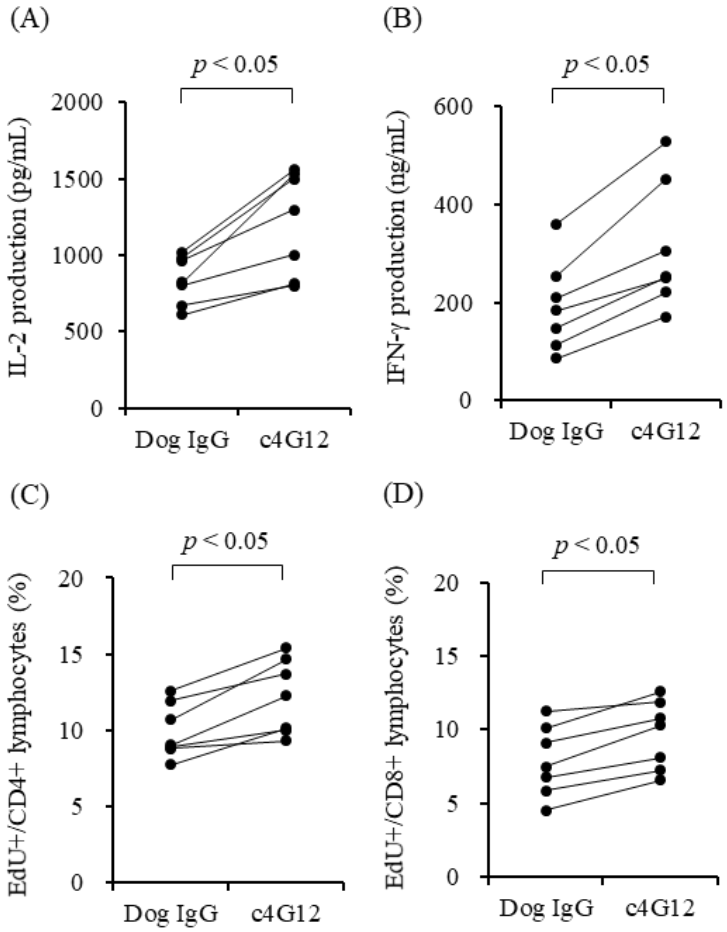

Figure III-4. Enhancement of cytokine production and cell proliferation of canine PBMCs by c4G12 treatment. Canine PBMCs were obtained from healthy beagle donors (n =7) and stimulated by 5 μg/mL SEB in the presence of 20 μg/mL c4G12. Dog IgG was used as a control antibody. For evaluation of cytokine production, the culture supernatant was harvested on day 3, and concentration of (A) IL-2 or (B) IFN-γ was measured by ELISA. To evaluate cell proliferation, nucleotide analogue EdU was added to the medium on day 2, and cells were harvested after incubation for another 2 h. The lymphocyte population was gated by FSC and SSC, and the incorporation of EdU in (C) $CD4^+$ or (D) $CD8^+$ cells was measured by a flow cytometer. Statistical analysis was performed with a Wilcoxon signed rank-sum test.

Table III-1. Binding properties of mAbs and recombinant receptors to cPD-L1-His.

	k_a ($\times 10^6$/Ms)	k_d ($\times 10^{-3}$/s)	K_D (nM)
4G12	2.42 ± 0.10	4.54 ± 0.19	1.88 ± 0.06
c4G12	3.14 ± 0.19	7.19 ± 0.26	2.30 ± 0.07
cPD-1			25.4 ± 4.89
cCD80			24.3 ± 0.89

The K_D of 4G12 or c4G12 was determined by fitting with the 1:1 kinetic binding model, and that of cPD-1-Ig or cCD80-Ig was determined by fitting with the two state reaction model in the SPR analysis. Data shown in the table are means ± SE of three independent experiments. k_a, association rate constant; k_d, dissociation rate constant.

DISCUSSION

Chimerization of mAb is a well-known, simple and effective way for the preparation of a therapeutic antibody that can be administrated repeatedly in other host species. Although humanized or fully humanized mAb has been developed and is considered a better option for therapeutic mAbs in humans, chimeric mAb is still a fascinating option for veterinary species because it does not require conformational analysis of animal antibodies or specialized mice genetically modified to produce animal antibodies. In fact, canine-chimerization of rat mAb was done quite simply in this study by combining the nucleotide sequences of rat variable regions with those of canine constant regions.

To prepare a therapeutic antibody that can be used in clinical studies, the comparison of therapeutic potential of anti-PD-L1 mAbs is essential as the first step. Among 3 rat mAbs, 6G7 and 4G12 showed sufficient blocking ability before chimerization. Because 6G7 is an IgM class antibody, it has 10 antigen binding sites which may contribute to the high binding avidity. As expected, canine chimeric IgG version of 6G7 (c6G7) had weakened blocking ability, possibly due to the decreased binding avidity with its only 2 binding sites. Chimerization itself seemed to have no or little effect on binding and blocking property because c4G12 had almost the same blocking ability and binding avidity as its original rat mAb. Therefore, c4G12 was selected as a drug candidate for the treatment of malignant cancers in dogs.

To prepare a large amount of the chimeric mAb for future clinical studies, establishment of high-producer cell lines is required. The dhfr/MTX method is a well-known strategy to generate high-producer cells of recombinant proteins. By using the dhfr-deficient CHO-DG44 cells and an expressing vector which encodes *dhfr* gene as a selection marker, stably expressing cells can be established in a selection medium lacking hypoxanthine and thymidine. Moreover, gene amplification and subsequent increase in the protein production can be achieved by culturing the cells in a medium containing a dhfr inhibitor, MTX. In this study, a CHO-DG44 cell line which can produce as much as 700 mg/L of c4G12 was successfully established. Using this cell line, more than 10 g of c4G12 has been expressed so far in our laboratory. Because c4G12 produced in this expression system enhanced multiple functions of canine immune cells *in vitro*, including cytokine productions and cell proliferation, the use of c4G12 in a clinical study is strongly encouraged.

SUMMARY

To prepare a therapeutic antibody for the use in clinical studies in dogs, rat mAb needs to be canine-chimerized to reduce its immunogenicity and to minimize effector functions such as ADCC and CDC. For this purpose, constant regions of canine IgGD heavy chain and lambda light chain were combined with variable regions of rat anti-PD-L1 mAb. Among anti-PD-L1 mAbs established in our laboratory, 6G7 and 4G12 had sufficient blocking effect on PD-1/PD-L1 binding before canine-chimerization. Canine chimeric IgG versions of 6G7 and 4G12, named c6G7 and c4G12, respectively, were produced in a transient expression system for further comparison. Because 6G7 is an IgM class mAb, c6G7 showed apparently attenuated blocking ability in PD-1/PD-L1 binding with its decreased binding sites. On the other hand, an IgG class mAb 4G12 retained sufficient binding and blocking ability even after the chimerization. Therefore, c4G12 was chosen as a candidate drug for future clinical studies.

To produce a large amount of the chimeric mAb, CHO-DG44 cell lines which stably express c4G12 were established by the dhfr/MTX method. The best producer cell line established in this study expressed up to 700 mg/L of chimeric mAb in the culture supernatant in 14 days of shaking culture. c4G12 produced in this expression system enhanced cytokine productions from canine PBMCs as well as the proliferation of $CD4^+$ and $CD8^+$ lymphocytes, encouraging the use of c4G12 in a future clinical study.

CHAPTER IV

Clinical efficacy of a therapeutic anti-PD-L1 monoclonal antibody in canine oral malignant melanoma and undifferentiated sarcoma

INTRODUCTION

Immunotherapy targeting immune checkpoint molecules, PD-1 and PD-L1, using therapeutic antibodies has shown great promise in cancer treatment in humans. For examples, nivolumab, a fully humanized IgG4 subclass mAb that targets PD-1, has been approved in Japan for the treatment of melanoma, NSCLC, RCC, classical Hodgkin's lymphoma, and head and neck cancer. Another anti-PD-1 mAb, pembrolizumab, has also been approved for melanoma and NSCLC. On the other hand, atezolizumab, an anti-PD-L1 mAb, has been approved for the treatment of urothelial carcinoma and NSCLC only in the United States [Iwai *et al*., 2017]. In addition to those cancers listed above, clinical benefit of PD-1/PD-L1 inhibitors has been reported for various malignant cancers including ovarian cancer, gastric cancer, breast cancer, and other types of lymphoma.

In dogs, recent reports from our group, as described in chapters I and II, and others demonstrated PD-L1 expression in various cancer types including OMM, HSA, MCT, and lymphoma, as well as the restoration of immune cell functions by PD-1/PD-L1 blockade using mAbs *in vitro* [Hartley *et al*., 2016; Shosu *et al*., 2016; Coy *et al*., 2017; Kumar *et al*., 2017]. However, the antitumor effect of the PD-1/PD-L1 inhibitors *in vivo* has not yet been fully elucidated because of the lack of therapeutic antibody that can be used in clinical experiments with dogs. In this chapter, to address this question and to build a proof-of-concept of our therapeutic strategy, a canine chimeric anti-PD-L1 mAb c4G12 was tested for its efficacy against OMM and undifferentiated sarcoma in dogs. This pilot clinical study provided the first evidence of safety and clinical benefit of c4G12 in dogs, suggesting that it could be a novel treatment for canine malignant cancers.

MATERIALS AND METHODS

Animals

Animal use throughout the clinical study was approved by the ethics committee, Faculty of Veterinary Medicine, Hokkaido University. Dogs with OMM or undifferentiated sarcoma seen at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, were enrolled in the pilot clinical study of the chimeric mAb after obtaining written informed consent from the owners of the dogs and veterinarians.

Preparation of c4G12 for the clinical study

Antibodies used in clinical studies must be highly purified to maximize the therapeutic potency and to avoid possible side effects. To reduce impurities and aggregates, culture supernatant containing c4G12 was obtained as described in chapter III and purified in a series of steps. First, c4G12 was purified from the supernatant by affinity chromatography using a HiScale 26/20 column packed with MabSelect SuRe LX (GE Healthcare). Additional purification by hydroxyapatite chromatography was performed using a BioScale CHT20-I prepacked column (Bio-Rad), and fractions containing aggregates were further purified by anion exchange chromatography using a HiScreen Q-Sepharose HP prepacked column (GE Healthcare). Throughout the purification steps, an ÄKTA avant 150 chromatography system (GE Healthcare) was used. The buffer was exchanged with PBS using a Vivaspin20 concentrator with 50 kDa molecular weight cut of membrane (Sartorius, Göttingen, Germany) and stored at 4 °C until use.

A pilot clinical study on dogs with OMM or undifferentiated sarcoma

Before starting the clinical study, the safety of c4G12 was confirmed in an experimental dog (13-year-old, female, beagle) by inoculating c4G12 intravenously at 2 mg/kg, every 2 weeks, 3 times in total. No allergic reactions or acute toxicity was found during 2 months of the observation period. Therefore, to evaluate the clinical efficacy of c4G12, a pilot clinical study was conducted at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University. The study period was from March 2016 to January 2017. Dogs with OMM or undifferentiated sarcoma were enrolled in the study if the PD-L1 expression was confirmed in the cancer cells. The PD-L1 expression in the primary tumor sections, obtained by surgical excision at prior surgery or biopsy, was assessed by IHC as described in chapter I using an anti-bovine PD-L1 mAb. Dogs previously treated with standard therapies such as surgery, radiation or chemotherapy

were included, while dogs with severe systemic illness or autoimmune disease were excluded from the study. c4G12 was diluted in 50 or 100 mL of saline depending on the body weight of the dog (100 mL of saline was used if > 20 kg) and administrated intravenously at 2 or 5 mg/kg, every 2 weeks, using a syringe pump over 1 h. During the treatment period, dogs were monitored with physical examination, complete blood count and serum chemistry at least every 2 weeks.

The tumor size was measured by a caliper and recorded every 2 weeks if measurable lesions were present on the body surface. At baseline (within 2 weeks prior to the first c4G12 administration) and every 6 weeks during the treatment, computed tomography (CT) was performed to evaluate the tumor burden and metastases. For dogs with OMM, tumor-node-metastasis-based staging was performed using the World Health Organization (WHO) staging scheme [Goldschmidt *et al*., 1998], at the time of study enrolment, by baseline CT and fine-needle aspiration/cytology of lymph nodes when required. The tumor burden was calculated as the sum of the longest diameters of all target measurable lesions. Tumors ≥10 mm in the longest diameter were considered as measurable lesions. A maximum of five target lesions were chosen from measurable lesions at baseline, with a maximum of two lesions per organ. Tumor response to the c4G12 treatment was defined as follows: complete response (CR), disappearance of all detectable tumor; partial response (PR), at least 30% reduction in the sum of the long diameters of target lesions; stable disease (SD), less than 20% increase or 30% reduction in the sum of diameters for at least 6 weeks; and progressive disease (PD), at least 20% increase in the sum of diameters. The longest diameters of new measurable lesions (up to five lesions in total and up to two lesions per organ) were included in the sum. Unequivocal progression of nontarget lesions was considered PD (according to modified criteria from response evaluation criteria in solid tumors in dogs v 1.0 [Nguyen *et al*., 2013] and unidimensional immune-related response criteria [Wolchok *et al*., 2009; Nishino *et al*., 2013]). If concomitant radiation therapy was given for local control, the lesions treated by radiation were excluded from the evaluation of the response to avoid overestimation. No potentially immunosuppressive medications (i.e., cytotoxic chemotherapy or immunosuppressive doses of steroids) were given concomitantly with the chimeric mAb treatment. Nonimmunosuppressive drugs, including NSAIDs, antibiotics, anodynes and antitussives were allowed for use during the study where clinically necessary. Adverse events were graded and recorded according to the veterinary cooperative oncology group - common terminology criteria for adverse events (VCOG-CTCAE) v 1.1 [VCOG, 2011].

Analysis of survival duration after confirmation of pulmonary metastasis

Survival from the confirmation of pulmonary metastasis to death was retrospectively investigated in dogs with OMM treated at the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University from 2013 to 2016. The dogs treated with standard therapy, such as definitive/palliative surgery, radiation and/or chemotherapy using cyclophosphamide or carboplatin at any time from diagnosis to death were included (n = 15, control group). Among dogs with OMM described in this chapter, all five dogs with stage IV disease had pulmonary metastases (n = 5, treatment group). Kaplan-Meier method was used to estimate the median survival of control group and treatment group. Kaplan-Meier curves were generated using a statistical analysis software EZR (Kanda, 2013), and a log-rank test was used to compere the survival patterns. A p value of less than 0.05 was considered statistically significant.

RESULTS

c4G12 treatment reduced tumor burden in dogs with OMM or undifferentiated sarcoma

Seven dogs histologically diagnosed with OMM and two dogs with undifferentiated sarcoma were enrolled in the pilot clinical study, after the confirmation of the PD-L1 expression in the primary cancers by IHC (Figure IV-1). The dogs were treated with 2 or 5 mg/kg of c4G12 by intravenous administration every 2 weeks. Five miniature dachshunds, a pug, a golden retriever, a toy poodle and a West Highland white terrier were included, with a median age at the time of enrolment of 12 years (ranging from 11 to 16 years). Among dogs with OMM, one had stage II disease (primary tumor 2-4 cm in diameter, no lymph node involvement), two had stage III disease (lymph node involvement but without distant metastasis), and four had stage IV disease (with distant metastases) at the time of enrolment. Four dogs were previously treated by surgery, and three had previous radiation therapy. During the antibody therapy, two dogs with lung metastases underwent concomitant palliative radiation therapy to control the primary oral cancers. Both dogs with undifferentiated sarcoma had multiple muscle metastases after surgical excision of primary tumors at the time of study enrolment (Table IV-1 and Table IV-2).

In one dog (no. 1) with stage II OMM, evident tumor regression was observed after 10 weeks of the treatment with 2 mg/kg of c4G12 (Figures IV-2A and B). However, the tumor began to grow slowly thereafter despite continuing treatment, thus the dose was increased to 5 mg/kg on week 24. Ten weeks after the dose change (week 34), the tumor again regressed dramatically (Figure IV-2C) with only traces of the mass seen on gross examination and CT images (Figures IV-2D-G, PR; approximately 81% reduction in the tumor burden at the maximum). In the other six dogs with OMM, the disease progressed with no evidence of tumor regression in detectable lesions (PD). The ORR (CR and PR) of OMM dogs was 14.3% (1/7) (Table IV-2).

Among two dogs with undifferentiated sarcoma, one (no. 9) clearly responded to the c4G12 therapy after 3 weeks of the treatment at 5 mg/kg (Figure IV-3), with an approximately 34% decrease in the tumor burden (PR). The other dog had progression of the disease and dropped out of the study on week 3 (PD). The ORR of undifferentiated sarcoma dogs was 50.0% (1/2) (Table IV-2).

No allergic reaction or autoimmune disease was seen throughout the study, in which a total of 63 doses were given to nine dogs. No systemic toxicity was noted on routine physical examination, complete blood count, or serum chemistry. Grade 1 diarrhoea was

observed in a dog with OMM during the treatment with 2 mg/kg (Table IV-2). However, it was transient and no medical intervention was needed. Although some other adverse events (mostly grade 1 or 2, changes in serum chemistry values etc.) were also noted during the study, they were not considered treatment-related, and additional medication or cancellation of the c4G12 treatment was not indicated.

Evidence of antitumor response in metastatic lesions of OMM: a case report

Additionally, another dog with OMM with pulmonary metastases was similarly treated with c4G12. The dog was a female, 11-year-old beagle that had been previously treated with surgery to remove the primary cancer. This case was not included in the pilot clinical study, because the treatment was given after the predefined observation period and no measurable lesion was present in the body at the time of the baseline measurement (all lesions were < 10 mm in the longest diameter). After 6 weeks, some lung metastatic legions clearly responded to the treatment, and almost all metastases disappeared on week 18 (Figure IV-4). No treatment-related adverse event was noticed during the treatment.

Prolongation of survival in dogs with OMM with pulmonary metastasis

Among dogs with OMM treated in the pilot clinical study, four dogs with pulmonary metastasis had progression of the disease as described above. However, one dog had unexpectedly long survival (no. 2, 222 days) after the confirmation of pulmonary metastasis. In addition, another dog with pulmonary metastases, that had responded to the treatment, also had long-term survival (censored at day 249). Because reliable survival data for dogs with stage IV OMM has not been reported in the literature to date, survival from the confirmation of pulmonary metastasis to death was retrospectively investigated in dogs with OMM treated at the Veterinary Teaching Hospital of Hokkaido University from 2013 to 2016, and the survival pattern of the treatment group was compared to that of historical control group. The control group comprised nine miniature dachshunds, two Labrador retrievers, a Chihuahua, a golden retriever, a beagle, a Pomeranian, a miniature schnauzer and a mixed breed. The median age at the time of confirmation of pulmonary metastasis was 14 years (range, 10-16 years, $n = 15$). The treatment group comprised two miniature dachshunds, a pug, a golden retriever, and a beagle, with a median age at the time of confirmation of pulmonary metastasis of 11 years (range, 10-14 years, $n = 5$). The survival pattern of the treatment group and that of the control group was analyzed by Kaplan-Meier method and the estimated median survival of the treatment and control groups were 96 days and 54 days, respectively (Figure IV-5, $p < 0.05$).

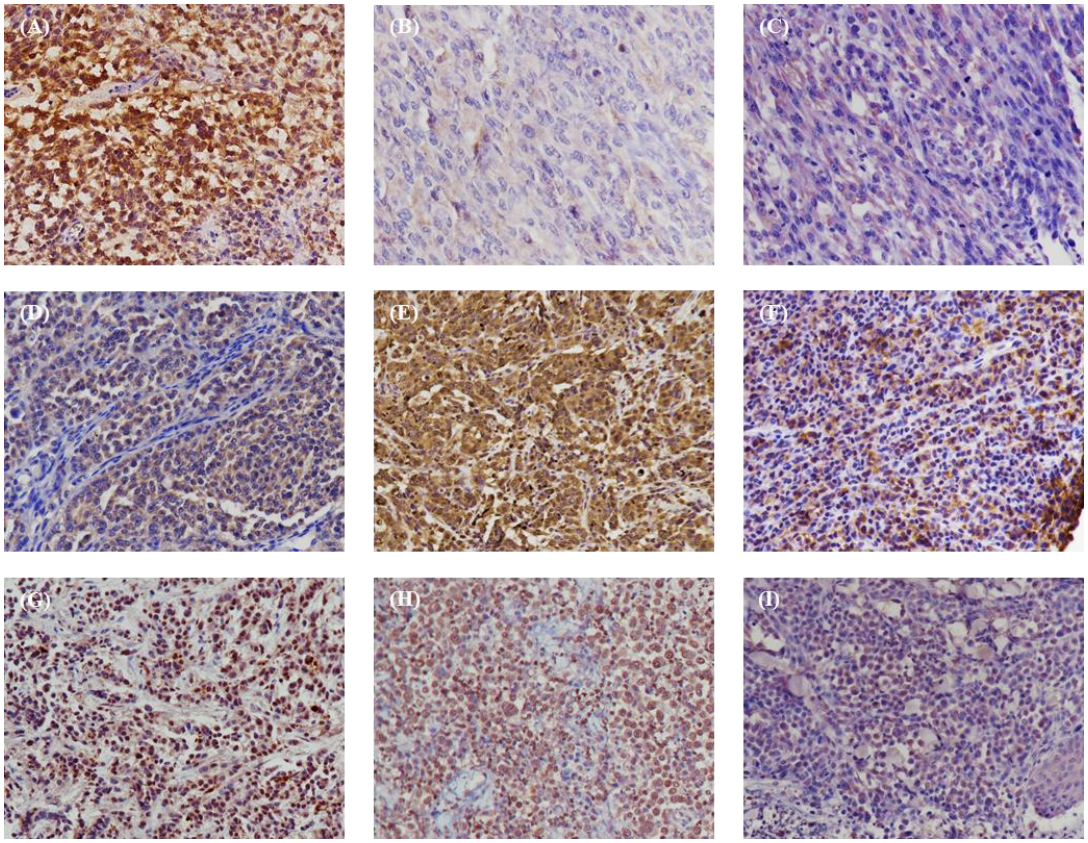

Figure IV-1. Expression of PD-L1 in tumor tissues from dogs enrolled in the pilot clinical study of c4G12. Tumor sections obtained by surgical excision at prior surgery or biopsy were tested for PD-L1 expression by IHC. (A-G) PD-L1 expression in OMM tissues obtained from dog no. 1-7, respectively. (H, I) PD-L1 expression in undifferentiated sarcoma tissues obtained from dog no. 8 and 9, respectively.

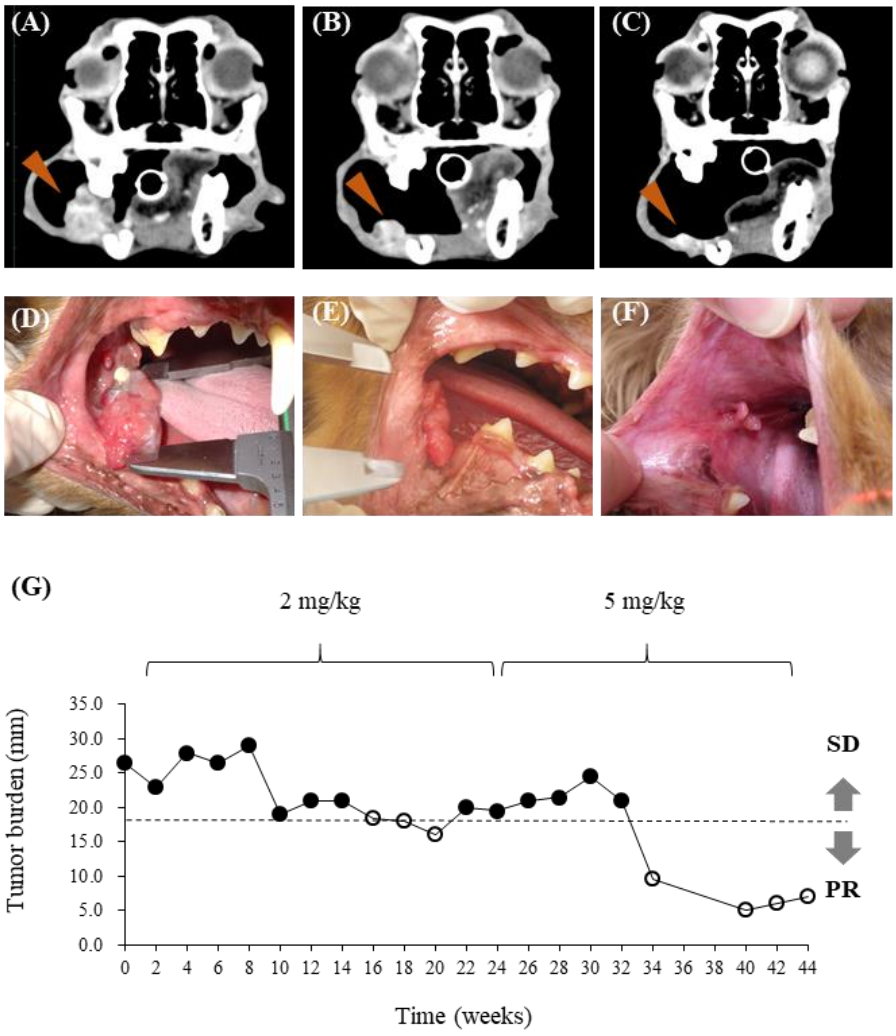

Figure IV-2. Antitumor effect of c4G12 in a dog with OMM. A dog with OMM was treated with 2 mg/kg (weeks 0-24) or 5 mg/kg (weeks 24-44) c4G12 every 2 weeks and the tumor burden was evaluated by gross examination and CT. (A, D) Tumor appearance at baseline (week 0). (B, E) Tumor appearance after 10 weeks of c4G12 treatment (C, F) Tumor appearance after 34 weeks of c4G12 treatment. Contrast-enhanced, and matched transverse CT images were shown. Arrowheads indicate the tumor lesions. (G) Changes in tumor burden. The longest diameter of target lesion was measured and recorded every 2 weeks. Reduction in tumor diameter by > 30% (time points indicated by open circles) was considered PR (see materials and methods for definition of response).

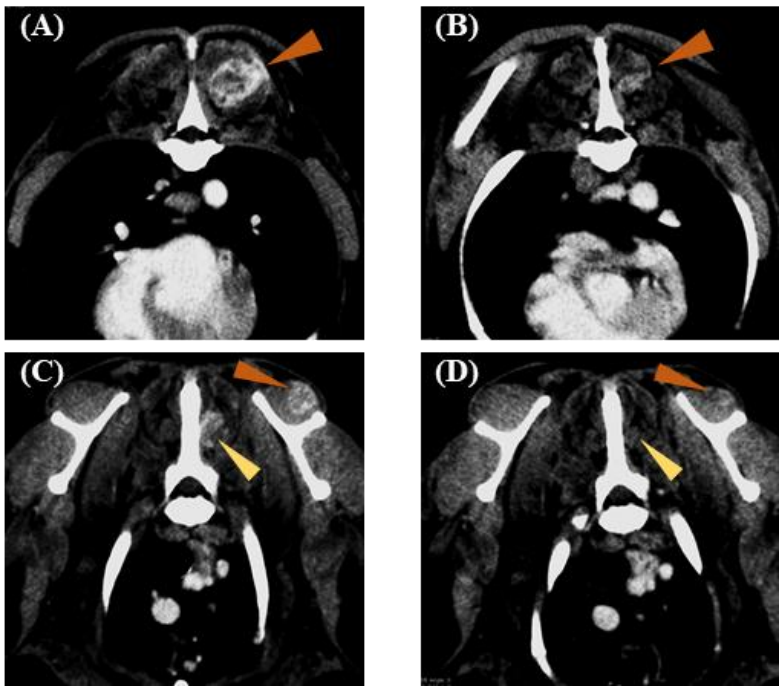

Figure IV-3. Antitumor effect of c4G12 in a dog with undifferentiated sarcoma. A dog with multiple metastatic lesions of undifferentiated sarcoma was treated with 5 mg/kg c4G12 every 2 weeks and the tumor burden was evaluated by CT. (A, C) Tumor appearance at baseline (week -2). (B, D) Tumor appearance after 3 weeks of c4G12 treatment. The tumors clearly responded to treatment on week 2, and the shrinkage was confirmed by CT on week 3. Contrast-enhanced, and matched transverse CT images were shown. Arrowheads indicate the tumor lesions.

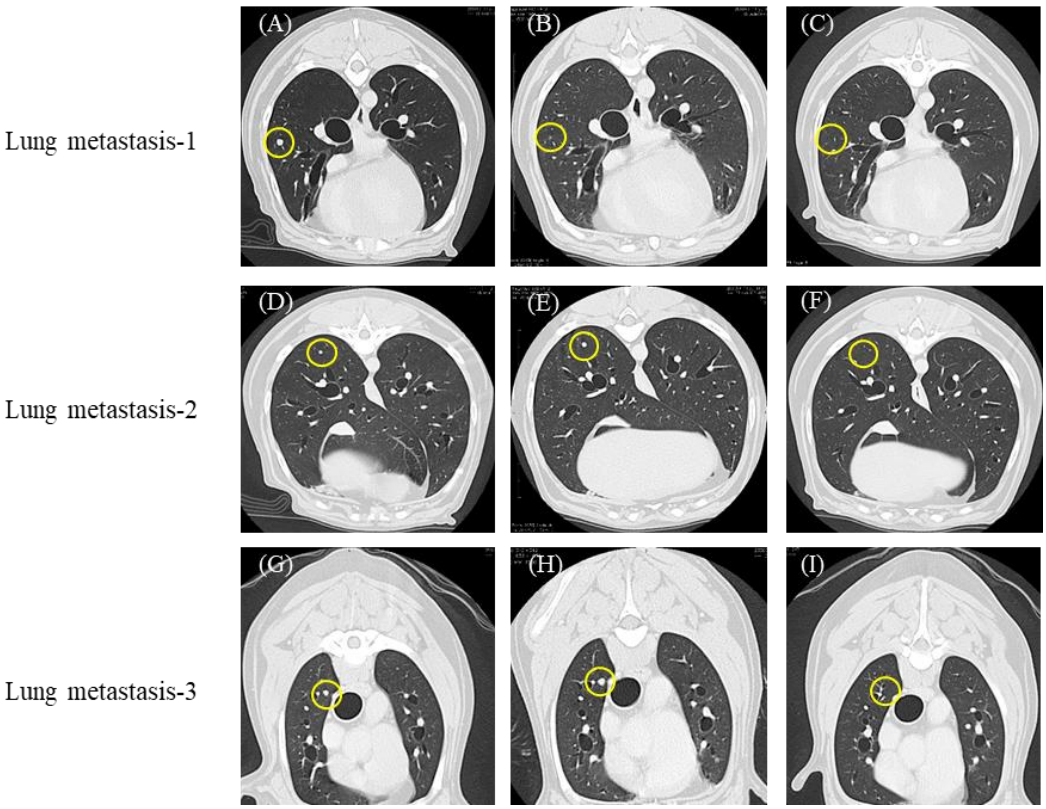

Figure IV-4. Antitumor effect of c4G12 in a dog with lung metastases of OMM. A dog with multiple pulmonary metastases of OMM was treated with 5 mg/kg (weeks 0-12) or 2 mg/kg (weeks 12-18) c4G12 every 2 weeks and the tumor size was evaluated by CT. (A, D, G) Tumor appearance at baseline (week 0). (B, E, H) Tumor appearance after 6 weeks of c4G12 treatment. (C, F, I) Tumor appearance after 18 weeks of c4G12 treatment. Contrast-enhanced, and matched transverse CT images were shown. Circles indicate the tumor lesions.

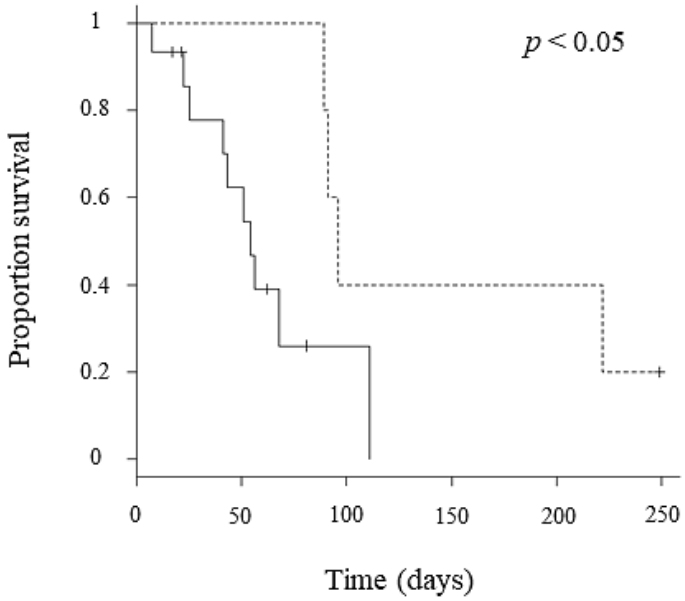

Figure IV-5. Survival in dogs with OMM after the confirmation of pulmonary metastasis. Survival (days) from the confirmation of pulmonary metastasis to death was recorded and plotted on the graph to generate Kaplan-Meier curves. The c4G12 treatment group (n = 5, dashed line) had longer survival compared to the historical control group (n = 15, black line) that had been treated by standard therapies at the Veterinary Teaching Hospital of Hokkaido University during 2013 to 2016. Marks on the line indicate censored data (5 dogs in the control group were censored at days 17, 21, 56, 62, and 81, and a dog in the treatment group was censored at day 249). Statistical analysis was performed with a log-rank test.

Table IV-1. Characteristics of dogs treated with c4G12 in the pilot study.

Dog no.	Breed	Age (years)	Sex	Primary tumor	WHO stage for OMM	Prior therapy
1	Miniature dachshund	11	Male	OMM	II	Surgery
2	Pug	11	Male, castrated	OMM	IV	Surgery
3	Miniature dachshund	16	Female	OMM	III	Radiation therapy
4	Miniature dachshund	14	Male, castrated	OMM	IV	Radiation therapy
5	Golden retriever	10	Male, castrated	OMM	IV	Surgery
6	Miniature dachshund	14	Male	OMM	IV	Radiation therapy
7	Miniature dachshund	14	Male, castrated	OMM	III	None
8	Toy poodle	11	Male, castrated	Undifferentiated sarcoma	-	Surgery, Chemotherapy
9	West Highland white terrier	12	Male, castrated	Undifferentiated sarcoma	-	Surgery

Prior therapy included definitive/palliative surgery, radiation, and chemotherapy with chlorambucil.

Table IV-2. Results of dogs treated with c4G12 in the pilot study.

Dog no.	Treatment duration (weeks)/ given doses	Dose of c4G12	Concomitant therapy	Best overall response	Survival after lung metastasis (days)	Treatment-related adverse event	Concurrent disease
1	44/22	Weeks 0-24: 2 mg/kg, then 5 mg/kg	None	PR	-	Diarrheoa, grade 1 (week 4)	None
2	32/16	Weeks 0-8: 2 mg/kg, then 5 mg/kg	Radiation therapy	PD	222	None	None
3	8/4	Weeks 0-2: 2 mg/kg, then 5 mg/kg	None	PD	-	None	None
4	6/3	2 mg/kg	None	PD	96	None	None
5	10/5	2 mg/kg	Radiation therapy	PD	91	None	None
6	12/6	5 mg/kg	None	PD	89	None	None
7	6/3	5 mg/kg	None	PD	-	None	None
8	3/2	5 mg/kg	None	PD	-	None	None
9	4/2	5 mg/kg	None	PR	-	None	Meningioma, Pulmonary fibrosis (suspected)

PR, partial response; PD, progressive disease. Adverse events which is possibly treatment-related are shown in the table.

DISCUSSION

OMM is a highly malignant cancer. When treated with surgery, MST for dogs with OMM depends on the stage. For stage I disease, MST is approximately 17 to 18 months, while for stage II and III, MSTs are 5 to 6 months and 3 months, respectively [MacEwen *et al*., 1986]. Radiation therapy can be applied for local control of cancer in addition to or instead of surgery, but chemotherapy in general yields insufficient tumor control. The lack of effective systemic therapy leads to a poor outcome in dogs with metastatic OMM, although the survival of dogs with stage IV OMM remains to be extensively studied. In this study, the survival of dogs with OMM with pulmonary metastasis was retrospectively investigated in our hospital and found to be less than 2 months (median 54 days). Development of alternative or additional systemic therapy is needed for the control of stage IV OMM.

As a candidate biological drug for canine malignant cancers, a canine chimeric anti-PD-L1 mAb, named c4G12, was developed. To date, there is no report on the safety of repeated administration of rat-dog chimeric mAb in dogs. c4G12 was well tolerated in dogs in the pilot clinical study, with no evidence of allergic reactions. Therefore, it was concluded that chimerization of antibody is a simple and effective strategy to prepare therapeutic mAbs for dogs, although it should be validated in future studies in which a larger number of dogs or different chimeric mAbs are included in the evaluation.

To the best of my knowledge, this is the first report in which canine chimeric anti-PD-L1 mAb was prepared and tested for its clinical efficacy in dogs with malignant cancers. Treatment with c4G12 induced an evident antitumor response in a dog with OMM and in another with undifferentiated sarcoma. The ORR in OMM was 14.3% (1/7) and that in undifferentiated sarcoma was 50.0% (1/2). Because the ORR of anti-PD-L1 mAb in melanoma patients was 17.3% (9/52) in a human clinical trial [Brahmer *et al*., 2012], these results seem consistent with human studies. In addition, in another dog with lung metastases of OMM, almost all metastatic lesions disappeared after 18 weeks of the treatment. Moreover, the c4G12 treatment significantly prolonged survival in dogs with OMM with pulmonary metastasis ($p < 0.05$). Considering that dogs with OMM with pulmonary metastasis have a poor prognosis with standard therapies, c4G12 could be a novel treatment option at least for a palliative purpose. Because the proof-of-concept has been established in this pilot clinical study, further clinical studies should be performed to fully elucidate the clinical benefit of c4G12.

In this study, dogs with PD-L1-positive cancers were selectively enrolled because, in human clinical trials using PD-1/PD-L1 inhibiting-mAbs, patients with PD-L1-positive

cancers tended to respond to the treatment, while patients with PD-L1-negative cancers had lower ORRs [Topalian *et al*., 2012; Larkin *et al*., 2015]. Although discussion continues as to whether measuring PD-L1 expression in cancers is a clinically useful predictive biomarker for the response to PD-1/PD-L1 inhibitors [Patel and Kurzrock, 2015], other PD-L1-positive cancers in dogs including OS, HSA, MCT, mammary adenocarcinoma, lymphoma and prostate adenocarcinoma [Shosu *et al*., 2016; Kumar *et al*., 2017], could also respond to c4G12. Clinical studies of c4G12 in these cancer types may be beneficial to find new treatment options for these highly malignant and refractory cancers.

Because no systemic toxicity or autoimmune disease was noted during the study, c4G12 seems to be safe and well tolerated in dogs despite repeated administration. A mild diarrhoea was noted in one dog with OMM although a causal relationship with the treatment remains unclear (possibly treatment-related). In humans, side effects possibly caused by PD-1/PD-L1 inhibiting-mAbs include infusion-related reactions, hypersensitivity, pneumonitis, colitis, diarrhea, and hypothyroidism [Brahmer *et al*., 2010; Brahmer *et al*., 2012; Topalian *et al*., 2012]. Because evidence of autoimmune disease was reported in PD-1 knockout mice [Nishimura *et al*., 1999; Nishimura *et al*., 2001], and treatment-related deaths due to pneumonitis have been reported in a human clinical trial [Topalian *et al*., 2012], a careful attention should be paid to these possibilities in future studies in dogs using c4G12.

The reason why antitumor responses were not induced in some dogs despite the PD-L1 expression is unclear. Recent studies showed the presence of some mechanisms for the resistance to the PD-1/PD-L1 blockade. For example, severely exhausted T cells, with high PD-1 expression and/or co-expression of other inhibitory receptors including T-cell immunoglobulin mucin 3, lymphocytes activation gene 3, and CTLA-4, cannot be fully reinvigorated by the blockade of the PD-1/PD-L1 pathway alone [Blackburn *et al*., 2008; Thommen *et al*., 2015]. Regulatory subsets of immune cells within tumor microenvironment, such as regulatory T cells and myeloid-derived suppressor cells, can also contribute to the resistance [Wang and Wu, 2017]. The use of drugs targeting these additional suppressive mechanisms should help enhance the therapeutic effectiveness of the PD-1/PD-L1 blockade. Another study, which attempted to find predictive biomarkers for the treatment outcome, demonstrated that higher nonsynonymous mutation burden in NSCLC was associated with improved sensitivity to the PD-1 blockade [Rizvi *et al*., 2015]. This suggested that the immunogenicity of cancer can vary among patients and is a key determinant of the response. For cancers with low immunogenicity, induction of tumor antigen-specific T cells using cancer vaccines, or transfusion of T cells genetically

modified to recognize tumor antigens, may improve the response rate of the PD-1/PD-L1 blockade.

In conclusion, a canine chimeric anti-PD-L1 mAb was successfully developed and its clinical efficacy was tested in dogs with OMM or undifferentiated sarcoma. The pilot clinical study demonstrated the safety and antitumor activity of c4G12. Some canine cancers, including malignant melanoma, are considered preferable preclinical models of human cancer research with their similar biological behaviors, resistance to chemo/radiotherapy, metastatic propensity and spontaneous occurrence in outbred and immunocompetent settings [Withrow *et al*., 2013]. Therefore, further studies may be beneficial not only for developing canine cancer treatment, but also to inform human studies using PD-1/PD-L1 inhibiting-mAbs.

SUMMARY

In human cancer treatment, the blockade of immune checkpoint by PD-1/PD-L1 inhibiting-mAbs is approved for several malignancies including melanoma, NSCLC, and RCC in the last 3 years. In dogs, our group and others have reported that PD-L1 is expressed in various types of canine cancers and PD-1/PD-L1 blockade by mAbs restores immune cell function *in vitro*. However, no clinical study had been conducted because of the lack of therapeutic antibody that can be used in dogs. In chapter III, a canine chimeric anti-PD-L1 mAb with sufficient blocking ability has been prepared for the use in a clinical study.

In this chapter, a pilot clinical study of the chimeric mAb, c4G12, was performed in dogs with OMM or undifferentiated sarcoma. Seven dogs with OMM and two with undifferentiated sarcoma were treated with c4G12 at 2 or 5 mg/kg, every 2 weeks. In one dog with OMM and another with undifferentiated sarcoma, the tumor burdens were decreased by more than 30% (PR). The ORRs for OMM dogs and undifferentiated sarcoma dogs were 14.3% (1/7) and 50.0% (1/2), respectively. In another dog with pulmonary metastases of OMM, the metastatic lesions were almost completely eliminated by 18 weeks of c4G12 treatment. Moreover, the MST for OMM dogs with pulmonary metastasis was significantly prolonged by c4G12 treatment ($p < 0.05$). No systemic toxicity or autoimmune disease was noticed during the observation period.

Taken together, this pilot clinical study demonstrated the safety and therapeutic efficacy of c4G12 in dogs with OMM or undifferentiated sarcoma. Further studies are warranted to fully clarify the clinical benefit of c4G12 for treatment of canine malignant cancers.

CONCLUSION

Cancer treatment in dogs has become more important along with their extended lifespan. Spontaneous cancers are common cause of death and the current treatment for them includes surgery, radiation, and chemotherapy. However, novel treatment options, such as immunotherapy, are needed to improve prognosis of some malignant cancers, especially for those effective systemic therapy is not available. For example, malignant melanoma that develops in oral cavity represents intractable cancers in dogs with its local invasiveness, metastatic propensity, and resistance to chemotherapy. In this study, the development of a novel immunotherapy was aimed against canine malignant cancers, including oral malignant melanoma (OMM), by targeting immune checkpoint molecules programmed cell death 1 (PD-1) and PD-ligand 1 (PD-L1).

PD-1 is an immunoinhibitory receptor that attenuates T-cell effector functions when it binds to its ligand, PD-L1. Aberrant expression of PD-L1 is reported in various types of malignant cancers in humans, and the PD-1/PD-L1 axis is considered an immune evasion mechanism. On the other hand, the blockade of the PD-1/PD-L1 pathway using monoclonal antibodies (mAbs) can induce antitumor activity in various types of malignant cancers in humans. For the success of PD-1/PD-L1 inhibiting-mAbs in clinical studies, immunotherapy has gained attention and now is considered the fourth therapeutic modality for human cancers. However, in dogs, no study had been performed on the PD-1/PD-L1 pathway and its association with cancers was unclear.

In chapter I, the sequences of canine *PD-1* and *PD-L1* genes were identified as the first step. The PD-1/PD-L1 pathway appeared to be immunosuppressive in dogs because functional motifs were conserved in deduced amino acid sequence of canine PD-1, and the PD-L1 blockade with an anti-PD-L1 mAb enhanced the immune cell function *in vitro*. The PD-1 and PD-L1 expressions were found in several canine cancers, confirming that the PD-1/PD-L1 axis could be an immune evasion mechanism in canine cancers. Most importantly, the treatment with anti-PD-L1 mAb enhanced the IFN-γ production from tumor-infiltrating mononuclear cells, encouraging the development of a therapeutic antibody that can be used for a future clinical study.

In chapter II, to clarify the cancer types that could respond to this therapeutic strategy, immunohistochemical analysis of the PD-L1 expression was further performed in various types of malignant cancers in dogs. Most tissue samples of OMM, osteosarcoma, hemangiosarcoma, mast cell tumor, and several other cancers expressed PD-L1, suggesting that these cancers could be candidates for immunotherapy using anti-PD-L1 antibody.

In chapter III, to prepare a therapeutic antibody that can be repeatedly administrated to dogs, blocking anti-PD-L1 mAbs were canine-chimerized using genetic engineering techniques and produced in a mammalian cell-based expression system. Three anti-PD-L1 mAbs were compared, and 4G12 was selected as the source of variable regions with its sufficient blocking ability. A stable high-producer cell line for canine-chimerized 4G12, named c4G12, was established using dihydrofolate reductase (dhfr)-deficient Chinese hamster ovary-DG44 cells and the dhfr/methotrexate method. The best cell line produced approximately 700 mg/L of chimeric mAb in the culture supernatant at the maximum. Because c4G12 showed immunostimulatory effects on canine peripheral blood mononuclear cells *in vitro*, the use of c4G12 in a future clinical study was warranted.

In chapter IV, a pilot clinical study was performed in the Veterinary Teaching Hospital of Hokkaido University to evaluate the safety and antitumor activity of c4G12 in dogs. Dogs with OMM or undifferentiated sarcoma were treated with c4G12 at 2 or 5 mg/kg, every 2 weeks. Evident cancer regression was found in a dog with OMM and another with undifferentiated sarcoma, with objective response rates of 14.3% (1/7) in OMM and 50.0% (1/2) in undifferentiated sarcoma. In an additional dog with metastatic OMM, lung metastatic lesions clearly responded to the treatment. Moreover, in dogs with pulmonary metastasis of OMM, the median survival time was significantly prolonged by the treatment, when compared to the historical control group that had been treated with standard therapies. Despite the repeated administration, no systemic toxicity or autoimmune disease was noticed during the observation period, demonstrating the safety of c4G12 in dogs.

Taken together, the blockade of the PD-1/PD-L1 pathway is a promising therapeutic strategy against various types of canine malignant cancers, and c4G12 is a candidate biological drug that deserves further investigation. Because the blockade of immune checkpoint molecules takes off the brake of antitumor immune responses, its combination with radiation, molecular-targeted drugs, cancer vaccines, or other immunotherapy may exert synergetic effect in cancer treatment. Therefore, further clinical studies are needed to fully elucidate the therapeutic potential of c4G12, and to find the way to elicit the maximum clinical benefit from the blockade of immune checkpoint molecules for canine cancers.

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my professors, Dr. Kazuhiko Ohashi, Dr. Satoru Konnai, and Dr. Shiro Murata, Laboratory of Infectious Diseases, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University (Sapporo, Japan), for their continuous mentoring, guidance, assistance and encouragement throughout the course of this work.

I would like to express my sincere appreciation to Dr. Yasuhiko Suzuki, Division of Bioresources, Research Center for Zoonosis Control, Hokkaido University, for his invaluable advice on the experiments and critical review on the manuscript.

I owe a deep debt of gratitude to Dr. Satoshi Takagi, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, for his pivotal help in the clinical research, practical advice on the experiments, and critical review on the manuscript.

I greatly appreciate Dr. Yumiko Kagawa, North Lab (Sapporo, Japan), for her comprehensive supervision on histological analyses and helpful suggestions. I would like to extend my sincere gratitude to Dr. Yukinari Kato, Department of Antibody Drug Development, Graduate School of Medicine, Tohoku University (Sendai, Japan), for his technical assistance and valuable advice.

I am deeply grateful to Dr. Mitsuyoshi Takiguchi, Dr. Kenji Hosoya, Dr. Yuki Hoshino, and veterinary staffs in the Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, for their kind assistance in sample collection and cooperation in the clinical research.

I greatly thank Dr. Motohiro Horiuchi and Dr. Akio Suzuki, Laboratory of Veterinary Hygiene, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, for their technical assistance.

Special deep thanks are extended to all members in Laboratory of Infectious Diseases, Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University for their excellent help and warm friendship.

I am very thankful to the Japan Society for the Promotion of Science (JSPS) for the fellowship that supported the current study. I would like to extend my sincere condolences on the death of experimental animals that were used for my research.

I would like to show my deep appreciation to my family and friends for their encouragement and support throughout the course of my education and research. Finally, I would like to thank all people who helped me to complete this study. Thank you so much.

REFERENCES

Adams VJ, **Evans KM**, **Sampson J**, **Wood JLN**. Methods and mortality results of a health survey of purebred dogs in the UK. *J. Small Anim. Pract.* 2010; **51**:512-524.

Arun SS, **Breuer W**, **Hermanns W**. Immunohistochemical examination of light-chain expression (lambda/kappa ratio) in canine, feline, equine, bovine and porcine plasma cells. *Zentralbl. Veterinarmed. A*. 1996; **43**:573-576.

Barroga EF, **Kadosawa T**, **Okumura M**, **Fujinaga T**. Establishment and Characterization of the Growth and Pulmonary Metastasis of a Highly Lung Metastasizing Cell Line from Canine Osteosarcoma in Nude Mice. *J. Vet. Med. Sci.* 1999; **61**:361-367.

Bergeron LM, McCandless EE, Dunham S, Dunkle B, Zhu Y, Shelly J, Lightle S, *et al.* Comparative functional characterization of canine IgG subclasses. *Vet. Immunol. Immunopathol.* 2014; **157**:31-41.

Blackburn SD, **Shin H**, **Freeman GJ**, **Wherry EJ**. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. *Proc. Natl. Acad. Sci. U. S. A.* 2008; **105**:15016-15021.

Blank C, Kuball J, Voelkl S, Wiendl H, Becker B, Walter B, Majdic O, *et al.* Blockade of PD-L1 (B7-H1) augments human tumor-specific T cell responses in vitro. *Int. J. Cancer*. 2006; **119**:317-327.

Bostock DE, **Crocker J**, **Harris K**, **Smith P**. Nucleolar organiser regions as indicators of post-surgical prognosis in canine spontaneous mast cell tumours. *Br. J. Cancer*. 1989; **59**:915-918.

Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, *et al.* Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: Safety, clinical activity, pharmacodynamics, and immunologic correlates. *J. Clin. Oncol.* 2010; **28**:3167-3175.

Brahmer JR, Tykodi SS, Chow LQM, Hwu WJ, Topalian SL, Hwu P, Drake CG, *et*

al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* 2012; **366**:2455-2465.

Brønden LB, **Nielsen SS**, **Toft N**, **Kristensen AT**. Data from the Danish Veterinary Cancer Registry on the occurrence and distribution of neoplasms in dogs in Denmark. *Vet. Rec.* 2010; **166**:586-590.

Brüggemann M, **Winter G**, **Waldmann H**, **Neuberger MS**. The immunogenicity of chimeric antibodies. *J. Exp. Med.* 1989; **170**:2153-2157.

Buchbinder EI, **Desai A**. CTLA-4 and PD-1 Pathways. *Am. J. Clin. Oncol.* 2016; **39**:98-106.

Butte MJ, **Keir ME**, **Phamduy TB**, **Sharpe AH**, **Freeman GJ**. Programmed Death-1 Ligand 1 Interacts Specifically with the B7-1 Costimulatory Molecule to Inhibit T Cell Responses. *Immunity*. 2007; **27**:111-122.

Chapon M, Randriamampita C, Maubec E, Badoual C, Fouquet S, Wang SF, Marinho E, *et al.* Progressive upregulation of PD-1 in primary and metastatic melanomas associated with blunted TCR signaling in infiltrating T lymphocytes. *J. Invest. Dermatol.* 2011; **131**:1300-1307.

Chemnitz JM, **Parry RV**, **Nichols KE**, **June CH**, **Riley JL**. SHP-1 and SHP-2 Associate with Immunoreceptor Tyrosine-Based Switch Motif of Programmed Death 1 upon Primary Human T Cell Stimulation, but Only Receptor Ligation Prevents T Cell Activation. *J. Immunol.* 2004; **173**:945-954.

Chen L, **Flies DB**. Molecular mechanisms of T cell co-stimulation and co-inhibtion. *Nat. Rev. Immunol.* 2013; **13**:227-242.

Coy J, **Caldwell A**, **Chow L**, **Guth A**, **Dow S**. PD-1 expression by canine T cells and functional effects of PD-1 blockade. *Vet. Comp. Oncol.* 2017; **4**:1-16.

D'Angelo SP, Shoushtari AN, Agaram NP, Kuk D, Qin LX, Carvajal RD, Dickson MA, *et al.* Prevalence of tumor-infiltrating lymphocytes and PD-L1 expression in the soft tissue sarcoma microenvironment. *Hum. Pathol.* 2015; **46**:357-365.

de Haij S, Woltman AM, Trouw LA, Bakker AC, Kamerling SW, van der Kooij SW, Chen L, *et al.* Renal tubular epithelial cells modulate T-cell responses via ICOS-L and B7-H1. *Kidney Int.* 2005; **68**:2091-2102.

Dickerson EB, Thomas R, Fosmire SP, Lamerato-Kozicki AR, Bianco SR, Wojcieszyn JW, Breen M, *et al.* Mutations of phosphatase and tensin homolog deleted from chromosome 10 in canine hemangiosarcoma. *Vet. Pathol.* 2005; **42**:618-632.

Dobson JM, **Samuel S**, **Milstein H**, **Rogers K**, **Wood JLN**. Canine neoplasia in the UK: estimates of incidence rates from a population of insured dogs. *J. Small Anim. Pract.* 2002; **43**:240-246.

Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* 2002; **8**:793-800.

Esch KJ, **Juelsgaard R**, **Martinez PA**, **Jones DE**, **Petersen CA**. Programmed Death 1-Mediated T Cell Exhaustion during Visceral Leishmaniasis Impairs Phagocyte Function. *J. Immunol.* 2013; **191**:5542-5550.

Eppihimer MJ, **Gunn J**, **Freeman GJ**, **Greenfield EA**, **Chernova T**, **Erickson J**, **Leonard JP**. Expression and regulation of the PD-L1 immunoinhibitory molecule on microvascular endothelial cells. *Microcirculation*. 2002; **9**:133-145.

Folkl A, **Wen X**, **Kuczynski E**, **Clark ME**, **Bienzle D**. Feline programmed death and its ligand: Characterization and changes with feline immunodeficiency virus infection. *Vet. Immunol. Immunopathol.* 2010; **134**:107-114.

Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, Fitz LJ, *et al.* Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J. Exp. Med*. 2000; **192**:1027-1034.

Ghebeh H, Mohammed S, Al-Omair A, Qattan A, Lehe C, Al-Qudaihi G, Elkum N, *et al.* 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*. 2006; **8**:190-198.

Ghiotto M, **Gauthier L**, **Serriari N**, **Pastor S**, **Truneh A**, **Nunès JA**, **Olive D**. PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1. *Int. Immunol.* 2010; **22**:651-660.

Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. *Cell*. 1981; **23**:175-182.

Goldschmidt MH, Dunstan RW, Stannard AA, von Tschamer C, Walder EJ, Yager JA. Histological classification of epithelial and melanocytic tumors of the skin of domestic animals. *World Health Organization International Histological Classification of Tumors in Domestic Animals. III.* 1998.

Goto S, Konnai S, Okagawa T, Nishimori A, Maekawa N, Gondaira S, Higuchi H, *et al.* Increase of cells expressing PD-1 and PD-L1 and enhancement of IFN-γ production via PD-1 / PD-L1 blockade in bovine mycoplasmosis. *Immun. Inflamm. Dis.* 2017:1-9.

Hartley G, Faulhaber E, Caldwell A, Coy J, Kurihara J, Guth A, Regan D, *et al.* Immune regulation of canine tumour and macrophage PD-L1 expression. *Vet. Comp. Oncol.* 2017; **15**:534-549.

Hitoshi N, **Ken-ichi Y**, **Jun-ichi M**. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene*. 1991; **108**:193-199.

Ikebuchi R, **Konnai S**, **Shirai T**, **Sunden Y**, **Murata S**, **Onuma M**, **Ohashi K**. Increase of cells expressing PD-L1 in bovine leukemia virus infection and enhancement of anti-viral immune responses in vitro via PD-L1 blockade. *Vet. Res.* 2011; **42**:1-15.

Ikebuchi R, Konnai S, Okagawa T, Yokoyama K, Nakajima C, Suzuki Y, Murata S, *et al.* Blockade of bovine PD-1 increases T cell function and inhibits bovine leukemia virus expression in B cells in vitro. *Vet. Res.* 2013; **44**:59.

Ikebuchi R, Konnai S, Okagawa T, Yokoyama K, Nakajima C, Suzuki Y, Murata S, *et al.* Influence of PD-L1 cross-linking on cell death in PD-L1-expressing cell lines and bovine lymphocytes. *Immunology*. 2014; **142**:551-561.

Iwai Y, **Ishida M**, **Tanaka Y**, **Okazaki T**, **Honjo T**, **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. U. S. A.* 2002; **99**:12293-12297.

Iwai Y, **Hamanishi J**, **Chamoto K**, **Honjo T**. Cancer immunotherapies targeting the PD-1 signaling pathway. *J. Biomed. Sci.* 2017; **24**:26.

Kadosawa T, **Nozaki K**, **Sasaki N**, **Takeuchi A**. Establishment and characterization of a new cell line from a canine osteosarcoma. *J. Vet. Med. Sci.* 1994; **56**:1167-1169.

Kanae Y, **Endoh D**, **Yokota H**, **Taniyama H**, **Hayashi M**. Expression of the PTEN tumor suppressor gene in malignant mammary gland tumors of dogs. *Am. J. Vet. Res.* 2006; **67**:127-133.

Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*. 2013; **48**:452-458.

Keir ME, **Butte MJ**, **Freeman GJ**, **Sharpe AH**. PD-1 and Its Ligands in Tolerance and Immunity. *Annu. Rev. Immunol.* 2008; **26**:677-704.

Kim JR, Moon YJ, Kwon KS, Bae JS, Wagle S, Kim KM, Park HS, *et al.* Tumor infiltrating PD1-positive lymphocytes and the expression of PD-L1 predict poor prognosis of soft tissue sarcomas. *PLoS One*. 2013; **8**:1-9.

Koenig A, **Bianco SR**, **Fosmire S**, **Wojcieszyn J**, **Modiano JF**. Expression and significance of p53, rb, p21/waf-1, p16/ink-4a, and PTEN tumor suppressors in canine melanoma. *Vet. Pathol.* 2002; **39**:458-472.

Kumar SR, **Kim DY**, **Henry CJ**, **Bryan JN**, **Robinson KL**, **Eaton AM**. Programmed death ligand 1 is expressed in canine B cell lymphoma and downregulated by MEK inhibitors. *Vet. Comp. Oncol.* 2017; **1**:1-10.

Kuus-Reichel K, **Grauer LS**, **Karavodin LM**, **Knott C**, **Krusemeier M**, **Kay NE**. Will immunogenicity limit the use, efficacy, and future development of therapeutic monoclonal antibodies? *Clin. Diagn. Lab. Immunol.* 1994; **1**:365-372.

Larkin J, Chiarion-Sileni V, Gonzalez R, Grob JJ, Cowey CL, Lao CD, Schadendorf D, *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N. Engl. J. Med.* 2015; **373**:23-34.

Latchman Y, Wood CR, Chernova T, Chaudhary D, Borde M, Chernova I, Iwai Y, *et al.* PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat. Immunol.* 2001; **2**:261-268.

Lee SJ, Jang BC, Lee SW, Yang YI, Suh SI, Park YM, Oh S, *et al.* Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-γ-induced upregulation of B7-H1 (CD274). *FEBS Lett.* 2006; **580**:755-762.

Levine RA, **Forest T**, **Smith C**. Tumor suppressor PTEN is mutated in canine osteosarcoma cell lines and tumors. *Vet. Pathol.* 2002; **39**:372-378.

London CA, Hannah AL, Zadovoskaya R, Chien MB, Kollias-Baker C, Rosenberg M, Downing S, *et al.* Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. *Clin. Cancer Res.* 2003; **9**:2755-2768.

Lussier DM, O'Neill L, Nieves LM, McAfee MS, Holechek SA, Collins AW, Dickman P, *et al.* Enhanced T-cell immunity to osteosarcoma through antibody blockade of PD-1/PD-L1 interactions. *J. Immunother.* 2015; **38**:96-106.

MacEwen EG, **Patnaik AK**, **Harvey HJ**, **Hayes AA**, **Matus R**. Canine oral melanoma: comparison of surgery versus surgery plus Corynebacterium parvum. *Cancer Invest.* 1986; **4**:397-402.

MacVean DW, **Monlux AW**, **Anderson PS**, **Silberg SL**, **Roszel JF**. Frequency of canine and feline tumors in a defined population. *Vet. Pathol.* 1978; **15**:700-715.

Maloney DG, Grillo-López AJ, White CA, Bodkin D, Schilder RJ, Neidhart JA, Janakiraman N, *et al.* IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma. *Blood*. 1997; **90**:2188-2195.

Massari F, Ciccarese C, Calió A, Munari E, Cima L, Porcaro AB, Novella G, *et al.* Magnitude of PD-1, PD-L1 and T Lymphocyte Expression on Tissue from Castration-Resistant Prostate Adenocarcinoma: An Exploratory Analysis. *Target. Oncol.* 2016; **11**:345-351.

Matsuyama-Kato A, Murata S, Isezaki M, Kano R, Takasaki S, Ichii O, Konnai S, *et al.* Molecular characterization of immunoinhibitory factors PD-1/PD-L1 in chickens infected with Marek's disease virus. *Virol. J.* 2012; **9**:94.

Merlo DF, Rossi L, Pellegrino C, Ceppi M, Cardellino U, Capurro C, Ratto A, *et al.* Cancer incidence in pet dogs: Findings of the animal tumor registry of Genoa, Italy. *J. Vet. Intern. Med.* 2008; **22**:976-984.

Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, Su X, *et al.* PD-L1 Expression in Triple-Negative Breast Cancer. *Cancer Immunol. Res.* 2014; **2**:361-370.

Murphy S, **Hayes AM**, **Blackwood L**, **Maglennon G**, **Pattinson H**, **Sparkes AH**. Oral malignant melanoma - the effect of coarse fractionation radiotherapy alone or with adjuvant carboplatin therapy. *Vet. Comp. Oncol.* 2005; **3**:222-229.

Nakae S, **Suto H**, **Iikura M**, **Kakurai M**, **Sedgwick JD**, **Tsai M**, **Galli SJ**. Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *J. Immunol.* 2006; **176**:2238-2248.

Nakaichi M, **Taura Y**, **Kanki M**, **Mamba K**, **Momoi Y**, **Tsujimoto H**, **Nakama S**. Establishment and characterization of a new canine B-cell leukemia cell line. *J. Vet. Med. Sci.* 1996; **58**:469-471.

Nguyen SM, **Thamm DH**, **Vail DM**, **London CA**. Response evaluation criteria for solid tumours in dogs (v1.0): A Veterinary Cooperative Oncology Group (VCOG) consensus document. *Vet. Comp. Oncol.* 2015; **13**:176-183.

Nishimori A, Konnai S, Okagawa T, Maekawa N, Ikebuchi R, Goto S, Sajiki Y, *et al.* *In vitro* and *in vivo* antivirus activity of an anti-programmed death-ligand 1 (PD-L1)

ratbovine chimeric antibody against bovine leukemia virus infection. *PLoS One*. 2017; **12**:1-18.

Nishimura H, **Nose M**, **Hiai H**, **Minato N**, **Honjo T**. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity*. 1999; **11**:141-151.

Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, Sasayama S, *et al.* Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. *Science*. 2001; **291**:319-322.

Nishino M, **Giobbie-Hurder A**, **Gargano M**, **Suda M**, **Ramaiya NH**, **Hodi FS**. Developing a common language for tumor response to immunotherapy: Immune-related response criteria using unidimensional measurements. *Clin. Cancer Res.* 2013; **19**:3936-3943.

Ogilvie GK, **Powers BE**, **Mallinckrodt CH**, **Withrow SJ**. Surgery and doxorubicin in dogs with hemangiosarcoma. *J. Vet. Intern. Med.* 1996; **10**:379-384.

Ohashi E, **Hong SH**, **Takahashi T**, **Nakagawa T**, **Mochizuki M**, **Nishimura R**, **Sasaki N**. Effect of Retinoids on Growth Inhibition of Two Canine Melanoma Cell Lines. *J. Vet. Med. Sci.* 2001; **63**:83-86.

Ohashi E, Inoue K, Kagechika H, Hong SH, Nakagawa T, Takahashi T, Mochizuki M, *et al.* Effect of natural and synthetic retinoids on the proliferation and differentiation of three canine melanoma cell lines. *J Vet Med Sci*. 2002; **64**:169-172.

Okagawa T, Konnai S, Nishimori A, Ikebuchi R, Mizorogi S, Nagata R, Kawaji S, *et al.* Bovine immunoinhibitory receptors contribute to suppression of Mycobacterium avium subsp. paratuberculosis-specific T-cell responses. *Infect. Immun.* 2016a; **84**:77-89.

Okagawa T, Konnai S, Deringer JR, Ueti MW, Scoles GA, Murata S, Ohashi K, *et al.* Cooperation of PD-1 and LAG-3 contributes to T-cell exhaustion in Anaplasma marginale-infected cattle. *Infect. Immun.* 2016b; **84**:2779-2790.

Okagawa T, Konnai S, Nishimori A, Maekawa N, Ikebuchi R, Goto S, Nakajima C,

et al. Anti-bovine programmed death-1 rat-bovine chimeric antibody for immunotherapy of bovine leukemia virus infection in cattle. *Front. Immunol.* 2017; **8**:1-12.

Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, Cachola KE, *et al.* Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat. Med.* 2007; **13**:84-88.

Patel SP, **Kurzrock R**. PD-L1 Expression as a Predictive Biomarker in Cancer Immunotherapy. *Mol. Cancer Ther.* 2015; **14**:847-856.

Patnaik AK, **Ehler WJ**, **MacEwen EG**. Canine cutaneous mast cell tumor: morphologic grading and survival time in 83 dogs. *Vet. Pathol.* 1984; **21**:469-474.

Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, Lee W, *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science.* 2015; **348**:124-128.

Rodig N, Ryan T, Allen JA, Pang H, Grabie N, Chernova T, Greenfield EA, *et al.* Endothelial expression of PD-L1 and PD-L2 down-regulates CD8+ T cell activation and cytolysis. *Eur. J. Immunol.* 2003; **33**:3117-3126.

Rue SM, **Eckelman BP**, **Efe JA**, **Bloink K**, **Deveraux QL**, **Lowery D**, **Nasoff M**. Identification of a candidate therapeutic antibody for treatment of canine B-cell lymphoma. *Vet. Immunol. Immunopathol.* 2015; **164**:148-159.

Saitou N, **Nei M**. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 1987; **4**:406-425.

Saltz LB, **Meropol NJ**, **Loehrer PJ**, **Needle MN**, **Kopit J**, **Mayer RJ**. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. *J. Clin. Oncol.* 2004; **22**:1201-1208.

Sandusky GE, **Caiuton WW**, **Wightman KA**. Diagnostic Immunohistochemistry of Canine Round Cell Tumors Materials and Methods. *Vet. Pathol*. 1987; **24**:24495-24499.

Shosu K, Sakurai M, Inoue K, Nakagawa T, Sakai H, Morimoto M, Okuda M, *et al.*

Programmed Cell Death Ligand 1 Expression in Canine Cancer. *In Vivo*. 2016; **30**:195-204.

Singer J, Fazekas J, Wang W, Weichselbaumer M, Matz M, Mader A, Steinfellner W, *et al.* Generation of a Canine Anti-EGFR (ErbB-1) Antibody for Passive Immunotherapy in Dog Cancer Patients. *Mol. Cancer Ther.* 2014; **13**:1777-1790.

Smith SH, **Goldschmidt MH**, **McManus PM**. A comparative review of melanocytic neoplasms. *Vet. Pathol.* 2002; **39**:651-678.

Sorenmo KU, **Goldschmidt MH**, **Shofer FS**, **Goldkamp C**, **Ferracone J**. Evaluation of cyclooxygenase-1 and cyclooxygenase-2 expression and the effect of cyclooxygenase inhibitors in canine prostatic carcinoma. *Vet. Comp. Oncol.* 2004; **2**:13-23.

Spodnick GJ, Berg J, Rand WM, Schelling SH, Couto G, Harvey HJ, Henderson RA, *et al.* Prognosis for dogs with appendicular osteosarcoma treated by amputation alone. *J. Small Anim. Pract.* 1993; **34**:94.

Szewczyk M, **Lechowski R**, **Zabielska K**. What do we know about canine osteosarcoma treatment? - review. *Vet. Res. Commun.* 2015; **39**:61-67.

Sznol M, **Chen L**. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. *Clin. Cancer Res.* 2013; **19**:1021-1034.

Takahashi T, **Kitani S**, **Nagase M**, **Mochizuki M**, **Nishimura R**, **Morita Y**, **Sasaki N**. IgG-mediated histamine release from canine mastocytoma-derived cells. *Int. Arch. Allergy Immunol.* 2001; **125**:228-235.

Tamura K, **Peterson D**, **Peterson N**, **Stecher G**, **Nei M**, **Kumar S**. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 2011; **28**:2731-2739.

Tang L, **Sampson C**, **Dreitz MJ**, **McCall C**. Cloning and characterization of cDNAs encoding four different canine immunoglobulin γ chains. *Vet. Immunol. Immunopathol.* 2001; **80**:259-270.

Targan SR, Hanauer SB, van Deventer SJ, Mayer L, Present DH, Braakman T, DeWoody KL, *et al.* A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. *N. Engl. J. Med.* 1997; **337**:1029-1035.

Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, Chen L, *et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin. Cancer Res.* 2014; **20**:5064-5074.

Thommen DS, Schreiner J, Müller P, Herzig P, Roller A, Belousov A, Umana P, *et al.* Progression of lung cancer is associated with increased dysfunction of T cells defined by coexpression of multiple inhibitory receptors. *Cancer Immunol. Res.* 2015; **3**:1344-1355.

Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, Sengupta S, *et al.* Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. *Cancer Res.* 2006; **66**:3381-3385.

Thompson RH, **Dong H**, **Lohse CM**, **Leibovich BC**, **Blute ML**, **Cheville JC**, **Kwon ED**. PD-1 is expressed by tumor-infiltrating immune cells and is associated with poor outcome for patients with renal cell carcinoma. *Clin. Cancer Res.* 2007; **13**:1757-1761.

Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* 2012; **366**:2443-2454.

Veterinary Co-operative Oncology Group (VCOG). Veterinary Co-operative Oncology Group - Common Terminology Criteria for Adverse Events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.0. *Vet. Comp. Oncol.* 2004; **2**:195-213.

Wang Q, **Wu X**. Primary and acquired resistance to PD-1/PD-L1 blockade in cancer treatment. *Int. Immunopharmacol.* 2017; **46**:210-219.

Welle MM, **Bley CR**, **Howard J**, **Rüfenacht S**. Canine mast cell tumours: A review of the pathogenesis, clinical features, pathology and treatment. *Vet. Dermatol.* 2008; **19**:321-339.

Withrow SJ, Page R, Vail DM. Withrow and MacEwen's Small Animal Clinical Oncology. *Elsevier Health Sciences*. 2013.

Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbé C, Maio M, *et al.* Guidelines for the evaluation of immune therapy activity in solid tumors: Immune-related response criteria. *Clin. Cancer Res.* 2009; **15**:7412-7420.

Wong RM, **Scotland RR**, **Lau RL**, **Wang C**, **Korman AJ**, **Kast WM**, **Weber JS**. Programmed death-1 blockade enhances expansion and functional capacity of human melanoma antigen-specific CTLs. *Int. Immunol.* 2007; **19**:1223-1234.

Wood CA, **Moore AS**, **Gliatto JM**, **Ablin LA**, **Berg RJ**, **Rand WM**. Prognosis for dogs with stage I or II splenic hemangiosarcoma treated by splenectomy alone: 32 cases (1991-1993). *J. Am. Anim. Hosp. Assoc.* 1998; **34**:417-421.

Wu C, **Zhu Y**, **Jiang J**, **Zhao J**, **Zhang XG**, **Xu N**. Immunohistochemical localization of programmed death-1 ligand-1 (PD-L1) in gastric carcinoma and its clinical significance. *Acta Histochem.* 2006; **108**:19-24.

Zettlmeissl G, **Gregersen JP**, **Duport JM**, **Mehdi S**, **Reiner G**, **Seed B**. Expression and characterization of human CD4: Immunoglobulin fusion proteins. *DNA Cell Biol.* 1990; **9**:347-353.

Zhao S, **Zhu W**, **Xue S**, **Han D**. Testicular defense systems: immune privilege and innate immunity. *Cell. Mol. Immunol.* 2014; **11**:428-437.

SUMMARY IN JAPANESE

和文要旨

腫瘍はイヌの死因の約 3 割を占め、特に高齢のイヌにおいて臨床上の大きな問題となっている。イヌの腫瘍に対しては主に外科療法、放射線療法、および化学療法が適用されるが、侵襲や副作用、がん種による感受性の違い等の理由により制限を受ける場合も多く、これらの治療法に加えて新たな治療戦略の開発が望まれている。近年ヒトの医療では、免疫抑制受容体 Programmed cell death 1 (PD-1) およびそのリガンド PD-ligand 1 (PD-L1) を標的とした抗体医薬に代表される免疫チェックポイント阻害薬が、種々の悪性腫瘍に対し著効を示し、免疫療法が第 4 の治療法としての地位を確立しつつある。

PD-1 は主に活性化 T 細胞上に発現する共刺激受容体であり、リガンド PD-L1 または PD-L2 と結合するとサイトカイン産生や細胞増殖等の T 細胞の機能を抑制する。種々のヒト腫瘍では腫瘍細胞上に PD-L1 が高発現していること、また腫瘍浸潤リンパ球において PD-1 が高発現していることが報告され、PD-1/PD-L1 経路は腫瘍の免疫回避機構の一つとして認識されている。一方で PD-1/PD-L1 経路による免疫抑制は可逆的であり、抗 PD-1 抗体や抗 PD-L1 抗体を用いてこれらの因子の結合を阻害すると T 細胞応答が再活性化し、さらには抗腫瘍効果が得られることも明らかになっている。特に抗 PD-1 抗体は、悪性黒色腫、非小細胞性肺癌、腎細胞癌、古典的ホジキンリンパ腫、頭頚部癌において臨床試験によりその抗腫瘍効果が実証され、国内での製造販売が承認されている。しかし、イヌ腫瘍においては PD-1/PD-L1 経路に関する報告はなく、経路阻害が腫瘍治療に応用可能であるかは不明である。そこで本研究では、イヌ腫瘍に対する新規免疫療法を樹立することを最終目的として、イヌ PD-1 および PD-L1 の発現解析・機能解析と腫瘍治療への応用について *in vitro* および *in vivo* で検討を行った。

第 1 章では、まずイヌ *PD-1* および *PD-L1* 遺伝子の同定を行い、得られた遺伝子情報をもとにこれら因子の組み換えタンパク質を作製した。次に当研究室にて樹立された抗ウシ PD-L1 抗体がイヌ PD-L1 と交差反応し、PD-1 との結合を阻害することが明らかとなったため、この抗体を用いてイヌ腫瘍における PD-L1 の発現解析および結合阻害による機能解析を行った。その結果、イヌ悪性黒色腫、肥満細胞腫、腎細胞癌において PD-L1 が高発現していることが免疫組織化学染色により示され、また PD-1/PD-L1 経路阻害試験で腫瘍浸潤単核球からの Interferon-γ の産生が亢進した。

第 2 章では、どのようながん種で PD-L1 阻害による治療効果を得られるかを明らかにするため、免疫組織化学染色法により、種々のイヌ悪性腫瘍における PD-L1 の発現解析を行った。全 110 検体を検査した結果、口腔内悪性黒色腫で 90% (36/40)、骨

肉腫で 70% (7/10)、血管肉腫で 60% (6/10)、グレード III 肥満細胞腫で 60% (3/5)、乳腺腺癌で 80% (4/5)、前立腺癌で 60% (3/5)の症例において PD-L1 が発現していた。一方で扁平上皮癌や組織球性肉腫を含む他 7 種の腫瘍においては、5 例ずつの検討で PD-L1 の発現は認められなかった。

第 3 章では、臨床研究で複数回イヌに投与可能な抗体を作出するため、ラット抗 PD-L1 抗体の定常領域をイヌ抗体の定常領域に入れ替えたラット-イヌキメラ抗 PD-L1 抗体を作製した。イヌ PD-1/PD-L1 結合に対し十分な結合阻害効果を示したラット抗 PD-L1 モノクローナル抗体 2 クローン (4G12、6G7) のうち、6G7 は IgM クラスの抗体であったため、イヌキメラ IgG 型への変換で結合阻害活性が減弱する可能性があった。そこで 4G12、6G7 両方をイヌキメラ IgG 化し、結合阻害活性を評価したところ、イヌキメラ化 4G12 (c4G12) は十分な結合阻害活性を保持していたのに対し、イヌキメラ化 6G7 (c6G7) では明らかな活性の低下が認められた。したがって c4G12 を治療用抗体の候補として選定し、大量発現に向けて高発現細胞株の樹立を行った。メトトレキサートを用いた遺伝子増幅により、c4G12 を高発現する CHO-DG44 細胞株を作製・選別して、14 日間の振盪培養を行ったところ最大 700 mg/L のキメラ抗体を培養上清中に産生した。この発現系により作製した c4G12 は、イヌ末梢血単核球からのサイトカイン産生を増大させ、リンパ球の増殖能を亢進した。

第 4 章では、イヌキメラ抗 PD-L1 抗体 c4G12 を用いて腫瘍罹患犬に対する治療試験を行った。北海道大学動物医療センターに来院した口腔内悪性黒色腫罹患犬 (n = 7) および未分化肉腫罹患犬 (n = 2) に対し、c4G12 を 2 mg/kg あるいは 5 mg/kg にて 2 週間隔で静脈内投与して腫瘍サイズの変化を記録した。治療の結果、悪性黒色腫罹患犬 1 頭および未分化肉腫罹患犬 1 頭において明らかな腫瘍の退縮が認められた（部分奏功）。さらに肺転移のある別の悪性黒色腫罹患犬において、18 週の抗体治療により肺転移巣がほぼ完全に消失した。悪性黒色腫罹患犬のうち肺転移を認めた 5 頭においては、標準治療を受けた対照群と比較して、生存期間が有意に延長された (中央生存期間 治療群 96 日； 対照群 54 日、$p < 0.05$)。なお臨床研究の観察期間内（2016 年 3 月－2017 年 1 月）において、治療に関連すると思われる副作用はほとんど認められなかった。

以上の結果より、イヌ腫瘍においても PD-1/PD-L1 経路は免疫応答を抑制する機構として機能していること、またイヌ腫瘍は PD-1/PD-L1 経路を免疫回避機構として利用している可能性が示された。特に口腔内悪性黒色腫、骨肉腫、血管肉腫、肥満細胞腫等では PD-L1 の発現が高率に認められたことから、PD-1/PD-L1 を標的とした免疫療法により治療効果を得られる可能性がある。さらにイヌキメラ抗 PD-L1 抗体 c4G12 を用いた臨床試験では悪性黒色腫の 14.3% (1/7)、未分化肉腫の 50.0% (1/2) で客観的奏効を認めたことより、c4G12 はこれらの腫瘍に対する新たな治療薬となりうることが明らかとなった。

本研究により、PD-1/PD-L1 経路を標的とした免疫チェックポイント阻害薬がイヌの種々の悪性腫瘍に対する新たな免疫療法として利用可能であることが強く示唆された。そこでさらなる臨床研究を行い、その治療効果をさらに詳細に検討していく必要がある。今後 c4G12 を含む PD-1/PD-L1 阻害薬が単剤、さらには放射線療法や他の治療法との併用としてイヌの悪性腫瘍の治療に応用されることが期待される。