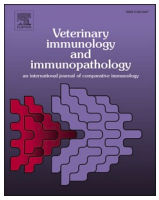
Veterinary Immunology and Immunopathology 261 (2023) 110609

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Molecular characterization of immunoinhibitory factors PD-1/PD-L1 in sheep

Wisa Tiyamanee [a], Satoru Konnai [a,b,c,*], Tomohiro Okagawa [b], Yutaro Nojima [a], Otgontuya Ganbaatar [a], Naoya Maekawa [b], Rie Hasebe [d], Yumiko Kagawa [e], Yukinari Kato [f,g], Yasuhiko Suzuki [a,c,h,i], Shiro Murata [a,b], Kazuhiko Ohashi [a,b,j]

[a] Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[b] Department of Advanced Pharmaceutics, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[c] Institute for Vaccine Research and Development (HU-IVReD), Hokkaido University, Sapporo, Japan
[d] Laboratory of Veterinary Hygiene, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[e] North Lab, Sapporo, Japan
[f] Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, Japan
[g] Department of Molecular Pharmacology, Tohoku University Graduate School of Medicine, Sendai, Japan
[h] Division of Bioresources, International Institute for Zoonosis Control, Hokkaido University, Sapporo, Japan
[i] Global Station for Zoonosis Control, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Sapporo, Japan
[j] International Affairs Office, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan

ARTICLE INFO

Keywords:
Sheep
Immunoinhibitory factors
PD-1
PD-L1

ABSTRACT

Sheep have been used as a large animal experimental model for studying infectious diseases. However, due to a lack of staining antibodies and reagents, immunological studies on sheep have not progressed. The immunoinhibitory receptor programmed death-1 (PD-1) is expressed on T lymphocytes. The interaction of PD-1 with its ligand PD-ligand 1 (PD-L1) delivers inhibitory signals and impairs proliferation, cytokine production, and cytotoxicity of T cells. We previously reported that the PD-1/PD-L1 pathway was closely associated with T-cell exhaustion and disease progression in bovine chronic infections using anti-bovine PD-L1 monoclonal antibodies (mAbs). Furthermore, we found that blocking antibodies against PD-1 and PD-L1 restore T-cell functions and could be used in immunotherapy of cattle. However, the immunological role of the PD-1/PD-L1 pathway in chronic diseases of sheep remains unknown. In this study, we identified cDNA sequences of ovine PD-1 and PD-L1 and examined the cross-activity of anti-bovine PD-L1 mAbs against ovine PD-L1 as well as the expression of PD-L1 in ovine listeriosis. The amino acid sequences of ovine PD-1 and PD-L1 share a high degree of identity and similarity with homologs from ruminants and other mammalian species. Anti-bovine PD-L1 mAb recognized ovine PD-L1 on lymphocytes in the flow cytometric assay. Furthermore, an immunohistochemical staining confirmed the PD-L1 expression on macrophages in the brain lesions of ovine listeriosis. These findings indicated that our anti-PD-L1 mAb would be useful for analyzing the ovine PD-1/PD-L1 pathway. Further research is needed to determine the immunological role of PD-1/PD-L1 in chronic diseases such as BLV infection through experimental infection of sheep.

1. Introduction

Programmed death-1 (PD-1) is an inhibitory receptor, also known as an immune checkpoint molecule, that is expressed on activated T cells and other lymphocytes (Agata et al., 1996). The binding of PD-1 and its ligand PD-ligand 1 (PD-L1) leads to the inhibition of T-cell receptor signaling and the subsequent effector functions of T cells, such as proliferation, cytokine production, and cytotoxic activity, which is called T-cell exhaustion (Carter et al., 2002; Freeman et al., 2000; Selenko-Gebauer et al., 2003). T-cell exhaustion caused by PD-1/PD-L1 interaction has been observed in human chronic and infectious diseases (Boni et al., 2007; Jurado et al., 2008; Muthumani et al., 2008; Penna et al., 2007). Many studies have demonstrated that blocking this interaction can restore T-cell activity when addressing immune exhaustion

* Corresponding author at: Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan.
E-mail address: konnai@vetmed.hokudai.ac.jp (S. Konnai).

https://doi.org/10.1016/j.vetimm.2023.110609
Received 5 March 2023; Received in revised form 2 May 2023; Accepted 10 May 2023
Available online 11 May 2023

via PD-1/PD-L1 (Barber et al., 2006; Day et al., 2006; Trautmann et al., 2006; Velu et al., 2009). Until now, there have been many therapeutic interventions approved by the Food and Drug Administration such as monoclonal antibody therapies targeting PD-1 (nivolumab, pembrolizumab, and cemiplimab) and PD-L1 (atezolizumab, durvalumab, and avelumab). Our previous studies on the PD-1/PD-L1 pathway in various animals revealed that this pathway mediates T-cell exhaustion on infectious diseases and cancers in cattle, dog, horse, and pig and anti-PD-1 and anti-PD-L1 monoclonal antibodies (mAbs) can restore T-cell function *in vitro* and *in vivo* (Ikebuchi et al., 2011, 2013; Okagawa et al., 2017; Nishimori et al., 2017; Maekawa et al., 2014, 2017; Ganbaatar et al., 2020, 2021).

Recently, experimental animal models have become widely used in biomedical research, including medical and veterinary fields, because *in vitro* models cannot fully mimic the complexity *in vivo* (Sartoretto et al., 2016). Among the animal models, sheep have been used as an experimental model for studying many types of infectious diseases and immunological disorders in human (Banstola and Reynolds, 2022; Gaudreault et al., 2022) as well as cattle (Begg et al., 2005; Porta et al., 2019). Sheep are one of the suitable experimental models for biomedical research due to their availability and societal acceptance as an experimental animal (Turner, 2007). Furthermore, sheep have interesting and outstanding biological features for many types of infectious diseases, and they reach the late stage of disease earlier than natural hosts (Porta et al., 2019). Thus, the use of sheep as an animal model will provide more opportunities for greater benefits in biomedical research to address the current state of immunological and infectious diseases, as they can provide faster results. However, a lack of staining antibodies and reagents affects to limited progress in immunological and infectious studies using the sheep models (Banstola and Reynolds, 2022). For example, a series of previous studies has reported the expression levels of *PD-1* and *PD-L1* mRNA in lungs and tracheobronchial lymph nodes of lambs infected with respiratory syncytial virus (Sow et al., 2011a, 2011b, 2012). Thus, there is potential interest in analyzing PD-1/PD-L1 to gain a deeper understanding of immune response in sheep, but protein expression analysis could not be performed due to the lack of the antibody reagents.

To fulfill the gap between the need for sheep as experimental models and the unavailability of reagents, in this study, we first identified the nucleotide sequence of ovine PD-1/PD-L1 mRNA, characterized these molecules using alignment and phylogenetic analyses, and then evaluated the possibility of anti-bovine PD-L1 mAbs for detecting and blocking ovine PD-L1.

2. Materials and methods

2.1. Sheep blood samples and cell preparation

Heparinized blood samples from Texel breed sheep were collected at the Experiment Farm, Field Science Center for Northern Biosphere, Hokkaido University (Sapporo, Japan). Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, UK), washed three times in phosphate-buffered saline (PBS), and suspended in PBS. All experimental procedures were conducted with the approval of the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (approval #20–0093).

2.2. Cloning of cDNA encoding of ovine PD-1 and PD-L1 (OvPD-1 and OvPD-L1)

Ovine PBMCs (4×10^6 cells) were cultured for 24 h with 20 ng/mL of phorbol 12-myristate acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) and 1 μg/mL of ionomycin (Sigma-Aldrich) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific) at 37 °C with 5% CO_2.

Total RNA was isolated from cultured PBMCs using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. Excess DNA was removed from RNA samples using deoxyribonuclease I (Thermo Fisher Scientific). cDNA was synthesized from 1 μg of total RNA using PrimeScript Reverse Transcriptase (Takara Bio, Otsu, Japan) and an oligo (dT) primer, according to the manufacturer's instructions.

Gene-specific primers were designed to amplify the OvPD-1 and OvPD-L1 genes, based on the sequences from sheep available on GenBank (XP_027819970 and XP_004004411) (Table 1). OvPD-1 and OvPD-L1 cDNAs were amplified from the cDNA templates by PCR using Ex Taq (Takara Bio) and the designed primers. The PCR products were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan) and cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). After being introduced to *Escherichia coli* HST08 Premium Competent Cells (Takara Bio), they were plated onto LB agar plates (Sigma-Aldrich) containing X-gal (Takara Bio) and ampicillin (Sigma-Aldrich). The purified plasmid clones were sequenced by a GenomeLab GeXP Genetic Analysis System (SCIEX, Framingham, MA, USA). The obtained sequences were aligned, and an unrooted neighbor-joining tree was created using the MEGA11 software (Molecular Evolutionary Genetics Analysis version 11) (Tamura et al., 2021).

2.3. Preparation of OvPD-1- and OvPD-L1-expressing cells

cDNA encoding OvPD-1 and OvPD-L1 were amplified by PCR using gene-specific primers with restriction enzyme cleavage sites and subcloned into the multicloning site of pEGFP-N2 (Clontech, Palo Alto, CA, USA). COS-7 cells were cultured at 37 °C and 5% CO_2 in RPMI 1640 medium (Sigma-Aldrich) which was supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Thermo Fisher Scientific). The cells were transfected with pEGPF-N2-OvPD-1 and pEGPF-N2-OvPD-L1 using Lipofectamine 2000 Reagent (Thermo Fisher Scientific) and cultivated for 48 h after transfection.

2.4. Expression and purification of soluble ovine PD-1 and PD-L1 proteins

The extracellular domain fragments of OvPD-1 and OvPD-L1 with their leader sequences were amplified by PCR using gene-specific primers with restriction enzyme cleavage sites (Table 1) to create the soluble OvPD-1 and OvPD-L1 proteins fused with rabbit IgG Fc region (OvPD-1-Ig and OvPD-L1-Ig) (Table 1). The amplicons were subcloned into the multicloning site of pCXN2.1(+) (kindly provided by Dr. T. Yokomizo, Juntendo University, Japan) (Niwa et al., 1991) with the

Table 1
Primer sequences used in this study.

Primer name	Sequence
Gene cloning	
PD-1 F	ATGGGGACCCCGCGGGCGCC
PD-1 R	TCAGAGGGGCCAGGAGCAGTGTCCA
PD-L1 F	ATGAGGATATATAGTGTCTTAACAT
PD-L1 R	TTACGTCTCCTCAAAATGTG
Plasmid construction for EGFP-tagged proteins	
PD-1 F *Bgl*II	GAAGATCTGTGGGGACCCCGCGGGCGCCG
PD-1 R *Sma*I	GACCCGGGGAGGGGCCAGGAGCAGTGTCC
PD-L1 F *Xho*I	CCGCTCGAGATGAGGATATATAGTGTCT
PD-L1 R *Sma*I	ATCCCGGGCGTCTCCTCAAAATGTGTAG
Plasmid construction for Ig fusion proteins	
PD-1 F *Nhe*I	ATAGCTAGCATGGGGACCCCGCGGGC
PD-1 R *Eco*RV	AGCTCGATATCGCCGATGACCAGGCTCTG
PD-L1 F *Nhe*I	GACGCTAGCATGAGGATATATAGTGTCT
PD-L1 R *Eco*RV	GCTCTGATATCCCTCGTTTTTGCTGGAT

Underlined; restriction enzyme recognition site

gene cassette encoding the Fc region of rabbit IgG. Transient cell lines expressing OvPD-1-Ig and OvPD-L1-Ig were established using an Expi293 Expression System (Thermo Fisher Scientific). Briefly, Expi293F cells were transfected with pCXN2.1(+)-OvPD-1-Ig and pCXN2.1(+)-OvPD-L1-Ig using Expifectamine (Thermo Fisher Scientific) and cultivated for 7 days in Expi293 medium (Thermo Fisher Scientific) at 37 °C and 125 rpm with 8% CO_2.

Purification of OvPD-1-Ig and OvPD-L1-Ig from the culture supernatants was achieved using affinity chromatography with an Ab-Capcher ExTra (ProteNova, Kagawa, Japan). The buffer of the purified fraction was exchanged for PBS by size exclusion chromatography with a PD-10 Desalting Column (GE Healthcare). The purity of OvPD-1-Ig and OvPD-L1-Ig was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reducing or nonreducing conditions using SuperSep Ace 5%−20% gradient polyacrylamide gel (FUJIFILM Wako Pure Chemical, Osaka, Japan) and 2 × Laemmli Sample Buffer (Bio-Rad, Hercules, CA, USA). Precision Plus Protein All Blue Standard (Bio-Rad) was used as a molecular-weight size marker. Quick-CBB (FUJIFILM Wako Pure Chemical) was used to visualized the proteins, and protein concentrations were determined using ultraviolet absorbance at 280 nm with a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific).

2.5. Binding assay of OvPD-1 and OvPD-L1

Binding of OvPD-1-Ig and OvPD-L1-Ig to COS-7 cells expressing OvPD-L1-EGFP and OvPD-1-EGFP was investigated using flow cytometry. OvPD-L1-EGFP cells or OvPD-1-EGFP cells were incubated at 37 °C for 30 min with 10 μg/mL of biotinylated OvPD-1-Ig or OvPD-L1-Ig, respectively. Biotinylated rabbit control IgG (Southern Biotech, Birmingham, AL, USA) was used as a negative control. Using a Lightning-Link Rapid Type A Biotin Conjugation Kit (Innova Biosciences, Cambridge, UK), OvPD-1-Ig, OvPD-L1-Ig, and rabbit control IgG were biotinylated. Cells were washed with PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich) and labeled using APC-conjugated streptavidin (BioLegend, San Diego, CA, USA) at 25 °C for 30 min. After rewashing, the cells were analyzed immediately by FACS Verse (BD Biosciences, San Jose, CA, USA).

2.6. Cross-reactivity assay of anti-bovine PD-L1 mAbs against OvPD-L1

OvPD-L1-EGFP cells were incubated with four clones of anti-bovine PD-L1 mAbs (4G12, 5A2, 6C11, 6G7) (Ikebuchi et al., 2014; Sajiki et al., 2018) at room temperature for 20 min to assess the binding ability of anti-bovine PD-L1 mAbs to OvPD-L1. Rat IgG_1 (R3–34, BD Biosciences), rat IgG_{2a} (R35–95, BD Biosciences), and rat IgM isotype controls (R4–22, BD Biosciences) were used for negative control staining. Cells were then washed with 1% BSA-PBS and labeled with APC-conjugated goat anti-rat immunoglobulin antibody (Southern Biotech) at room temperature for 20 min. After rewashing, the cells were analyzed immediately by FACS Verse (BD Biosciences).

Flow cytometry was performed to analyze stimulated ovine PBMCs. To assess the binding ability of anti-bovine PD-L1 mAbs to ovine immune cells, ovine PBMCs (4×10^6 cells) were cultured for 24 h with 20 ng/mL of PMA (Sigma-Aldrich) and 1 μg/mL of ionomycin (Sigma-Aldrich), as described above. To prevent nonspecific reactions, stimulated PBMCs were incubated at room temperature for 15 min in PBS supplemented with 10% goat serum (Thermo Fisher Scientific). Cells were washed and stained with anti-PD-L1 mAbs (5A2, rat IgG_1; 4G12, rat IgG_{2a}; 6C11, rat IgG_{2a}; 6G7, rat IgM) (Ikebuchi et al., 2014; Sajiki et al., 2018) at room temperature for 30 min. Moreover, rat IgG_1 (R3–34, BD Biosciences) and rat IgG_{2a} isotype control (R35–95, BD Biosciences) were used for negative control stainings. Cells were washed with PBS containing 1% BSA (Sigma-Aldrich) and labeled with APC-conjugated anti-rat Ig antibody (Southern Biotech) at room temperature for 30 min. After rewashing, the cells were analyzed immediately by FACS Verse (BD Biosciences).

2.7. Blockade assay of OvPD-1/OvPD-L1 interaction

Blocking assays were performed in microplates using OvPD-1-Ig and OvPD-L1-Ig to analyze the ability of anti-PD-L1 mAbs to block PD-1/PD-L1 binding. MaxiSorp Immuno Plates (Thermo Fisher Scientific) were coated with OvPD-1-Ig (1 μg/mL) in carbonate-bicarbonate buffer (Sigma-Aldrich) and blocked using SuperBlock T20 (PBS) Blocking Buffer (Thermo Fisher Scientific). Biotinylated OvPD-L1-Ig was pre-incubated with anti-PD-L1 mAb 6G7 (rat IgM) (Ikebuchi et al., 2014), 6C11 (rat IgG_{2a}) (Sajiki et al., 2018), 4G12 (rat IgG_{2a}) (Ikebuchi et al., 2014), rat IgM isotype control (Acris Antibodies, Herford, Germany), or rat IgG_{2a} isotype control (R35–95, BD Biosciences) at various concentrations (0, 1.25, 2.5, 5.0, 7.5, and 10 μg/mL) at 37 °C for 30 min. The preincubated reagents were added to the microplates and incubated at 37 °C for another 30 min. OvPD-L1-Ig binding was detected using horseradish peroxidase-conjugated Neutravidin (Thermo Fisher Scientific) and TMB One Component Substrate (Bethyl Laboratories, Montgomery, TX, USA). Further, optical density at 450 nm was measured by a microplate reader MTP-900 (Corona Electric, Hitachinaka, Japan). Three independent experiments were carried out in duplicate.

2.8. Immunohistochemical assay of PD-L1

Brain tissue was obtained from a sheep that naturally infected with *Listeria monocytogenes* in the Experiment Farm, Field Science Center for Northern Biosphere, Hokkaido University (Sapporo, Japan). This animal exhibited clinical neurological abnormalities and was diagnosed by pathological analyses as encephalitis due to intracerebral infection with *Listeria monocytogenes*. The tissues were fixed in formalin, embedded in paraffin wax, and cut into 4-μm-thick sections. The dried sections were deparaffinized in xylene before being hydrated with graded alcohols. Microwave heating for 10 min resulted in antigen retrieval using citrate buffer (0.37 g/mL citric acid and 2.4 g/mL trisodium citrate dihydrate). Endogenous peroxidase activity was blocked by incubating the sections for 15 min in methanol containing 0.3% hydrogen peroxide. The sections were incubated at room temperature for 30 min with anti-PD-L1 mAb (6C11, rat IgG_{2a}) (Sajiki et al., 2018), followed by incubation with Histofine simple stain MAXPO (rat) (Nichirei, Tokyo, Japan) at room temperature for 30 min. The reaction was visualized with 3-diaminobenzidine tetrahydrochloride. Moreover, immunostained sections were examined by an optical microscope.

2.9. Statistical analyses

The Wilcoxon signed-rank test or Turkey's test was used to identify significant differences. All statistical tests were performed using the ASTATSA (https://astatsa.com/) statistical analysis program. The statistical significance level was set as $p < 0.05$.

3. Results

3.1. Molecular cloning and sequence analysis of ovine PD-1/PD-L1

To begin, we identified cDNA sequences encoding OvPD-1 and OvPD-L1. Figs. 1A and 2A show the putative amino acid sequences of OvPD-1 and OvPD-L1, respectively. OvPD-1 and OvPD-L1 are type I transmembrane proteins with a putative signal peptide, an extracellular region, a transmembrane region, and an intracellular region. A conserved domain search revealed an immunoglobulin variable (IgV)-like domain in the extracellular regions of OvPD-1. Moreover, extracellular regions of OvPD-L1 were found to have Ig domains. The OvPD-1 had two structural motifs in its intracellular region: an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Phylogenetic analyses revealed that OvPD-1

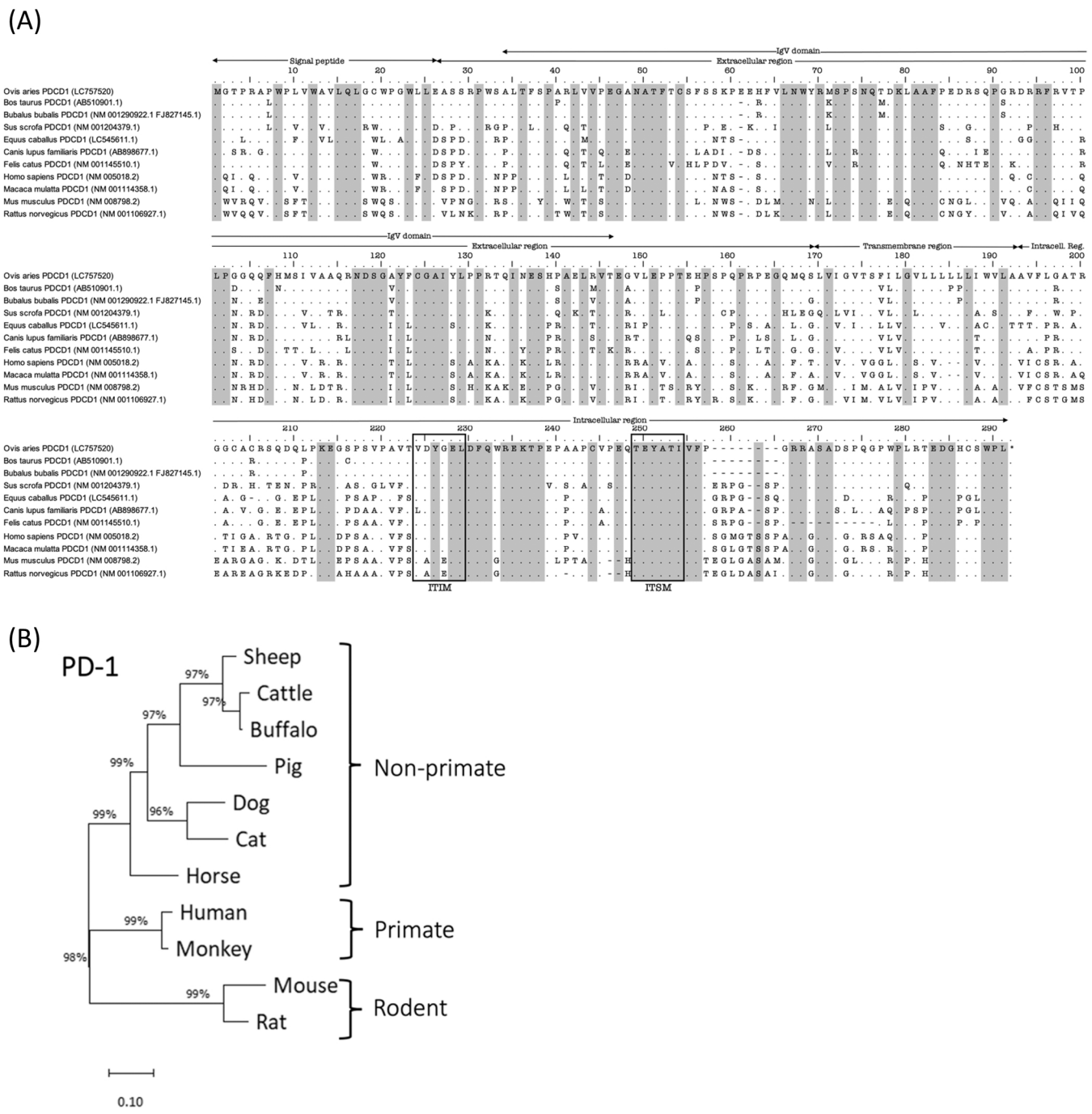


Fig. 1. Analysis of ovine PD-1 amino acid sequence. (A) Multiple alignment of the PD-1 predicted amino acid sequences of sheep, cattle, buffalo, pig, dog, cat, horse, human, monkey, mouse, and rat; GenBank accession numbers are shown in parentheses to the right of the species name. Lines represent the predicted signal peptide, IgV domain, intracellular region, transmembrane region, and intracellular region. ITIM and ITSM are encircled by rectangles. (B) Phylogenetic tree analysis of ovine PD-1 sequences in relation to other vertebrate species. The bootstrap consensus tree was inferred from 1000 replicates with neighbor-joining method using the MEGA11 software. The scale indicates the divergence time.

and OvPD-L1 were clustered into the non-primate group (Figs. 1B and 2B). OvPD-1 had 92.2%, 72.7%, 71.3%, 70.8%, 71.0%, 65.6%, and 51.7% amino acid similarities to cattle, pig, horse, dog, cat, human, and mouse, respectively (Table 2). Furthermore, OvPD-L1 had 94.1%, 81.7%, 78.9%, 77.5%, 75.6%, 74.2%, and 66.2% amino acid similarities to cattle, pig, horse, dog, cat, human and mouse, respectively (Table 2).

3.2. Interaction of OvPD-1 and OvPD-L1

Expi293 Expression System was used to develop soluble recombinant OvPD-1-Ig and OvPD-L1-Ig to analyze the interactions of the OvPD-1 and OvPD-L1 proteins. OvPD-1-Ig and OvPD-L1-Ig were successfully purified from culture supernatants and dimerization was confirmed (Fig. 3A). OvPD-1-Ig and OvPD-L1-Ig have predicted molecular weights of 42.4 kDa and 50.8 kDa under reduced conditions while 84.7 kDa and 101.7 kDa under nonreduced condition. Posttranslational modifications, such as glycosylation, would cause these proteins to have larger molecular weights than predicted.

Flow cytometry was performed to examine the interactions of OvPD-1-Ig or OvPD-L1-Ig with OvPD-L1-EGFP- or OvPD-1-EGFP-expressing

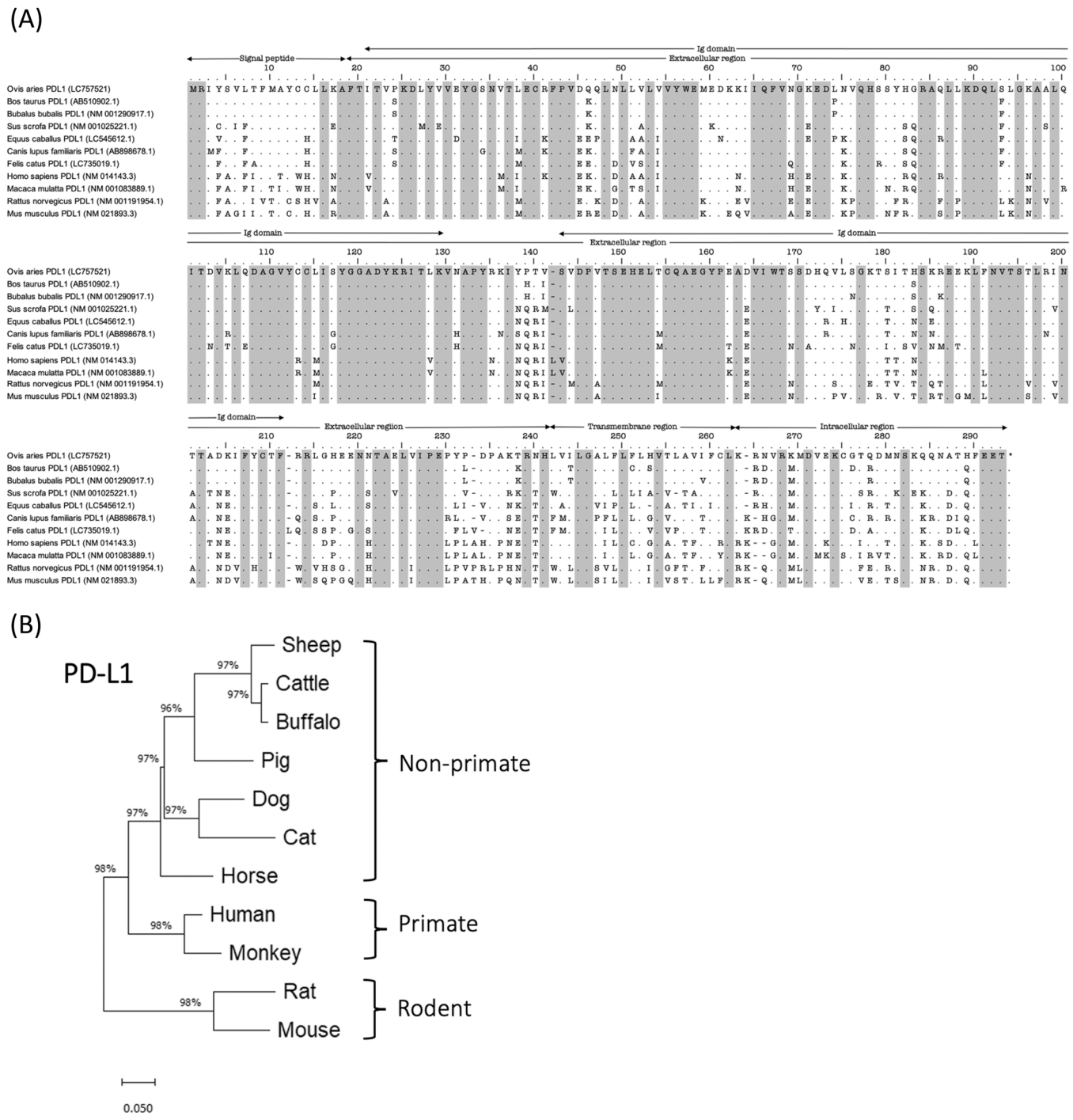


Fig. 2. Analysis of ovine PD-L1 amino acid sequence. (A) Multiple alignment of the PD-L1 predicted amino acid sequences of sheep, cattle, buffalo, pig, horse, dog, cat, human, monkey, rat, and mouse; GenBank accession numbers are shown in parentheses to the right of the species name. Lines represent the predicted signal peptide, Ig domain, extracellular region, transmembrane region and intracellular region. (B) Phylogenetic tree analysis of the ovine PD-L1 sequences in relation to other vertebrate species. The bootstrap consensus tree was inferred from 1000 replicates with neighbor-joining method using the MEGA11 software. The scale indicates the divergence time.

cells (Fig. 3B and 3C). The flow cytometric assay revealed that OvPD-1-Ig binding to OvPD-L1-EGFP-expressing cells is dependent on the expression level of OvPD-1-EGFP (Fig. 3B). OvPD-L1-Ig also binds to OvPD-1-EGFP-expressing cells in an expression-dependent manner (Fig. 3C).

3.3. Cross-reactivity of anti-bovine PD-L1 mAbs against OvPD-L1

We evaluated the cross-reactivity of our previously established anti-bovine PD-L1 mAbs (Ikebuchi et al., 2014; Sajiki et al., 2018) against OvPD-L1 and found that three of the four tested mAbs (4G12, 6C11, and 6G7) detected OvPD-L1-EGFP overexpressed on COS-7 cells (Fig. 4A). Of all the tested mAbs, 6C11 had the strongest fluorescent intensity to OvPD-L1-EGFP-expressing cells (Fig. 4A). We also tested the reactivity of 4G12, 6C11, and 6G7 mAbs against stimulated ovine PBMCs and found that 6C11 showed the strongest fluorescent intensity to stimulated PBMCs (Fig. 4B). Furthermore, the flow cytometric assay using the mAb 6C11 detected PD-L1 expressed in fresh ovine PBMCs which did not

Table 2
Similarities of amino acid sequences of PD-1 and PD-L1 among mammalian species.

Species	vs. Sheep (PD-1) (%)	vs. Sheep (PD-L1) (%)
Cattle	92.2	94.1
Pig	72.7	81.7
Horse	71.3	78.9
Dog	70.8	77.5
Cat	71.0	75.6
Human	65.6	74.2
Mouse	51.7	66.2

receive any stimulation (Fig. 4C).

3.4. Inhibition of OvPD-1/OvPD-L1 binding by anti-PD-L1 mAbs

We investigated the blocking activity of anti-bovine PD-L1 mAbs on the interaction of OvPD-1/OvPD-L1. Although 6C11 had strongest cross-reactivity against OvPD-L1, it does not significantly block OvPD-1 and OvPD-L1 binding. In contrast, 4G12 and 6G7 blocked OvPD-1 and OvPD-L1 binding in a dose-dependent manner (Fig. 5).

3.5. Immunohistochemical analysis of PD-L1 in Listeria monocytogenes

PD-L1 expression is upregulated in many types of infectious diseases in cattle and pigs (Ikebuchi et al., 2011; Sajiki et al., 2019; Okagawa et al., 2016a, 2016b; Goto et al., 2017, 2020; Ganbaatar et al., 2021). We hypothesized that PD-L1 expression is also upregulated in infectious disease of sheep. Therefore, we performed immunohistochemistry using the mAb 6C11 to analyze the expression of PD-L1 in an encephalitis lesion of sheep infected with *L. monocytogenes*. In ovine listeriosis, various immune cells accumulate in the brain lesions and macrophages are involved in the bacterial transfer from the periphery to the central nervous system and in the pathogenesis of encephalitis (Oevermann et al., 2009). PD-L1 was clearly detected in macrophages infiltrating the encephalitis lesion of this animal (Fig. 6), confirming the utility of 6C11 mAb for immunohistochemistry of PD-L1 protein in ovine tissue specimens.

4. Discussion

As therapeutic intervention for cancer and chronic disease, immunotherapy targeting immune checkpoint molecules has been widely used over the last decade. Many immune checkpoint molecules such as for example PD-1, CTLA-4, LAG-3, and TIM-3 have been identified and studied for cancer immunotherapy in human (He and Xu, 2020). In veterinary field, these molecules have been also studied in various infectious diseases in livestock (Ganbaatar et al., 2020, 2021; Goto et al., 2017; Ikebuchi et al., 2011, 2013; Nakamura et al., 2023; Okagawa et al., 2012, 2016a,b; Watari et al., 2019). Moreover, many studies revealed that the PD-1/PD-L1 pathway plays essential roles on T-cell exhaustion during infections (Buchbinder and Desai, 2016; Jubel et al., 2020). Therefore, this study first focused on PD-1/PD-L1 to gain a deeper understanding of immune checkpoint molecules of sheep.

Furthermore, immunotherapy with anti-PD-1 and anti-PD-L1 mAbs is effective in the treatment of different types of cancers in human and dogs (Robert et al., 2015; Weber et al., 2015; Brahmer et al., 2015; Garon et al., 2015; Maekawa et al., 2017, 2021). However, there have been numerous reports about limitations in clinical effects and observation of adverse effects by anti-PD-1 and PD-L1 mAbs due to a lack of

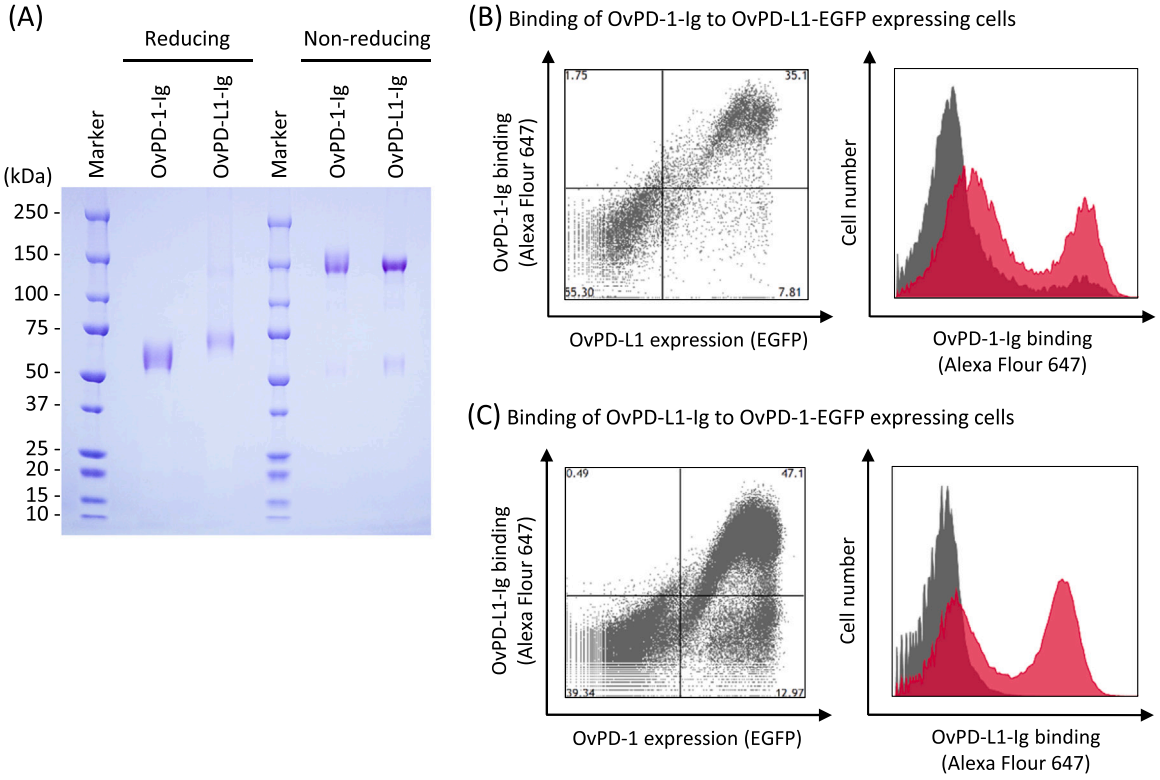


Fig. 3. Purification and interaction of OvPD-1 and OvPD-L1. (A) Expression and purification of ovine PD-1 and PD-L1 proteins. OvPD-1-Ig and OvPD-L1-Ig proteins were expressed by an Expi293 Expression System and purified from culture supernatant using affinity chromatography with an Ab-Capcher ExTra. SDS-PAGE and CBB staining were performed to evaluate the protein purity. (B) Binding of OvPD-1-Ig to OvPD-L1-EGFP-expressing cells was shown in a dotplot (left) and a histogram (right). In the histogram, the red area shows the binding of OvPD-1-Ig, whereas the gray area shows the binding of rabbit IgG (negative control). (C) Binding of OvPD-L1-Ig to OvPD-1-EGFP-expressing cells was shown in a dotplot (left) and a histogram (right). In the histogram, the red area shows the binding of OvPD-L1-Ig, whereas the gray area shows the binding of rabbit IgG (negative control).

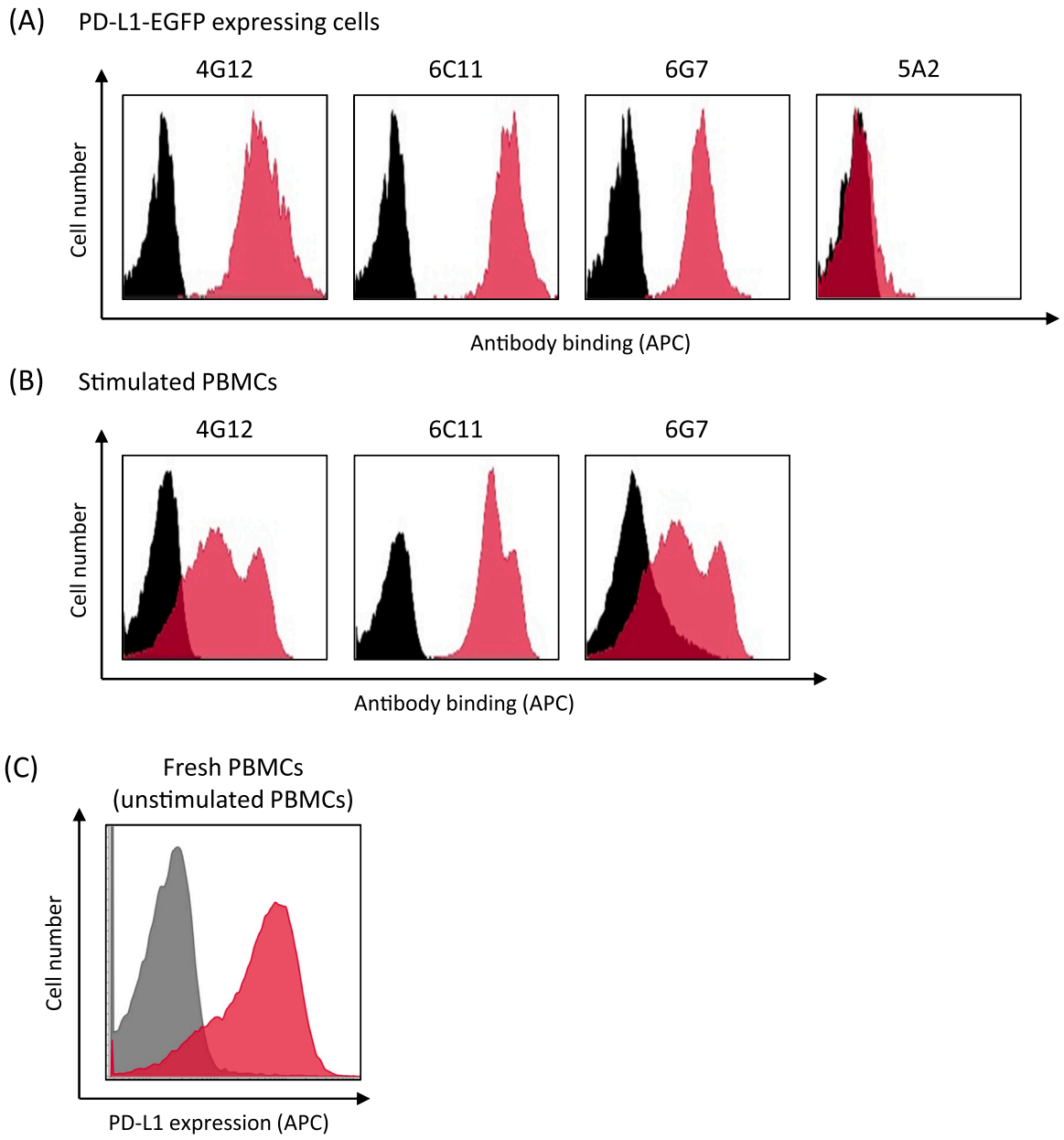


Fig. 4. Cross-reactivities of anti-bovine PD-L1 mAbs against OvPD-L1. (A–C) Cross-reactivities of four anti-bovine PD-L1 mAbs (5A2; rat IgG_1, 4G12 and 6C11; rat IgG_{2a}, 6G7; rat IgM) against OvPD-L1 were determined by flow cytometry using OvPD-L1-EGFP-expressing cells and ovine PBMCs. Isotype-matched control antibodies were used as negative control. In the histograms, the red area shows the binding of anti-bovine PD-L1 mAb, while the gray area shows the binding of isotype control. (A) Cross-reactivity of anti-bovine PD-L1 mAbs against OvPD-L1-expressing cells. (B) Cross-reactivity of the mAbs against PBMCs cultured for 24 h under PMA/ionomycin stimulation. (C) PD-L1 expression in fresh ovine PBMCs detected by flow cytometric analysis using anti-bovine PD-L1 mAb (6C11).

thorough understanding of these molecules (Higano et al., 2009; Kantoff et al., 2010; Topalian et al., 2012; Weber et al., 2017). We intend to further clarify the role of the PD-1/PD-L1 pathway by using sheep as a suitable animal experimental model, but basic information about ovine PD-1/PD-L1 pathway is still limited. Thus, we first identify and characterize the ovine PD-1 and PD-L1 sequences for further immunological studies in sheep.

In this study, we found that cDNA of ovine PD-1 and PD-L1 has high similarity of amino acid sequence to other species. Moreover, the ITIM and ITSM were conserved as the structural motifs of PD-1 that play roles in negative regulation in T-cell function (Chatterjee et al., 2013; Chemnitz et al., 2004; Okazaki et al., 2001). Thus, we hypothesized that ovine PD-1 and PD-L1 would function similarly to other species. Flow cytometric analyses showed that ovine PD-1 and PD-L1 can bind to each other in an expression-dependent manner, just like other species (Ganbaatar et al., 2020, 2021, Maekawa et al., 2014, 2016; Lin et al., 2008).

The results of cross-reactivities of anti-bovine PD-L1 mAbs against OvPD-L1 revealed that one of mAbs (6C11) strongly bound to OvPD-L1 in expressing cells and stimulated PBMCs. In comparison to 6C11 mAbs, other anti-bovine PD-L1 mAbs (4G12 and 6G7) showed moderate cross-reactivities. Furthermore, anti-bovine PD-L1 mAbs can interfere the binding of OvPD-1 and OvPD-L1 (Fig. 5), implying the ability of the mAbs to block this interaction.

We observed obvious accumulation of $PD\text{-}L1^+$ macrophages in the encephalitis lesion of sheep infected with *L. monocytogenes*. Macrophages are observed in the encephalitis lesions of ovine listeriosis from acute to chronic stages (Oevermann et al., 2009). In addition, infected macrophages appear to be involved in the bacterial transfer from the periphery to the central nervous system. Therefore, upregulation of

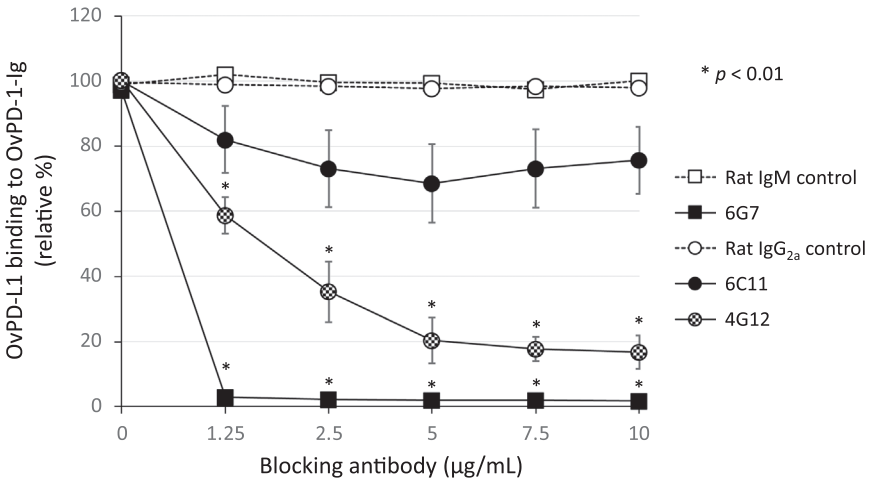


Fig. 5. Inhibition of ovine PD-1/PD-L1 binding by anti-PD-L1 mAbs. The blocking effect of anti-bovine PD-L1 mAbs on the binding of OvPD-L1-Ig to OvPD-1-Ig. Biotinylated OvPD-L1-Ig was preincubated with various concentrations of anti-PD-L1 mAb (6G7, 6C11, and 4G12) and then incubated in microwell plates coated with OvPD-1-Ig. Rat IgG_{2a} and IgM control antibodies were used as isotype-matched negative controls. Each value represents the relative binding of ovine PD-L1-Ig preincubated with blocking antibodies compared to that preincubated without the blocking antibody. Each value is the average determined from three independent experiments. Significant differences between each treatment were identified using Turkey's test. Asterisk (*) indicates $p < 0.01$.

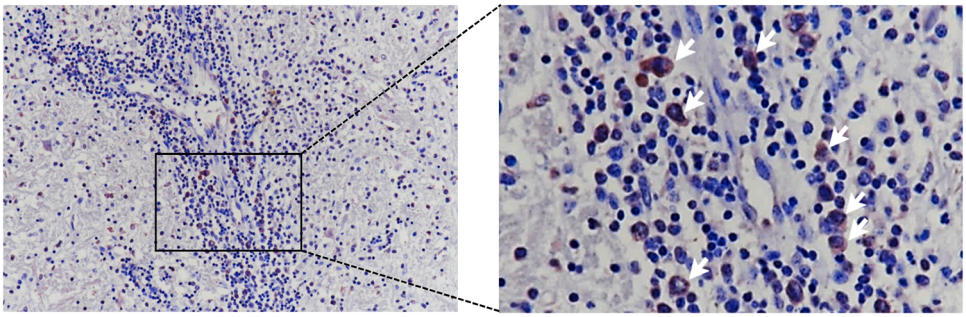


Fig. 6. Immunohistochemistry analysis of PD-L1 in *Listeria monocytogenes* infection in sheep. Immunohistochemical staining of PD-L1 in brain lesion of sheep with listeriosis using an anti-bovine PD-L1 mAbs (6C11) were performed. PD-L1 positive macrophages are shown with white arrows.

PD-L1 may be involved in immune evasion of infected macrophages during ovine listeriosis.

However, this study did not examine T-cell response are suppressed by the PD-1/PD-L1 signaling in sheep. Further investigations are required in the expression kinetics of PD-1 and PD-L1 and their association with T-cell exhaustion in infectious diseases of sheep. According to previous studies, blocking PD-1/PD-L1 binding with mAbs restores T-cell function and improves disease prognosis in humans, dogs, and cattle (John et al., 2013; Maekawa et al., 2017, 2021; Nishimori et al., 2017; Okagawa et al., 2017; Sajiki et al., 2020). Thus, additional experiments are needed to analyze the restoration of T-cell functions by PD-1/PD-L1 blockade in sheep, including the measurement of T-cell cytokines.

Lack of available reagents has limited immunological analysis of ruminants to a very limited extent. It would be very beneficial to develop detection antibodies that could be used for the analysis of such animals. This study provides an opportunity for immunological analysis focused on the PD-1/PD-L1 pathway in sheep. The achievements of this study will lead to progress in immunological analysis of diseases in sheep and increase their usefulness as model animals.

Funding

This work was supported by JSPS KAKENHI grant number 19KK0172, 22H02503, 22K19232 [to S.K.], 19K15993, and 22K15005 [to T.O.], grants from the Project of the NARO, Bio-oriented Technology Research Advancement Institution (Research Program on Development of Innovative Technology 26058 BC [to S.K.] and Special Scheme Project on Regional Developing Strategy, Grant 16817557 [to S.K.]), grants from Regulatory Research Projects for Food Safety, Animal Health and Plant Protection (JPJ008617.17935709 [to S.K.]), and supported by the Japan Agency for Medical Research and Development (AMED) under Grants JP223fa627005 [to S.K.], JP21am0101078 [to Y.K.] and JP22ama121008 [to Y.K.]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration of Competing Interest

All authors declare that they have no competing interests.

Acknowledgments

We are grateful to Dr. Hideyuki Takahashi, Dr. Yasuyuki Mori, and Dr. Tomio Ibayashi for their valuable advice and discussions. We would like to thank Enago (http://www.enago.jp) for the English language review.

References

Agata, Y., Kawasaki, A., Nishimura, H., Ishida, Y., Tsubata, T., Yagita, H., Honjo, T., 1996. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int. Immunol. 8, 765–772. https://doi.org/10.1093/intimm/8.5.765.

Banstola, A., Reynolds, J.N.J., 2022. The sheep as a large animal model for the investigation and treatment of human disorders. Biology 11, 1251. https://doi.org/10.3390/biology11091251.

Barber, D.L., Wherry, E.J., Masopust, D., Zhu, B., Allison, J.P., Sharpe, A.H., Freeman, G. J., Ahmed, R., 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 439, 682–687. https://doi.org/10.1038/nature04444.

Begg, D.J., O'Brien, R., Mackintosh, C.G., Griffin, J.F.T., 2005. Experimental infection model for Johne's disease in sheep. Infect. Immun. 73, 5603–5611. https://doi.org/10.1128/IAI.73.9.5603-5611.2005.

Boni, C., Fisicaro, P., Valdatta, C., Amadei, B., Vincenzo, P.D., Giuberti, T., Laccabue, D., Zerbini, A., Cavalli, A., Missale, G., Bertoletti, A., Ferrari, C., 2007. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. J. Virol. 81, 4215–4225. https://doi.org/10.1128/jvi.02844-06.

Brahmer, J., Reckamp, K.L., Baas, P., Crinò, L., Eberhardt, W.E.E., Poddubskaya, E., Antonia, S., Pluzanski, A., Vokes, E.E., Holgado, E., Waterhouse, D., Ready, N., Gainor, J., Frontera, O.A., Havel, L., Steins, M., Garassino, M.C., Aerts, J.G., Domine, M., Paz-Ares, L., Reck, M., Baudelet, C., Harbison, C.T., Lestini, B., Spigel, D.R., 2015. Nivolumab versus Docetaxel in advanced squamous-cell non–small-cell lung cancer. N. Engl. J. Med 373, 123–135. https://doi.org/10.1056/nejmoa1504627.

Buchbinder, E.I., Desai, A., 2016. CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition. Am. J. Clin. Oncol. 39, 98–106. https://doi.org/10.1097/COC.0000000000000239.

Carter, L.L., Fouser, L.A., Jussif, J., Fitz, L., Deng, B., Wood, C.R., Collins, M., Honjo, T., Freeman, G.J., Carreno, B.M., 2002. PD-1:PD-L inhibitory pathway affects both CD4 + and CD8+ T cells and is overcome by IL-2. Eur. J. Immunol. 32, 634–643. https://doi.org/10.1002/1521-4141(200203)32:3<634::AID-IMMU634>3.0.CO;2-9.

Chatterjee, P., Patsoukis, N., Freeman, G.J., Boussiotis, V.A., 2013. Distinct roles of PD-1 Itsm and ITIM in regulating interactions with SHP-2, ZAP-70 and Lck, and PD-1-mediated inhibitory function. Blood 122, 191. https://doi.org/10.1182/blood.v122.21.191.191.

Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H., Riley, J.L., 2004. 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. 173, 945–954. https://doi.org/10.4049/jimmunol.173.2.945.

Day, C.L., Kaufmann, D.E., Kiepiela, P., Brown, J.A., Moodley, E.S., Reddy, S., Mackey, E. W., Miller, J.D., Leslie, A.J., DePierres, C., Mncube, Z., Duraiswamy, J., Zhu, B., Eichbaum, Q., Altfeld, M., Wherry, E.J., Coovadia, H.M., Goulder, P.J.R., Klenerman, P., Ahmed, R., Freeman, G.J., Walker, B.D., 2006. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature 443, 350–354. https://doi.org/10.1038/nature05115.

Freeman, G.J., Long, A.J., Iwai, Y., Bourque, K., Chernova, T., Nishimura, H., Fitz, L.J., Malenkovich, N., Okazaki, T., Byrne, M.C., Horton, H.F., Fouser, L., Carter, L., Ling, V., Bowman, M.R., Carreno, B.M., Collins, M., Wood, C.R., Honjo, T., 2000. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J. Exp. Med. 192, 1027–1034. https://doi.org/10.1084/jem.192.7.1027.

Ganbaatar, O., Konnai, S., Okagawa, T., Nojima, Y., Maekawa, N., Minato, E., Kobayashi, A., Ando, R., Sasaki, N., Miyakoshi, D., Ichii, O., Kato, Y., Suzuki, Y., Murata, S., Ohashi, K., 2020. PD-L1 expression in equine malignant melanoma and functional effects of PD-L1 blockade. PLoS One 15, e0234218. https://doi.org/10.1371/journal.pone.0234218.

Ganbaatar, O., Konnai, S., Okagawa, T., Nojima, Y., Maekawa, N., Ichikawa, Y., Kobayashi, A., Shibahara, T., Yanagawa, Y., Higuchi, H., Kato, Y., Suzuki, Y., Murata, S., Ohashi, K., 2021. Programmed death-ligand 1 expression in swine chronic infections and enhancement of interleukin-2 production via programmed death-1/programmed death-ligand 1 blockade. Immun. Inflamm. Dis. 9, 1573–1583. https://doi.org/10.1002/iid3.510.

Garon, E.B., Rizvi, N.A., Hui, R., Leighl, N., Balmanoukian, A.S., Eder, J.P., Patnaik, A., Aggarwal, C., Gubens, M., Horn, L., Carcereny, E., Ahn, M.J., Felip, E., Lee, J.S., Hellmann, M.D., Hamid, O., Goldman, J.W., Soria, J.C., Dolled-Filhart, M., Rutledge, R.Z., Zhang, J., Lunceford, J.K., Rangwala, R., Lubiniecki, G.M., Roach, C., Emancipator, K., Gandhi, L., 2015. Pembrolizumab for the treatment of non–small-cell lung cancer. N. Engl. J. Med. 372, 2018–2028. https://doi.org/10.1056/nejmoa1501824.

Gaudreault, N.N., Cool, K., Trujillo, J.D., Morozov, I., Meekins, D.A., McDowell, C., Bold, D., Carossino, M., Balaraman, V., Mitzel, D., Kwon, T., Madden, D.W., Artiaga, B.L., Pogranichniy, R.M., Roman-Sosa, G., Wilson, W.C., Balasuriya, U.B.R., García-Sastre, A., Richt, J.A., 2022. Susceptibility of sheep to experimental co-infection with the ancestral lineage of SARS-CoV-2 and its alpha variant. Emerg. Microbes Infect. 11, 662–675. https://doi.org/10.1080/22221751.2022.2037397.

Goto, S., Konnai, S., Okagawa, T., Nishimori, A., Maekawa, N., Gondaira, S., Higuchi, H., Koiwa, M., Tajima, M., Kohara, J., Ogasawara, S., Kato, Y., Suzuki, Y., Murata, S., Ohashi, K., 2017. 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. 5, 355–363. https://doi.org/10.1002/iid3.173.

Goto, S., Konnai, S., Hirano, Y., Kohara, J., Okagawa, T., Maekawa, N., Sajiki, Y., Watari, K., Minato, E., Kobayashi, A., Gondaira, S., Higuchi, H., Koiwa, M., Tajima, M., Taguchi, E., Uemura, R., Yamada, S., Kaneko, M.K., Kato, Y., Yamamoto, K., Toda, M., Suzuki, Y., Murata, S., Ohashi, K., 2020. Upregulation of PD-L1 expression by prostaglandin E_2 and the enhancement of IFN-γ by anti-PD-L1 antibody combined with a COX-2 inhibitor in *Mycoplasma bovis* infection. Front. Vet. Sci. 7, 12. https://doi.org/10.3389/fvets.2020.00012.

He, X., Xu, C., 2020. Immune checkpoint signaling and cancer immunotherapy. Cell. Res. 30, 660–669. https://doi.org/10.1038/s41422-020-0343-4.

Higano, C.S., Schellhammer, P.F., Small, E.J., Burch, P.A., Nemunaitis, J., Yuh, L., Provost, N., Frohlich, M.W., 2009. Integrated data from 2 randomized, double-blind, placebo-controlled, phase 3 trials of active cellular immunotherapy with Sipuleucel-T in advanced prostate cancer. Cancer 115, 3670–3679. https://doi.org/10.1002/cncr.24429.

Ikebuchi, R., Konnai, S., Shirai, T., Sunden, Y., Murata, S., Onuma, M., Ohashi, K., 2011. 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. 42, 103. https://doi.org/10.1186/1297-9716-42-103.

Ikebuchi, R., Konnai, S., Okagawa, T., Yokoyama, K., Nakajima, C., Suzuki, Y., Murata, S., Ohashi, K., 2013. Blockade of bovine PD-1 increases T cell function and inhibits bovine leukemia virus expression in B cells in vitro. Vet. Res. 44, 59. https://doi.org/10.1186/1297-9716-44-59.

Ikebuchi, R., Konnai, S., Okagawa, T., Yokoyama, K., Nakajima, C., Suzuki, Y., Murata, S., Ohashi, K., 2014. Influence of PD-L1 cross-linking on cell death in PD-L1-expressing cell lines and bovine lymphocytes. Immunology 142, 551–561. https://doi.org/10.1111/imm.12243.

John, L.B., Devaud, C., Duong, C.P.M., Yong, C.S., Beavis, P.A., Haynes, N.M., Chow, M. T., Smyth, M.J., Kershaw, M.H., Darcy, P.K., 2013. Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. Clin. Cancer Res. 19, 5636–5646. https://doi.org/10.1158/1078-0432.CCR-13-0458.

Jubel, J.M., Barbati, Z.R., Burger, C., Wirtz, D.C., Schildberg, F.A., 2020. The role of PD-1 in acute and chronic infection. Front. Immunol. 11, 487. https://doi.org/10.3389/fimmu.2020.00487.

Jurado, J.O., Alvarez, I.B., Pasquinelli, V., Martínez, G.J., Quiroga, M.F., Abbate, E., Musella, R.M., Chuluyan, H.E., García, V.E., 2008. Programmed death (PD)-1:PD-ligand 1/PD-ligand 2 pathway inhibits T cell effector functions during human tuberculosis. J. Immunol. 181, 116–125. https://doi.org/10.4049/jimmunol.181.1.116.

Kantoff, P.W., Higano, C.S., Shore, N.D., Berger, E.R., Small, E.J., Penson, D.F., Redfern, C.H., Ferrari, A.C., Dreicer, R., Sims, R.B., Xu, Y., Frohlich, M.W., Schellhammer, P.F., 2010. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N. Engl. J. Med. 363, 411–422. https://doi.org/10.1056/nejmoa1001294.

Lin, D.Y., Tanaka, Y., Iwasaki, M., Gittis, A.G., Su, H.P., Mikami, B., Okazaki, T., Honjo, T., Minato, N., Garboczi, D.N., 2008. The PD-1/PD-L1 complex resembles the antigen-binding Fv domains of antibodies and T cell receptors. Proc. Natl. Acad. Sci. U. S. A. 105, 3011–3016. https://doi.org/10.1073/pnas.0712278105.

Maekawa, N., Konnai, S., Ikebuchi, R., Okagawa, T., Adachi, M., Takagi, S., Kagawa, Y., Nakajima, C., Suzuki, Y., Murata, S., Ohashi, K., 2014. Expression of PD-L1 on canine tumor cells and enhancement of IFN-γ production from tumor-infiltrating cells by PD-L1 blockade. PLoS One 9, e98415. https://doi.org/10.1371/journal.pone.0098415.

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., 2016. Immunohistochemical analysis of PD-L1 expression in canine malignant cancers and PD-1 expression on lymphocytes in canine oral melanoma. PLoS One 11, e0157176. https://doi.org/10.1371/journal.pone.0157176.

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., 2017. A canine chimeric monoclonal antibody targeting PD-L1 and its clinical efficacy in canine oral malignant melanoma or undifferentiated sarcoma. Sci. Rep. 7, 8951. https://doi.org/10.1038/s41598-017-09444-2.

Maekawa, N., Konnai, S., Nishimura, M., Kagawa, Y., Takagi, S., Hosoya, K., Ohta, H., Kim, S., Okagawa, T., Izumi, Y., Deguchi, T., Kato, Y., Yamamoto, S., Yamamoto, K., Toda, M., Nakajima, C., Suzuki, Y., Murata, S., Ohashi, K., 2021. PD-L1 immunohistochemistry for canine cancers and clinical benefit of anti-PD-L1 antibody in dogs with pulmonary metastatic oral malignant melanoma. NPJ Precis. Oncol. 5, 10. https://doi.org/10.1038/s41698-021-00147-6.

Muthumani, K., Choo, A.Y., Shedlock, D.J., Laddy, D.J., Sundaram, S.G., Hirao, L., Wu, L., Thieu, K.P., Chung, C.W., Lankaraman, K.M., Tebas, P., Silvestri, G., Weiner, D.B., 2008. Human immunodeficiency virus type 1 Nef induces programmed death 1 expression through a p38 mitogen-activated protein kinase-dependent mechanism. J. Virol. 82, 11536–11544. https://doi.org/10.1128/jvi.00485-08.

Nakamura, H., Konnai, S., Okagawa, T., Maekawa, N., Sajiki, Y., Watari, K., Kamitani, K., Saito, M., Kato, Y., Suzuki, Y., Murata, S., Ohashi, K., 2023. Combined immune checkpoint blockade enhances antiviral immunity against bovine leukemia virus. J. Virol. 97, e0143022 https://doi.org/10.1128/jvi.01430-22.

Nishimori, A., Konnai, S., Okagawa, T., Maekawa, N., Ikebuchi, R., Goto, S., Sajiki, Y., Suzuki, Y., Kohara, J., Ogasawara, S., Kato, Y., Murata, S., Ohashi, K., 2017. *In vitro* and in vivo antivirus activity of an anti-programmed death-ligand 1 (PD-L1) rat-bovine chimeric antibody against bovine leukemia virus infection. PLoS One 12, e0174916. https://doi.org/10.1371/journal.pone.0174916.

Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108, 193–200. https://doi.org/10.1016/0378-1119(91)90434-d.

Oevermann, A., Di, P.S., Doherr, M.G., Abril, C., Zurbriggen, A., Vandevelde, M., 2009. Neuropathogenesis of naturally occurring encephalitis caused by *Listeria monocytogenes* in ruminants. Brain Pathol. 20, 378–390. https://doi.org/10.1111/j.1750-3639.2009.00292.x.

Okagawa, T., Konnai, S., Ikebuchi, R., Suzuki, S., Shirai, T., Sunden, Y., Onuma, M., Murata, S., Ohashi, K., 2012. Increased bovine Tim-3 and its ligand expressions during bovine leukemia virus infection. Vet. Res. 43, 45. https://doi.org/10.1186/1297-9716-43-45.

Okagawa, T., Konnai, S., Nishimori, A., Ikebuchi, R., Mizorogi, S., Nagata, R., Kawaji, S., Tanaka, S., Kagawa, Y., Murata, S., Mori, Y., Ohashi, K., 2016a. Bovine immunoinhibitory receptors contribute to suppression of *Mycobacterium avium* subsp. *paratuberculosis*-specific T-cell responses. Infect. Immun. 84, 77–89. https://doi.org/10.1128/IAI.01014-15.

Okagawa, T., Konnai, S., Deringer, J.R., Ueti, M.W., Scoles, G.A., Murata, S., Ohashi, K., Brown, W.C., 2016b. Cooperation of PD-1 and LAG-3 contributes to T-cell exhaustion in *Anaplasma marginale*-infected cattle. Infect. Immun. 84, 2779–2790. https://doi.org/10.1128/IAI.00278-16.

Okagawa, T., Konnai, S., Nishimori, A., Maekawa, N., Ikebuchi, R., Goto, S., Nakajima, C., Kohara, J., Ogasawara, S., Kato, Y., Suzuki, Y., Murata, S., Ohashi, K., 2017. Anti-bovine programmed death-1 rat-bovine chimeric antibody for

immunotherapy of bovine leukemia virus infection in cattle. Front. Immunol. 8, 650. https://doi.org/10.3389/fimmu.2017.00650.

Okazaki, T., Maeda, A., Nishimura, H., Kurosaki, T., Honjo, T., 2001. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proc. Natl. Acad. Sci. U. S. A 98, 13866–13871. https://doi.org/10.1073/pnas.231486598.

Penna, A., Pilli, M., Zerbini, A., Orlandini, A., Mezzadri, S., Sacchelli, L., Missale, G., Ferrari, C., 2007. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. Hepatology 45, 588–601. https://doi.org/10.1002/HEP.21541.

Porta, N.G., Alvarez, I., Archilla, G.S., Ruiz, V., Abdala, A., Trono, K., 2019. Experimental infection of sheep with bovine leukemia virus (BLV): minimum dose of BLV-FLK cells and cell-free BLV and neutralization activity of natural antibodies. Rev. Argent. Microbiol. 51, 316–323. https://doi.org/10.1016/j.ram.2019.01.004.

Robert, C., Schachter, J., Long, G.V., Arance, A., Grob, J.J., Mortier, L., Daud, A., Carlino, M.S., McNeil, C., Lotem, M., Larkin, J., Lorigan, P., Neyns, B., Blank, C.U., Hamid, O., Mateus, C., Shapira-Frommer, R., Kosh, M., Zhou, H., Ibrahim, N., Ebbinghaus, S., Ribas, A., 2015. Pembrolizumab versus Ipilimumab in advanced melanoma. N. Engl. J. Med. 372, 2521–2532. https://doi.org/10.1056/nejmoa1503093.

Sajiki, Y., Konnai, S., Okagawa, T., Nishimori, A., Maekawa, N., Goto, S., Ikebuchi, R., Nagata, R., Kawaji, S., Kagawa, Y., Yamada, S., Kato, Y., Nakajima, C., Suzuki, Y., Murata, S., Mori, Y., Ohashi, K., 2018. Prostaglandin E_2 induction suppresses the Th1 immune responses in cattle with Johne's disease. Infect. Immun. 86, e00910–17. https://doi.org/10.1128/IAI.00910-17.

Sajiki, Y., Konnai, S., Okagawa, T., Nishimori, A., Maekawa, N., Goto, S., Watari, K., Minato, E., Kobayashi, A., Kohara, J., Yamada, S., Kaneko, M.K., Kato, Y., Takahashi, H., Terasaki, N., Takeda, A., Yamamoto, K., Toda, M., Suzuki, Y., Murata, S., Ohashi, K., 2019. Prostaglandin E_2-induced immune exhaustion and enhancement of antiviral effects by anti-PD-L1 antibody combined with COX-2 inhibitor in bovine leukemia virus infection. J. Immunol. 203, 1313–1324. https://doi.org/10.4049/jimmunol.1900342.

Sajiki, Y., Konnai, S., Cai, Z., Takada, K., Okagawa, T., Maekawa, N., Fujisawa, S., Kato, Y., Suzuki, Y., Murata, S., Ohashi, K., 2020. Enhanced immunotherapeutic efficacy of anti–PD-L1 antibody in combination with an EP4 antagonist. Immunohorizons 4, 837–850. https://doi.org/10.4049/immunohorizons.2000089.

Sartoretto, S.C., Uzeda, M.J., Miguel, F.B., Nascimento, J.R., Ascoli, F., Calasans-Maia, M. D., 2016. Sheep as an experimental model for biomaterial implant evaluation. Acta Ortop. Bras. 24, 262–266. https://doi.org/10.1590/1413-785220162405161949.

Selenko-Gebauer, N., Majdic, O., Szekeres, A., Höfler, G., Guthann, E., Korthäuer, U., Zlabinger, G., Steinberger, P., Pickl, W.F., Stockinger, H., Knapp, W., Stöckl, J., 2003. B7-H1 (programmed death-1 ligand) on dendritic cells is involved in the induction and maintenance of T cell anergy. J. Immunol. 170, 3637–3644.

Sow, F.B., Gallup, J.M., Olivier, A., Krishnan, S., Patera, A.C., Suzich, J., Ackermann, M. R., 2011a. Respiratory syncytial virus is associated with an inflammatory response in lungs and architectural remodeling of lung-draining lymph nodes of newborn lambs. Am. J. Physiol. Lung Cell Mol. Physiol. 300, L12–L24. https://doi.org/10.1152/ajplung.00169.2010.

Sow, F.B., Gallup, J.M., Krishnan, S., Patera, A.C., Suzich, J., Ackermann, M.R., 2011b. Respiratory syncytial virus infection is associated with an altered innate immunity and a heightened pro-inflammatory response in the lungs of preterm lambs. Respir. Res. 12, 106. https://doi.org/10.1186/1465-9921-12-106.

Sow, F.B., Gallup, J.M., Derscheid, R., Krishnan, S., Ackermann, M.R., 2012. Ontogeny of the immune response in the ovine lung. Immunol. Invest. 41, 304–316. https://doi.org/10.3109/08820139.2011.631657.

Tamura, K., Stecher, G., Kumar, S., 2021. MEGA11: molecular evolutionary genetics analysis version 11. Mol. Biol. Evol. 38, 3022–3027. https://doi.org/10.1093/molbev/msab120.

Topalian, S.L., Hodi, F.S., Brahmer, J.R., Gettinger, S.N., Smith, D.C., McDermott, D.F., Powderly, J.D., Carvajal, R.D., Sosman, J.A., Atkins, M.B., Leming, P.D., Spigel, D.R., Antonia, S.J., Horn, L., Drake, C.G., Pardoll, D.M., Chen, L., Sharfman, W.H., Anders, R.A., Taube, J.M., McMiller, T.L., Xu, H., Korman, A.J., Jure-Kunkel, M., Agrawal, S., McDonald, D., Kollia, G.D., Gupta, A., Wigginton, J.M., Sznol, M., 2012. Safety, activity, and immune correlates of anti–PD-1 antibody in cancer. N. Engl. J. Med 366, 2443–2454. https://doi.org/10.1056/nejmoa1200690.

Trautmann, L., Janbazian, L., Chomont, N., Said, E.A., Gimmig, S., Bessette, B., Boulassel, M.R., Delwart, E., Sepulveda, H., Balderas, R.S., Routy, J.P., Haddad, E.K., Sekaly, R.P., 2006. Upregulation of PD-1 expression on HIV-specific $CD8^+$ T cells leads to reversible immune dysfunction. Nat. Med. 12, 1198–1202. https://doi.org/10.1038/nm1482.

Turner, A.S., 2007. Experiences with sheep as an animal model for shoulder surgery: strengths and shortcomings. J. Shoulder Elb. Surg. 16, 158S–163S. https://doi.org/10.1016/j.jse.2007.03.002.

Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., Vanderford, T.H., Chennareddi, L., Silvestri, G., Freeman, G.J., Ahmed, R., Amara, R.R., 2009. Enhancing SIV-specific immunity in vivo by PD-1 blockade. Nature 458, 206–210. https://doi.org/10.1038/nature07662.

Watari, K., Konnai, S., Maekawa, N., Okagawa, T., Suzuki, Y., Murata, S., Ohashi, K., 2019. Immune inhibitory function of bovine CTLA-4 and the effects of its blockade in IFN-γ production. BMC Vet. Res. 15, 380. https://doi.org/10.1186/s12917-019-2082-7.

Weber, J.S., D'Angelo, S.P., Minor, D., Hodi, F.S., Gutzmer, R., Neyns, B., Hoeller, C., Khushalani, N.I., Miller, W.H., Lao, C.D., Linette, G.P., Thomas, L., Lorigan, P., Grossmann, K.F., Hassel, J.C., Maio, M., Sznol, M., Ascierto, P.A., Mohr, P., Chmielowski, B., Bryce, A., Svane, I.M., Grob, J.J., Krackhardt, A.M., Horak, C., Lambert, A., Yang, A.S., Larkin, J., 2015. Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol. 16, 375–384. https://doi.org/10.1016/S1470-2045(15)70076-8.

Weber, J.S., Hodi, F.S., Wolchok, J.D., Topalian, S.L., Schadendorf, D., Larkin, J., Sznol, M., Long, G.V., Li, H., Waxman, I.M., Jiang, J., Robert, C., 2017. Safety profile of nivolumab monotherapy: a pooled analysis of patients with advanced melanoma. J. Clin. Oncol. 35, 785–792. https://doi.org/10.1200/JCO.2015.66.1389.