Research for the development of novel control methods for bovine leukemia virus infection

牛白血病ウイルス感染症に対する
新規制御法開発のための研究

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CONTENTS

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AGID	agar gel immunodiffusion
AL	aleukemic
ANOVA	analysis of variance
APC	allophycocyanin
ATL	adult T-cell leukemia
BCR	B-cell receptor
BLV	bovine leukemia virus
Boch4G12	anti-bovine PD-L1 rat-bovine chimeric antibody 4G12
B-SBL	B-cell-type sporadic bovine leukosis
cEBL	classic enzootic bovine leukosis
CFSE	carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese hamster ovary
ConA	concanavalin A
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Cy	cyanin
DDW	double distilled water
DHFR	dihydrofolate reductase
EBL	enzootic bovine leukosis
EDTA	ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FLK	fetal lamb kidney
FLK-BLV	BLV-infected fetal lamb kidney
HBV	hepatitis B virus
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HTLV-1	human T-cell leukemia virus type 1
IFN	interferon
Ig	immunoglobulin
IgH	immunoglobulin heavy chain

LAG-3	lymphocyte activation gene 3
LN	lymph node
LTR	long terminal repeat
mAb(s)	monoclonal antibody(antibodies)
MHC	major histocompatibility complex class
MTX	methotrexate
NK	natural killer
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing 0.05% Tween 20
PCR	polymerase chain reaction
PCR-DB	polymerase chain reaction directly from whole blood
PD-1	programmed death 1
PD-1-Ig	Fc fusion protein of PD-1
PD-L1	programmed death ligand 1
PD-L2	programmed death ligand 2
PD-L2-Ig	Fc fusion protein of PD-L2
PE	phycoerythrin
pEBL	polyclonal enzootic bovine leukosis
PL	persistent lymphocytosis
RACE	rapid amplification of cDNA ends
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
SBL	sporadic bovine leukosis
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TBE	Tris-borate-ethylenediaminetetraacetic acid
TIM-3	T-cell immunoglobulin and mucin domain 3
T-SBL	T-cell-type sporadic bovine leukosis

NOTES

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PREFACE

Bovine leukemia virus (BLV) belongs to the *Deltaretrovirus* genus of the *Retroviridae* family, and commonly infects host B cells. During BLV infection, approximately 60–70% of infected cattle become asymptomatic carriers at what is called the aleukemic (AL) stage. However, after a few months to years of this asymptomatic period, nearly 30% of infected cattle develop persistent lymphocytosis (PL), and then <5% develop lymphoma, which is a lethal form of this disease (Fig. 1A) [Gillet *et al.*, 2007; Gutiérrez *et al.*, 2014]. The clinical condition in BLV-infected cattle is characterized by an increase in the number of circulating B-lymphocytes (>10,000 cells/μl in peripheral blood), and it has been found that the lymphoma occurs predominantly in adult cattle >3–5 years old [Gutiérrez *et al.*, 2014; Tsutsui *et al.*, 2016].

BLV is transmitted into a new animal through the transfer of BLV-positive cells in the blood or milk and probably via blood-sucking insects [Ooshiro *et al.*, 2013]. Moreover, BLV infection occurs from mother-to-child *in utero* or in the birth canal to the low or moderate extent [Rodríguez *et al.*, 2011; Mekata *et al.*, 2015]. Experimental transmissions of BLV have been reported in various types of species, such as rabbit, rats, chickens, pigs, goats, and sheep; however, only sheep develop leukemia and, thus, are often used as a model of this disease [Gillet *et al.*, 2007; Merimi *et al.*, 2009; Lairmore, 2014].

Lymphoma occurred in a small fraction of BLV-infected cattle as a lethal stage of the infection is called enzootic bovine leukosis (EBL), but sporadic bovine leukosis (SBL) is not associated with BLV and has an unknown etiology. Both of them are characterized as neoplastic lymphocytosis and systemic lymphoma, and finally leads to the death of the animals. Although EBL has been eradicated in certain European countries [Nuotio *et al.*, 2003; Acaite *et al.*, 2007; Maresca *et al.*, 2015], BLV infection is still prevalent worldwide. The prevalence of BLV varies in each country; 81.9% in Iran [Morovati *et al.*, 2012], 37.4% in Canada [VanLeeuwen *et al.*, 2005], 0% to 65% (average 19.8%) in Colombia [Benavides *et al.*, 2013], 5.3% to 87.8% (average 26.2%) in Thailand [Lee *et al.*, 2016], 77.6% to 85.7% in Brazil [Rajão *et al.*, 2014], and 14.0 to 42.3% in Russia [Ruzina *et al.*, 2013]. In Japan, where the number of EBL cases has been increasing recently, a nationwide survey conducted from 2009 to 2011 indicated high seroprevalence of BLV in both dairy and beef cattle (40.9% and 28.7%,

respectively) [Murakami *et al*., 2013]. Since the Act on Domestic Animal Infectious Diseases Control in Japan dictates that cattle with bovine leukemia found in farms or meat hygiene inspection centers must be slaughtered, BLV infection exerts a severe influence on farm management.

Even though all infected cattle do not develop bovine lymphoma, BLV infection can cause an economic loss for farmers. In several studies, it has been demonstrated that BLV infection was linked to decreased milk production in dairy cattle although most of infected cattle are asymptomatic [Norby *et al*., 2016; Yang *et al*., 2016]. Moreover, the reduction in cellular immune responses, such as down-regulation of type 1 helper T cell cytokines, is observed in BLV-infected cattle [Kabeya *et al*., 2001]. In addition, the number of regulatory T cells and percentages of cells producing transforming growth factor-β were increased in BLV-infected cattle, inducing the functional declines of $CD4^+$ T cells and natural killer (NK) cells during the infection [Ohira *et al*., 2016]. Thus, those immune disorders in both of adaptive and innate immunity play major roles in disease progression of BLV infection and may lead to increased susceptibility to other infections.

Several reports have elucidated the mechanisms of immunosuppression during BLV infection, but their details remain unknown [Frie *et al*., 2015]. Recent studies have investigated the contribution of immunoinhibitory molecules to ensuing immune disorder in BLV-infected cattle. Cell surface immunoinhibitory receptors, such as programmed death1 (PD-1), lymphocyte activation gene3 (LAG-3), T cell immunoglobulin and mucin domain 3 (TIM-3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), are negative regulators of T cell activation and proliferation. These receptors which are expressed on activated T cells and B cells induce immune suppression through binding to their ligands, for example, PD-ligand 1 (PD-L1) and 2 (PD-L2) for PD-1 signaling, which are involved in the dysfunction of antigen-specific T cells called “T-cell exhaustion” (Fig. 1B) [Keir *et al*., 2008; Wherry, 2011].

The PD-1/PD-Ls interaction normally works as a negative feedback system inhibiting excessive immune responses, with PD-1 knockout mice developing autoimmune diseases such as lupus-like arthritis and glomerulonephritis [Nishimura *et al*., 1999]. However, this mechanism is often exploited by several chronic diseases to avoid immune elimination by the hosts. For instance, the expression of PD-L1 has been reported in many human cancers, including melanoma, squamous cell carcinoma, urothelial carcinoma, and solid tumors in the lung, ovary, kidney, pancreas, stomach,

and colon [Dong *et al*., 2002; Strome *et al*., 2003; Thompson *et al*., 2004; Wu *et al*., 2006; Inman *et al*., 2007; Nakanishi *et al*., 2007; Nomi *et al*., 2007]. In addition, PD-1 upregulation has been demonstrated in tumor-infiltrating T lymphocytes [Ahmadzadeh *et al*., 2009]. Importantly, it has been shown that PD-L1 expression is strongly correlated with a progressive stage and worse prognosis, indicating its relationship with disease outcome [Thompson *et al*., 2004; Wu *et al*., 2006; Inman *et al*., 2007; Nakanishi *et al*., 2007; Nomi *et al*., 2007].

PD-1 and PD-Ls also play a key role in the failure to eliminate pathogens during chronic infections. Exhausted T cells in mice which are chronically infected with lymphocytic choriomeningitis virus express high levels of PD-1, explaining the mechanism of T-cell dysfunction in this infection [Barber *et al*., 2006]. In addition, the upregulation of PD-1 has been reported on $CD8^+$ T cells, which are specific to human immunodeficiency virus (HIV), hepatitis B virus (HBV), and human T-cell leukemia virus type 1 (HTLV-1) [Day *et al.*, 2006; Trautmann *et al*., 2006; Boni *et al*., 2007; Kozako *et al*., 2009; Kozako *et al*., 2011]. The PD-1 pathway may also contribute to insufficient clearance during bacterial and parasitic infections. For instance, during *Helicobacter pylori* infection, which causes chronic gastritis, PD-L1 is upregulated on human gastric epithelial cells, suggesting that it contributes to the inhibition of the T-cell response against *H. pylori* [Das *et al*., 2006]. In addition, the upregulation of PD-L2 in macrophages has been reported in *Taenia crassiceps* [Terrazas *et al*., 2005], *Schistosoma mansoni* [Smith *et al*., 2004] and *Mycobacterium tuberculosis* infections [Jurado *et al*., 2008; Mendoza-Coronel *et al*., 2011]. Thus, the PD-1 pathway appears to be a common mechanism of immune evasion in many chronic diseases.

Several studies revealed that upregulation of the immunoinhibitory receptors on T cells is closely associated with disease progression of BLV infection, suggesting that they play critical roles in the suppression of cellular immune responses during the infection [Shirai *et al*., 2011; Okagawa *et al*., 2012; Ikebuchi *et al*., 2013; Konnai *et al*., 2013; Suzuki *et al*., 2015; Konnai *et al*., 2017]. In addition, PD-L1 and major histocompatibility complex class (MHC) II, which are ligands of LAG-3, were also upregulated on B cells from BLV-infected cattle [Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2013]. Therefore, the pathways of PD-1/PD-Ls or other immunoinhibitory molecules are important to lead to immunosuppression in cattle during BLV infection.

Recently, the blockade of the immunoinhibitory receptors has emerged as a successful treatment concept, reactivating antigen-specific T-cell response (Fig. 1B)

[Ott *et al.*, 2013]. Ipilimumab and tremelimumab are fully human monoclonal antibodies (mAbs) targeting CTLA-4 and have exhibited antitumor activity in patients with malignant melanoma, which lead to complete or partial response in some patients [Hodi *et al*., 2010; Robert *et al.*, 2011]. Furthermore, fully human mAbs targeting PD-1, BMS-936558 (also known as Nivolumab), and PD-L1, BMS-936559, have shown striking response against melanoma and other cancers in large phase I studies [Brahmer *et al.*, 2012; Topalian *et al.*, 2012a]. A chimeric antibody is a genetically engineered antibody which is composed of heterogeneous variable regions and host IgG constant regions, and can reduce host response against antibody itself when administered into hosts, contributing its long-term use for the treatment. Although there is no human chimeric mAb against immunoinhibitory molecules, an anti-human CD20 mouse-human chimeric mAb, Rituximab, exerted antitumor activities in patients with malignant B-cell lymphoma and is now commercially available [Maloney *et al.*, 1997]. On the other hand, a recombinant protein which binds to the immunoinhibitory receptors is another approach to prevent the interaction between those molecules, and currently GlaxoSmithKline conducts phase I study of Fc fusion protein of human PD-L2 (PD-L2-Ig), AMP-224, as the third agent targeting PD-1/PD-Ls interaction. [Topalian *et al.*, 2012b; Sunshine *et al.*, 2015]. Since previous reports revealed the strong association of PD-1 pathway with BLV infection, PD-1/PD-L1 blockade can be applied for the treatment in this infection.

Although BLV infection exerts many negative influence, there is no effective vaccine and therapeutic method against this infection currently, and hence novel methods are required for the control of BLV in the field. Therefore, in this study, several strategies for the development of novel control methods of BLV infection were proposed and investigated. For this goal, in Chapter I, a rapid diagnosis method was established for the early detection and eradication of BLV infection, which contributes to the prevention of virus spreading. In Chapter II, a field survey of cattle having bovine leukemia was conducted to clarify current status on EBL onset in Japan, providing the immunophenotypic features which might be conducive to understand the mechanism of tumor development. Finally, in Chapter III, two biomedicines for cattle targeting ligands of PD-1 were established with the aim of developing novel therapeutic methods against cattle at the PL and EBL stages of the BLV infection.

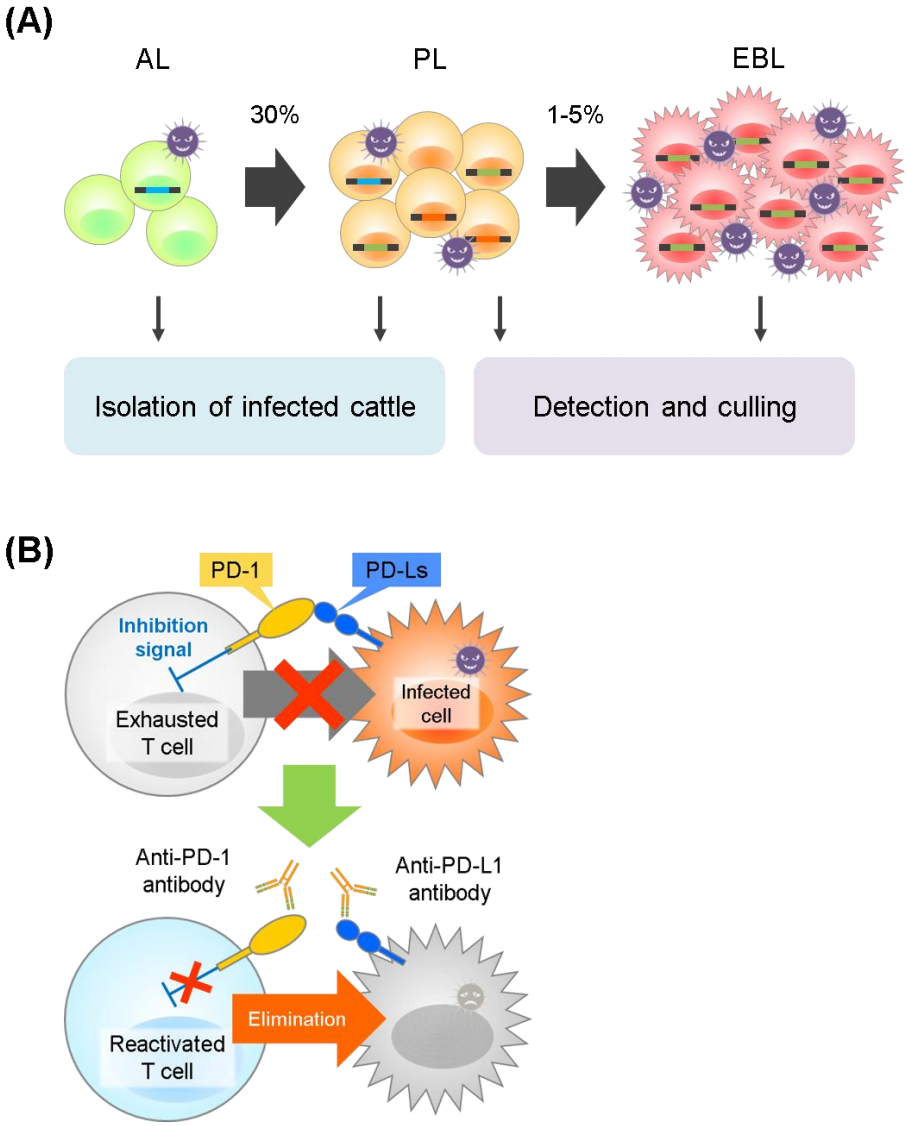

Figure 1 Disease progression during BLV infection and T-cell exhaustion by PD-1/PD-Ls interaction. (A) Stages of disease progression during BLV infection. After cattle are infected with BLV, virus genome is inserted into host genome as provirus. Although most of infected cattle become asymptomatic carriers (AL), approximately 30% of them develop lymphocytosis (PL), and 1 to 5% develop B-cell lymphoma (EBL). In the early stage of infection, the isolation of infected cattle is effective to prevent virus spreading, but once developed lymphoma, the cattle should be slaughtered. (B) Molecular mechanism of T-cell exhaustion during chronic infection. PD-1 expressed on T cells impair their effector function through the interaction with PD-Ls. The blockade of the PD-1 pathway can restore the exhausted status and reactivate the response of antigen-specific T cells, resulting in the elimination of pathogens.

CHAPTER I

Direct polymerase chain reaction from blood and tissue samples for rapid diagnosis of BLV infection

INTRODUCTION

BLV is an oncogenic retrovirus which mainly infects B cells in cattle and sheep. Because EBL which occurs in 1−5% of infected cattle is lethal disorder, BLV infection leads to negative effects on farm management. However, there is no effective therapeutic strategy or vaccine against BLV infection available. Thus, currently, the only countermeasure for the possible development of EBL is early detection and eradication of BLV-infected cattle before BLV transmission. In Denmark, a country-wide government initiative of detecting and culling all BLV-infected cattle resulted in the elimination of BLV in the country [Gillet *et al*., 2007]. However, in many countries, including Japan, BLV is widely spread with high prevalence rates.

Screening for BLV-infected cattle is critical to freeing a farm or region of BLV infection. However, most of BLV-infected cattle do not show clinical symptoms, and hence field veterinarians cannot detect them through routine examination practices [Florins *et al*., 2007]. Thus, molecular biological techniques are required for the detection of BLV. The major methods for identifying an infected carrier are agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA), which detect antibodies specific to a BLV antigen in serum or milk, and nested polymerase chain reaction (PCR) and real-time PCR, which amplify the BLV provirus inserted in host genome. In Sweden, where is free of BLV, ELISA test is applied to BLV detection in milk samples in an ongoing surveillance program [Emanuelson *et al*., 1992]. However, problems for AGID and ELISA tests are that their sensitivity and specificity are poor compared to those of PCR test [Trono *et al*., 2001; Kohara *et al*., 2006], and that the detection of anti-BLV antibody using serum from calves <6 months old is not appropriate because of the presence of maternal antibodies. Thus, nested PCR and real-time PCR are now regarded as the most sensitive methods for detecting the active infection.

Although PCR methods are suitable for the diagnosis of BLV infection, contamination of a small amount of DNA templates is frequent, in particular, in nested PCR or in screening of thousands of samples. Nested PCR is a labor-intensive method when a large number of cattle should be screened since it requires many experimental steps to obtain results, such as DNA purification, first round PCR, second round PCR and electrophoresis. To reduce those experimental steps and risks for cross contamination of samples, in this chapter, a novel PCR method for amplifying BLV

provirus without DNA extraction and second round PCR was developed, named PCR directly from whole blood, PCR-DB. This method facilitates the diagnosis of BLV infection without special techniques and is more suitable than nested PCR for first screening of thousands of cattle.

MATERIALS AND METHODS

Blood samples

Bovine blood samples were obtained from several farmers and veterinarians for the diagnosis of BLV infection at the Hokkaido University Veterinary Teaching Hospital (Sapporo, Japan). Informed consent was obtained from each owner, and approval for all procedures was obtained from the Institutional Animal Care and Use Committee of Hokkaido University (Experiment No. 11-0059). DNA was purified from 500 µl blood samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol and finally suspended in 50 µl DNA Rehydration Solution (Promega).

Cells

Bovine B-lymphoma cell line, KU-1 cells, which are infected with BLV [Onuma *et al*., 1986] were maintained at 37°C in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cell Culture Technologies, Gravesano, Switzerland) and 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). To compare the sensitivity of PCR-DB with that of nested PCR, blood samples not infected with BLV that contained various numbers of KU-1 cells (10^2–10^7 cells/1,000 µl of blood; 10^{-1}–10^4 cells/µl) were used as PCR-DB templates, and DNA purified from those blood samples was used as nested PCR templates.

PCR-DB

Amplification of BLV provirus from whole blood used KOD FX Neo (Toyobo, Osaka, Japan), which is superior for the amplification from crude samples, and a specific primer pair, PV2-F 5′-ACT TTC AGA CCC CCT TGA CTG ACA-3′ and PV2-R 5′-AAA CCT CTG CCC TGG TGA TTA AGG-3′. This primer pair was designed by Primer3 to amplify BLV provirus (GeneBank accession number: both K02120 and AF033818), and the intron region of the provirus (3308−3580 in K02120) was amplified by the primer pair. Briefly, each of 30 µl reaction mixture containing 0.4 mM dNTPs, 0.5 µM of primers, 1 U of KOD FX Neo and 1 µl of whole blood (10-, 50- or 100-fold dilutions in double distilled water (DDW); Fig. I-2). Amplifications were

performed under the following conditions: one lysis cycle at 94 °C for 2 min and then 45 cycles of template denaturation at 94 °C for 15 sec followed by annealing and extension at 68 °C for 50 sec. All experiments were carried out in duplicate or sextuplicate. The *β-globin* gene was amplified as an internal control using the following primer pairs: 5′-TGC TGA CTG CTG AGG AGA AGG CTG-3′ and 5′-GTC CTC ACA CGC CCA GGT GCA TTT C-3′. The amplicons were confirmed by electrophoresis in an ethidium bromide-stained 2% Tris- acetate-EDTA (TAE) agarose gel.

Nested PCR

To amplify the long terminal repeat (LTR) in the BLV provirus, nested PCR was performed using rTaq (TaKaRa Bio, Otsu, Japan) as previously described [Ikebuchi *et al*., 2011]. Briefly, the *BLV LTR* gene was amplified using primer pairs, BLV-LTR1 5′-TGT ATG AAA GAT CAT GCC GAC-3′ and BLV-LTR533 5′-AAT TGT TTG CCG GTC TCT-3′ for the first PCR, and 1.5 µl of DNA extracted from whole blood or BLV-uninfected blood with KU-1 cells were used as templates. And then, 1.5 µl of the first PCR products were re-amplified using BLV-LTR256 5′-GAG CTC TCT TGC TCC CGA GAC-3′ and BLV-LTR453 5′-GAA ACA AAC GCG GGT GCA AGC CAG-3′ for the second PCR. The conditions for both PCRs were shown as follows: incubation at 94 °C for 5 min, followed by amplification of template for 35 cycles of 94 °C 30 sec, 55 °C 30 sec and 72 °C 30 sec with the final extension at 72 °C for 7 min [Konnai *et al*., 2013a]. The *β-globin* gene was amplified as an internal control using the following primer pairs: PCO3 5′-ACA CAA CTG TGT TCA CTA GC-3′ and PCO4 5′-CAA CTT CAT CCA CGT TCA CC-3′ [Tajima *et al*., 2003]. The amplicons were confirmed by electrophoresis in an ethidium bromide-stained 2% TAE agarose gel.

Real-time PCR

To confirm the provirus loads of BLV- infected cattle diagnosed by nested PCR, real-time PCR was performed using a real-time PCR system (LightCycler 480 system II; Roche Diagnostics, Mannheim, Germany), SYBR Premix Dimer-Eraser (TaKaRa Bio), and the primers PV2-F and PV2-R for the BLV gene and PCO3 and PCO4 for *β-globin* gene. Amplification of DNA samples from whole blood was performed as follows: one cycle at 95 °C for 30 sec, followed by a 3-step PCR procedure consisting of 5 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C for 45 cycles. To obtain a standard

curve, serial dilutions of the standard plasmid containing from 10^7 to 10^1 copies were prepared and stored at −20°C until use.

Tumor samples

To diagnose bovine leukemia by using PCR-DB, tumor cells from three cattle with clinically diagnosed as lymphoma were collected: case No. 1 (Holstein-Friesian, 4 months old, EBL), case No. 2 (Japanese Black, 5 years old, EBL) and case No. 3 (Holstein-Friesian, 2 years old, SBL, thymic lymphosarcoma). In order to identify cell types of the lymphomas for supporting the clinical diagnosis, the tumor cells were stained with an antibody specific to B cell markers and analyzed by flow cytometry as described previously [Ikebuchi *et al*., 2013; Ikebuchi *et al*., 2014a]. In brief, double staining was performed using anti-IgM (IL-A30; Bio-Rad, Hercules, CA, USA) pre-labeled with Zenon Alexa Fluor 488 (Thermo Fisher Scientific) and the following antibodies: anti-WC4 (CC55; CD19-like; Bio-Rad) and anti-CD21 (GB25A; WSU Monoclonal Antibody Center, Pullman, WA, USA). Alexa Fluor 647-conjugated anti-mouse IgG (Thermo Fisher Scientific) was used for bound antibody detection (anti-WC4 and anti-CD21). Concisely, tumor cells were incubated with anti-WC4 and anti-CD21 as the first antibody, Alexa Fluor 647-conjugated anti-mouse IgG as the second antibody, and anti-IgM pre-labeled with Zenon Alexa Fluor 488 as the third antibody. Binding of the antibodies was detected using FACS Verse (BD Biosciences, San Jose, CA, USA) and FCS Express 4 (De Novo Software, Glendale, CA, USA). BLV infection was diagnosed from blood and tumor cells by nested PCR as described above. Approximately 1–2 mm^3 of tumor tissue suspended in 1 ml of phosphate-buffered saline (PBS, pH7.2) was used as a template for PCR-DB.

RESULTS

PCR-DB amplification of BLV provirus from whole blood

An optimal primer pair for the polymerase of PCR-DB was designed following the manufacturer's protocol. The PV2 primer pair was determined from seven ones based on their ability to show the most sensitive and reproducible results (data not shown). To confirm the amplification of BLV provirus from whole blood, PCR-DB was performed using blood samples of cattle for which BLV infection had been previously diagnosed by a conventional method. Single bands approximately 270 bp were found using the PV2 primer pair, with the results of PCR-DB amplification completely consistent with those of nested PCR (Fig. I-1). The amplification of the *β-globin* gene was observed as bands of approximately 100 bp in all samples, although a second band of approximately 400 bp was also produced in PCR-DB using the *β-globin* primer pair (data not shown).

PCR-DB results are reproducible with sample dilution

To compare the sensitivity of nested PCR and PCR-DB methods, BLV provirus was amplified from KU-1 cells mixed in blood from healthy cattle (Fig. I-2A). In sextuplicate reactions, the provirus was detected by nested PCR even from samples containing 0.1 or 1 KU-1 cell per 1 μl of blood (average level of undetectable cell numbers: 0.083 cells/μl; Fig. I-2B). PCR-DB amplification using DNA samples as templates was also performed to confirm the effects of its primers and polymerase on assay sensitivity. The results showed a similar sensitivity to that of nested PCR (0.067 cells/μl; Fig. I-2b). However, the results of PCR-DB used undiluted blood samples indicated less sensitivity and reproducibility. On the other hand, PCR-DB performed under variable conditions, with blood samples diluted 10-, 50- and 100-fold with DDW, showed improved reproducibility, though the sensitivity was lower than that for undiluted samples (0.55, 5.5 and 25 cells/μl, respectively; Fig. I-2C).

PCR-DB diagnosis of most BLV-infected cattle which showed detectable provirus loads

A total of 225 bovine blood samples were tested by nested PCR and PCR-DB. Whole blood was diluted 50-fold with DDW for PCR-DB, representing the middle condition tested in Fig. I-2C. Using both methods, 37 samples were positive, and 176

samples were negative, with no samples which were positive by PCR-DB and negative by nested PCR, thus indicating a PCR-DB specificity of 100% (Table I-1). The provirus in 12 samples was detected only using nested PCR and not with PCR-DB, indicating a PCR-DB sensitivity of 75.5%. Because those false-negative results are probably due to the very low levels of the infection, provirus loads of clinical samples which were positive by nested PCR were measured by real-time PCR. The results showed that all provirus loads of those 12 samples were quite low, suggesting that the animals were asymptomatic carriers at the aleukemic stage (Fig. I-3).

PCR-DB directly detected BLV provirus in tumor samples

To investigate whether PCR-DB can be used to diagnose BLV infection from tumor tissues, the provirus in tumor samples was amplified using PCR-DB. In the three sample cases, two cases were BLV-positive tumor tissues, and all PCR-DB results were entirely consistent with those of nested PCR (Fig. I-4). Flow cytometry analysis showed that case No. 1, a BLV-positive tumor, expressed several B-cell markers, such as IgM, WC4 (CD19-like) and CD21 on the cell membrane, and on the other hand, case No. 3, which was BLV-negative, did not show B-cell phenotypes (data not shown). These results strongly supported the clinical diagnosis, cases No.1 and No.2 as EBL and case No. 3 as thymic lymphosarcoma of SBL.

Table I-1 Amplification of BLV provirus in clinical blood samples.

PCR Result	Nested PCR Positive	Nested PCR Negative	Total
PCR-DB Positive	37	0	37
PCR-DB Negative	12	176	188
Total	49	176	225

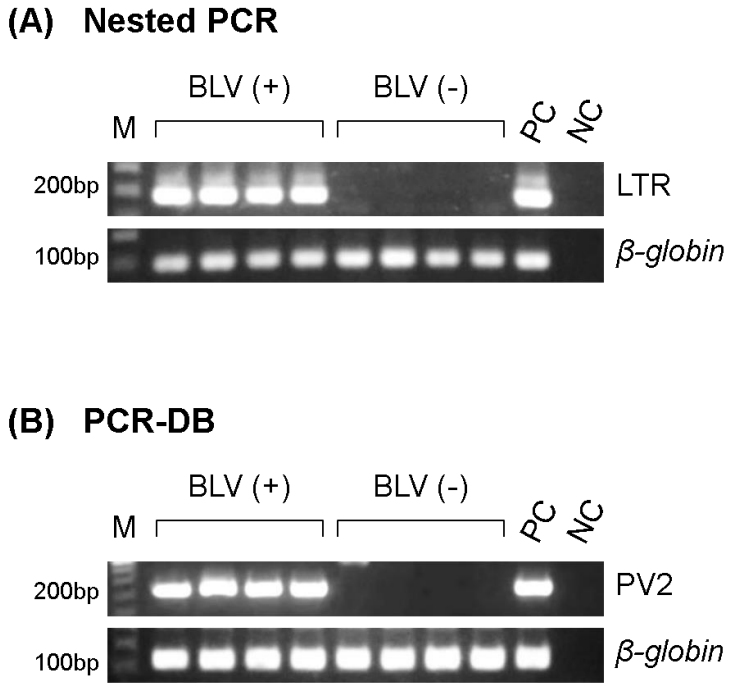

Figure I-1 Amplification of the BLV provirus from whole blood. Amplification of BLV provirus was performed by (A) nested PCR and (B) PCR-DB. Blood samples were collected from cattle with (n = 4) and without (n = 4) BLV infections diagnosed in advance. DNA samples purified from individual blood samples or whole blood were used as templates for nested PCR and PCR-DB, respectively. The *β-globin* gene was amplified as an internal control. For both of nested PCR and PCR-DB, DNA samples purified from BLV-positive cattle were used as a positive control (PC), and DDW was used as a negative control (NC).

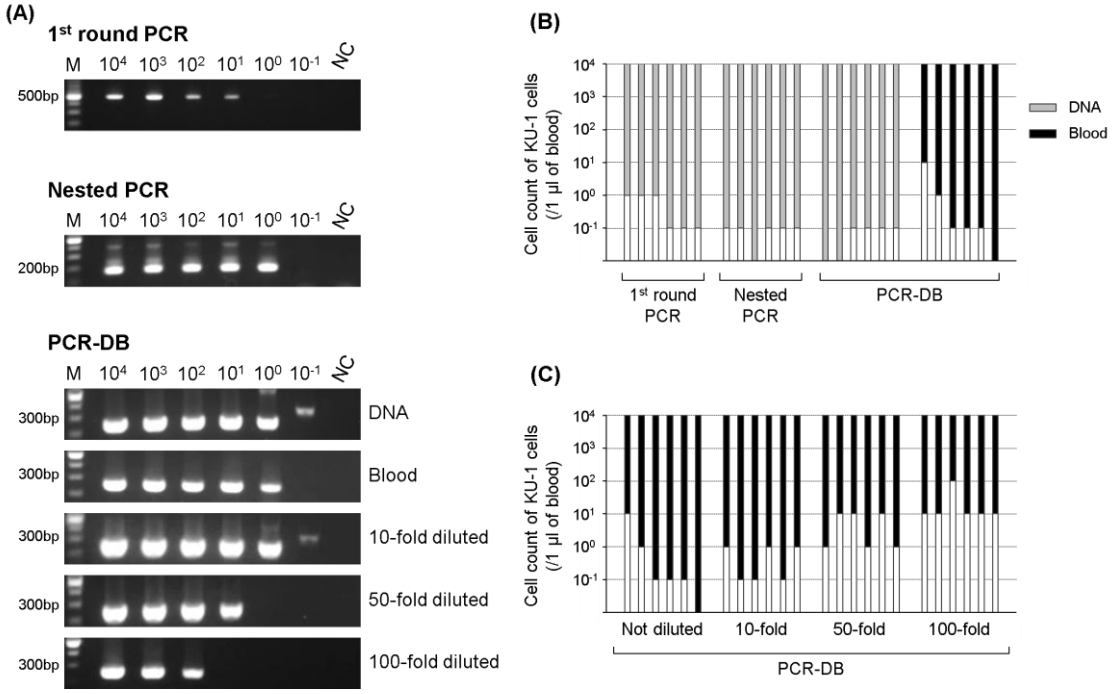

Figure I-2 Comparison of sensitivity of nested PCR and PCR-DB. To assess the sensitivity of each PCR method, DNA samples purified from BLV-uninfected blood containing KU-1 cells (10^{-1}–10^4 cells/µl) were used as nested PCR templates, and those blood samples were used as PCR-DB templates (1-, 10-, 50- or 100-fold diluted in DDW). (A) Representative images of electrophoresis indicating amplicons generated by each PCR condition. The numbers along the top indicate KU-1 cell counts per 1 µl blood. (B and C) The results of detectable samples using each PCR condition. White bars indicate KU-1-cell counts which were undetectable in electrophoresis, while the gray (DNA) and black (blood) bars indicate the templates used for each PCR condition. All amplification procedures were performed in sextuplicate.

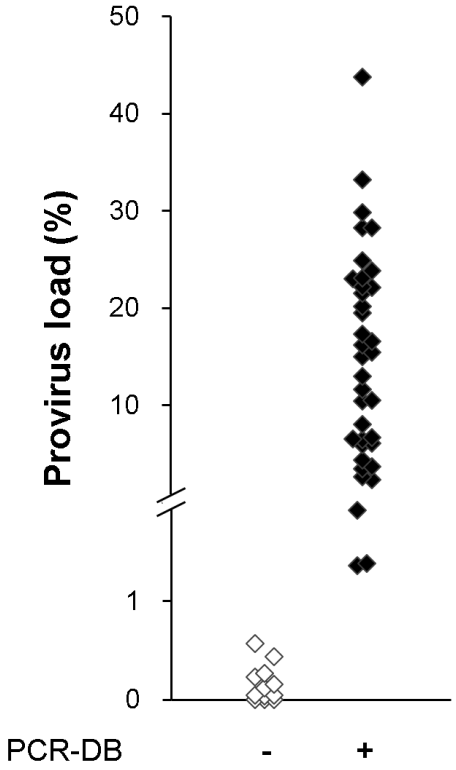

Figure I-3 BLV provirus loads for clinical samples which were positive by nested PCR. DNA samples extracted from BLV-infected cattle which were diagnosed by nested PCR were used for evaluation for determining provirus loads by real-time PCR (*n* = 49). The Y-axis indicates the rates of BLV copies in 100 cells as determined by copy numbers of *β-globin* amplicon. The dot colors depict the PCR-DB results (white: negative; black: positive). Each amplification procedure was performed in duplicate.

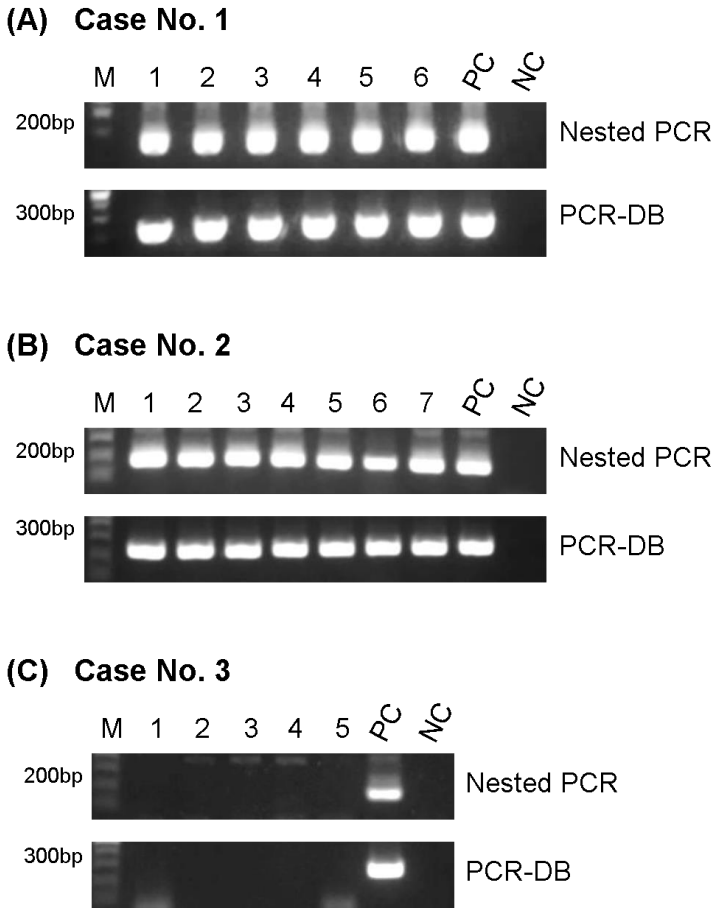

Figure I-4 Direct detection of BLV provirus in tumor samples. Amplification of the BLV provirus in blood and tumor samples collected from three cattle with bovine leukemia was performed by nested PCR and PCR-DB. Whole blood with 50-fold dilution and tumor suspensions were used as templates for PCR-DB. Each amplification procedure was performed in duplicate. LN, lymph node. (A) Case No. 1 (Holstein-Friesian, 4 months old): 1, blood; 2, thymus; 3, spleen; 4, gastric LN; 5, mesenteric LN; and 6, inguinal LN. (B) Case No. 2 (Japanese Black, 5 years old): 1, blood; 2, spleen; 3, heart; 4, superficial cervical LN; 5, mesenteric LN; 6, mediastinal LN; and 7, renal LN. (C) Case No. 3 (Holstein-Friesian, 2 years old): 1, blood; 2, cervical thymus; 3, thoracic thymus; 4, superficial cervical LN; and 5, bronchial LN.

DISCUSSION

In this study, novel diagnosis method named PCR-DB was developed for amplifying the BLV provirus directly from whole blood. This method showed high specificity and reproducibility with diluted blood samples, while the results of undiluted ones were less reproducibly probably because of the presence of endogenous PCR inhibitors in the blood samples, and of blood viscosity which made it difficult to measure the sample volume accurately. Although the sensitivity of PCR-DB was lower than that of nested PCR, all clinical samples detected only by nested PCR and not with PCR-DB showed very low levels of provirus loads, suggesting that they were from carriers at early AL stage. Thus, this study demonstrates the usefulness of PCR-DB for rapid diagnosis of BLV infection.

In previous studies, several researchers have reported the amplification of DNA directly from whole blood using PCR methods for the diagnosis of bacterial or viral infections, including *Mycoplasma haemofelis* [Watanabe *et al*., 2008], *Bartonella quintana* [Morton *et al*., 2013] and hepatitis B virus [Cheng *et al*., 2007]. Moreover, in another report, PCR-DB was used for the screening of G_{M1} gangliosidosis-related gene mutation in dogs, demonstrating that this technique can also be a good method for the screening of hereditary disease, not only for detecting infectious diseases [Yamato *et al*., 2004]. These reports suggest that PCR-DB has the potential to be applied in multiple clinical situations as a novel, rapid and viable method of diagnosis.

One of the conventional methods to identify BLV-infected cattle is a serological test (AGID, ELISA, etc.) which detects antibodies against BLV antigen in serum and milk. Those tests are appropriate for screening thousands of cattle, because sample preparation is easy compared to PCR tests which require DNA extraction, and a rapid and cost-effective diagnosis kit is currently available. However, in some cases, serological tests show several problems, such as poorer sensitivity than that of PCR testing, false positive reaction, detection of maternal antibodies, and inability to apply for the diagnosis using tissue or semen samples [Trono *et al*., 2001; Choi *et al*., 2002; Kohara *et al*., 2006; Asadpour *et al*., 2012]. Although Sweden achieved the eradication of BLV-infected cattle only using an ELISA test [Emanuelson *et al,* 1992], PCR testing will enable a more definitive surveillance program to eliminate BLV infection.

There are many advantages of using PCR-DB to detect the BLV provirus: 1) the specificity of PCR-DB was 100% as calculated by diagnosing clinical samples; 2) this

method does not require a special or expensive thermal cycler like real-time PCR; 3) it is neither labor intensive nor time-consuming; 4) because of its rapid and simple procedure, there is less possibility of contamination; and 5) less than 10 μl of blood is enough to run the PCR-DB assay. The biggest challenge with PCR-DB is the existence of PCR inhibitors in whole blood, which contains IgG, hemoglobin and lactoferrin [Al-Soud *et al*., 2000; Al-Soud *et al*., 2001]. The results in this study demonstrate that endogenous PCR inhibitors do not impact the reproducibility of the PCR reaction if the blood samples are appropriately diluted. Thus, PCR-DB is a suitable method for clinical veterinarians to perform BLV diagnosis in a typical veterinarian's office.

Nested PCR using purified genomic DNA is likely the best method for detecting BLV infection, because the provirus in blood from several cattle which showed a few number of BLV copy was not detected by PCR-DB. Although the amplification of low-copy provirus may be difficult by using PCR-DB, the sensitivity of PCR-DB can be improved by simply increasing the template blood volume. As demonstrated in Fig. I-2C, PCR-DB using 10-fold diluted blood samples showed higher sensitivity than those of other dilutions, keeping adequate reproducibility. Therefore, PCR-DB is appropriate for the first screening diagnosis in an individual farm or region to systematically eradicate BLV-infected cattle. This technique is preferred, because the use of nested PCR for diagnosing a large number of cattle is quite labor intensive and time-consuming. PCR-DB represents the best practical way for eliminating BLV infection through periodic screening and for the isolation or culling of all infected animals at intervals of several months or a few years.

PCR-DB amplification of BLV provirus was also evaluated using tumor tissues. In the preliminary data, it has been shown that the number of cells in a PCR reaction buffer influenced the stability of the results and that excessive cell numbers inhibited the reaction (data not shown). Thus, suspending tumor cells in PBS or other suitable solutions are important in order to adjust templates to the appropriate PCR conditions. In tumors, PCR-DB may be a better method for the diagnosis of BLV infection rather than nested PCR or other methods, because the most rapid and definitive method is required by clinical veterinarians for diagnosing BLV infection in cattle with lymphoma, and because serological tests are not suitable for tissue diagnosis.

BLV infection is difficult to eliminate due to its long latent period. However, Denmark did so by only counting of peripheral blood cells without serological tests [Gillet *et al*., 2007]. Thus, other countries can eliminate this infection more definitively

using PCR-DB. In conclusion, this PCR-DB assay is a highly simplified, cost-effective and rapid method (results can be obtained within 3 h) which serves as a new alternative way to diagnosis BLV infection without DNA purification.

SUMMARY

BLV infection induces lethal leukemia in cattle and causes significant financial harm to farmers and farm management. There is no effective therapy or vaccine; thus, the diagnosis and elimination of BLV-infected cattle are the most effective method to eradicate this infection. Clinical veterinarians need a simpler and more rapid method for the diagnosis of BLV infection, because both nested PCR and real-time PCR are labor intensive, time-consuming, and require specialized molecular biological techniques and expensive equipment. This study describes a novel PCR method for amplifying the BLV provirus from whole blood, PCR-DB, eliminating the step for DNA extraction. Although the sensitivity of PCR-DB measured by bovine blood which contains BLV-infected cell lines was lower than that of nested PCR, the PCR-DB technique showed high specificity and reproducibility. Among 225 clinical samples, 49 samples were positive by nested PCR, and 37 samples were positive by PCR-DB. There were no false positive samples; thus, PCR-DB sensitivity and specificity were 75.5% and 100%, respectively. In those clinical samples, the provirus loads of the samples detected by nested PCR and not PCR-DB were quite low. Moreover, PCR-DB also stably amplified the BLV provirus from tumor tissue samples. PCR-DB method exhibited good reproducibility and excellent specificity and is suitable for first screening of thousands of cattle, thus serving as a viable alternative to nested PCR and real-time PCR.

CHAPTER II

A novel classification of bovine leukemia based on immunophenotypic analysis

INTRODUCTION

Bovine leukemia is a fatal disorder in cattle characterized by neoplastic lymphocytosis and systemic lymphoma. There are two types of bovine leukemia, EBL and SBL, based on their epidemiology. EBL which is associated with BLV infection occurs in a small fraction of infected cattle and predominantly in adult cattle. SBL is not a transmissible cancer and further subdivided into juvenile, thymic, and cutaneous forms depending on the ages and tumor-developing sites [Grimshaw *et al*., 1979]. The juvenile form occurs in calves of ≤2 years old (usually 6-month old) and typically shows systemic lymphoma. The thymic form develops in calves from 6 months to 2 years old and is characterized by strong lymphoproliferation of thymic tissue. The cutaneous form has been found in cattle between 1 and 3 years old and shows multifocal lymphoproliferation in the skin. However, there are several reports on atypical SBL cases, such as intermediate cases which involve the overlapping of the juvenile and thymic forms, and multicentric lymphadenopathy in adult cattle of >3 years old which are negative for BLV [Asahina *et al*., 1995; Grünberg *et al*., 2013]. Therefore, the classification of bovine leukemia remains inconsistent.

EBL is characterized by systemic B-cell lymphoma associated with BLV infection, whereas SBL includes tumors of both B-cell and T-cell origin. The diagnosis of bovine leukemia is based on the observation of lymphadenopathy through palpation and rectal examination during routine examination practices, but many clinical cases have been found in meat hygiene inspection centers after the cattle are slaughtered [Bartlett *et al*., 2014]. The cell origin in the tumor-developing sites is often determined by immunohistochemical analysis to confirm cell-marker expression, and BLV association is usually determined by the detection of anti-BLV antibody by ELISA or virus genome by PCR. Quantitative analyses, such as flow cytometry and real-time PCR, should be useful for accurate evaluation of the expression level of cell markers and BLV provirus loads, but those methods are less frequently used clinically. The detection of monoclonality in B-cell proliferations using clonal rearrangement of the immunoglobulin heavy chain (IgH) gene is an effective way to diagnose B-cell lymphoma, and it is established not only for humans [Pan *et al*., 1994; Ilyas *et al*., 1995] but also for dogs [Burnett *et al*., 2003; Tamura *et al*., 2006; Valli *et al*., 2006; Rütgen *et al*., 2010], cats [Werner *et al*., 2005], and pigs [Sinkora *et al*., 2003]. In cattle, one study used a PCR-based IgH analysis to estimate the amount of founder clones in follicles of

ileal Peyer's patch [Niku *et al*., 2002], but no study has investigated the diagnosis of bovine B-cell lymphoma using this method.

Recently, the number of EBL cases has been increasing in Japan whose prevalence rate of BLV is >40% in dairy cattle. In Chapter I, PCR-DB was established for early detection of BLV infection, and its application for tumor samples succeeded with identical results to nested PCR. However, there is no common definition to classify bovine leukemia occurred in the field into EBL, thus application range of PCR-DB for tumor diagnosis is still speculative. Moreover, in recent years, a few papers reported that EBL onset even in juvenile calves has been observed in Japan [Inoue *et al*., 2013; Oguma *et al*., 2017], even though EBL occurs predominantly in adult cattle. To clarify the current status of bovine leukemia in Japan, in this chapter, quantitative analyses and PCR-based IgH analysis for evaluation of cell-marker expression, BLV provirus loads, and B-cell clonality were performed using clinical samples from cattle diagnosed as having bovine leukemia. Surprisingly, this work not only found many cases of early onset of EBL but also identified several atypical EBL types previously unreported. Thus, this study reports a novel characteristic of bovine leukemia which was identified in the field in Japan. Those findings should contribute to a deeper understanding of immunophenotypic features of bovine leukemia and perhaps of the mechanism underlying tumor development during disease progression of BLV infection.

MATERIALS AND METHODS

Blood and tissue samples

Peripheral blood and tissues, such as the spleen, lymph nodes, and solid tumors in several organs, in cattle with lymphoma were collected from livestock hygiene centers and meat hygiene inspection centers in Japan. Blood samples from BLV-infected or uninfected cattle were obtained from several farms, and BLV infection was diagnosed at the Hokkaido University Veterinary Teaching Hospital by amplifying the *BLV LTR* gene, as described in Chapter I. Peripheral blood mononuclear cells (PBMCs) were purified by density-gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, UK). Tumor samples were minced with scissors into small pieces, and the single-cell suspension was collected and washed twice with PBS. Genomic DNA was extracted from 1 to 5×10^6 PBMCs or tumor cells using the Wizard Genomic DNA Purification Kit (Promega).

Cell-marker expression

Cells were stained with antibodies specific to markers of T or B cells, as described in Chapter I with some modification. Briefly, double staining was performed using anti-IgM (IL-A30; Bio-Rad) pre-labeled with Zenon Alexa Fluor 488 (Thermo Fisher Scientific) and the following antibodies: anti-CD5 (CACT105A; WSU Monoclonal Antibody Center), anti-WC4 (CC55; CD19 like; Bio-Rad), anti-CD21 (GB25A; WSU Monoclonal Antibody Center), and anti-CD3 (MM1A; WSU Monoclonal Antibody Center). Alexa Fluor 647-conjugated anti-mouse IgG (Thermo Fisher Scientific) was used for the detection of antibody binding (anti-CD5, anti-WC4, anti-CD21, and anti-CD3). In contrast, cells were stained with anti-CD79a (HM57; Bio-Rad) and anti-BLV-gp51 (BLV1; WSU Monoclonal Antibody Center) pre-labeled with Zenon Alexa Fluor 647 (Thermo Fisher Scientific) after the treatment with FOXP3 Fix/Perm Buffer (BioLegend, San Diego, CA, USA) and FOXP3 Perm Buffer (BioLegend). To induce the expression of BLV antigens, cells were cultivated overnight in RPMI-1640 medium (Sigma-Aldrich) containing 10% heat-inactivated FBS (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific) before the staining of anti-BLV-gp51. Fixable Viability Dye eFluor1780 (Thermo Fisher Scientific) was used for a live/dead staining. Binding of the antibodies was detected using FACS Verse (BD Biosciences) and FCS Express 4 (De

Novo Software).

B-cell clonality

Genomic DNA extracted from blood and tissue samples was used as a template for PCR-based IgH analysis. The gene encoding the IgH region was amplified using the following primer pairs: J_H primer 5′-AGA CTA GTG AAG ACT CTC GGG TGT G-3′ and V_H primer 5′-AGC TCG AGA TGA ACC CAC TGT G-3′ for the first PCR, and J_H primer and CDR3 fw2 primer 5′-C(G/T)G AGG AC(A/T) CGG CCA CAT A-3′ for the second PCR [Saini *et al.,* 1999]. The amplification was performed in a reaction mixture containing 3 μl of 10 × Ex Taq Buffer (TaKaRa Bio), 2.4 μl of a 2.5 mM dNTP Mixture (TaKaRa Bio), 0.15 μl of TaKaRa Ex Taq (TaKaRa Bio), and 1 μl each of primers in ≤30 μl in DDW. The PCR condition of the first or second cycle was as follows: one cycle at 96°C for 2 min, followed by a three-step procedure consisting of 20 sec at 96°C, 30 sec at 61°C, and 45 sec at 72°C for 35 cycles (the first PCR) or 20 sec at 96°C, 30 sec at 56°C, and 20 sec at 72°C for 35 cycles (the second PCR). The amplicons were confirmed by electrophoresis in an ethidium bromide-stained 3% Tris-borate-EDTA (TBE) agarose gel. To confirm identity of the amplicon in PCR-based IgH analysis, one amplified product was purified by using the FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan), cloned into the pGEM-T Easy vector (Promega), and sequenced using the CEQ 8000 DNA Analysis System (Beckman Coulter, Fullerton, CA, USA).

BLV provirus loads

The *BLV Tax* gene was amplified using DNA samples extracted from blood and tissue samples of cattle with lymphoma. The amplification was performed in a reaction mixture containing 5 μl of a 2×Cycleave PCR Reaction Mix (TaKaRa Bio), 0.5 μl of Probe/Primer Mix for BLV (TaKaRa Bio), 1 μl of a DNA template, and 3.5 μl of PCR-grade water (TaKaRa Bio) using a real-time PCR system (LightCycler 480 system II; Roche Diagnostics), according to the manufacturer's instructions. Serial dilution of the BLV-positive control (TaKaRa Bio) was used for the generation of calibration curves to determine the provirus loads. Each result is expressed as the number of BLV copies per 50 ng of genomic DNA determined using a NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific).

Statistical analysis

Differences between multiple groups were examined for statistical significance using the Kruskal-Wallis test followed by the Steel-Dwass test. A *P*-value of <0.05 was considered as indicating statistical significance. Linear discriminant analysis was performed using the data on cell-marker expression to look for linear combinations of quantitative variables. The discrimination was derived by maximizing the separation of the groups in the data. To visualize the diversity of the data, multiple discriminant analysis was performed, and the results are presented as scatter plots using three principal components of the scores.

RESULTS

Sample collection and phenotypic analysis of cattle with lymphoma

To examine immunophenotypic features of bovine lymphoma, 176 samples were collected from 50 cattle which were clinically diagnosed as having bovine leukemia in livestock hygiene centers and meat hygiene inspection centers in Japan (Table II-1). The samples were subjected to following three analyses: flow cytometry analysis for cell-marker expression, PCR-based IgH analysis for B-cell clonality, and quantitative real-time PCR for BLV provirus loads. First, the expression of the cell markers was determined by evaluating not only the percentages of positive cells but also the numbers of cell populations (Fig. II-1) because single cell population suggests that the cells express similar patterns of cell markers, whereas multiple population showed that there are several populations having different marker expressions. Thus, the results were indicative of an extent of tumorigenesis. Second, the amplification of the gene encoding the IgH region of interest was performed to investigate B-cell clonality, and the results of the amplification were divided into high- or low-clonality. A clear DNA band in electrophoresis indicated monoclonal or oligoclonal B-cell expansion (high-clonality: Fig. II-2, lanes 1–4), whereas a smear indicated the presence of polyclonal B cells (low-clonality: Fig. II-2, lanes 5–9 and 11–14). In some cases, there were unclear results which were difficult to classify into high- or low-clonality (Fig. II-2, lane 10). Determining DNA sequence of the amplicon indicated that the clear DNA band found in a high-clonality sample consisted of single clone of B cells, but not of two or three B-cell clones of similar clone sizes (data not shown). Third, BLV provirus loads were quantified as copy numbers of the *BLV Tax* gene in 50 ng of genome DNA.

Classification and diagnosis of cattle with lymphoma

All of the cattle with lymphoma were classified into five groups on the basis of the results of cell-marker expression, B-cell clonality, and BLV provirus loads: classic EBL (cEBL), polyclonal EBL (pEBL), B-cell-type SBL (B-SBL), T-cell-type SBL (T-SBL), and nontypeable cases (Table II-2). cEBL was defined as a monoclonal or oligoclonal B-cell lymphoma associated with BLV infection, whereas B-SBL was defined as B-cell lymphoma unrelated to BLV. The criteria of the association with BLV infection was set as ≥2,000 copies per 50 ng DNA (400 copies per 10 ng DNA) because according to a previous study, the average BLV copy number in whole blood from

BLV-infected cattle without lymphoma was 330 copies per 10 ng DNA, whereas that of EBL cattle was 2,800 copies per 10 ng DNA [Somura *et al*., 2014]. A novel type of EBL found in this study, pEBL, was similar to cEBL except for low B-cell clonality, which suggested that pEBL was characterized by neoplastic proliferation of polyclonal B cells. T-SBL was categorized as T-cell lymphoma regardless of BLV infection, and the nontypeable cases included non-T- or non-B-cell tumors or simply a non-neoplastic lymphadenopathy (data not shown). The diagnosis of cattle with lymphoma resulted in 52.0% with cEBL, 16.0% with pEBL, 8.0% with B-SBL, and 6.0% with T-SBL (Table II-2).

Difference in the susceptibility to cEBL onset between breed and age

To examine the relationship between the onset of bovine leukemia and background information of cattle, the breed and age of the cattle were compared between each type of lymphoma. In the Japanese Black breed, 69.0% of the cattle were diagnosed as cEBL, followed by 13.8% as pEBL and 3.4% each as B-SBL and T-SBL (Fig. II-3, left). In contrast, in Holsteins, the percentage of cEBL was 23.5%, whereas the percentages of pEBL, B-SBL, and T-SBL were 17.6%, 17.6%, and 5.9%, respectively. These data suggested that Japanese Black is more susceptible to EBL onset than Holstein, and conversely that Holstein might be susceptible to SBL onset. Regarding the age of the cattle, B-SBL and T-SBL were mainly found in juvenile calves, and pEBL seemed to occur in cattle of >1 year old (Fig. II-3, right). As suspected, many cEBL cases were found in juvenile cattle of <3 years old, and surprisingly, the frequency of early onset of cEBL was equal to that of adult cattle. Thus, this study found that most of juvenile cattle which were clinically diagnosed as EBL were classified into cEBL, hence that early onset of EBL truly occurred in Japanese cattle.

Lack of lymphocytosis in peripheral blood of cEBL cattle

As one of the remarkable points, there were non-negligible numbers of cEBL cattle which did not show lymphocytosis, defined as lymphocyte counts of >10,000 cells per 1 μl blood (Fig. II-4A). It was not caused by the failure in counting lymphocytes by an automated hemocytometer, because the numbers of lymphocytes strongly correlated with those of WBCs (Fig. II-4B). Furthermore, compared with the cattle that showed lymphocytosis, the PBMCs from the cattle lacking lymphocytosis exhibited an immature tumor phenotype which was defined as multiple cell population

and low B-cell clonality (Table II-3). The other sampling sites, such as lymph nodes and solid tumors in organs, from the non-lymphocytosis cattle showed mature tumor phenotypes (data not shown). Therefore, these results suggested the possibility that certain BLV-infected cattle developed B-cell lymphoma without going through the PL stage.

Immunophenotyping of bovine B-cell lymphoma

The diagnosis of three types of B-cell lymphoma mainly depended on B-cell clonality and provirus loads, not on individual cell markers. To detect differences in expression patterns, the expression levels of each cell marker in cEBL were compared with those of the other B-cell lymphomas and healthy controls. All of the B-cell lymphomas were clearly distinguished from a healthy phenotype by their high CD79a expression and low CD3 expression (Fig. II-5A). Moreover, the expressions of WC4 in cEBL and pEBL were significantly lower than those in healthy controls, whereas the expression of CD21 was quite low in B-SBL compared to all other groups. The difference between cEBL and pEBL was determined by the significantly low levels of IgM expression in pEBL; however, cEBL still appeared to be divided into two populations: a major IgM^+ group (IgM positive cells ≥50%) and a minor IgM^- group (IgM positive cells <50%). The expression patterns of three B-cell markers, IgM, WC4, and CD21, were distinctly different among three types of B-cell lymphoma. To clarify their immunophenotypic character, dual and triple expressions of these cell markers were compared with each other (Fig. II-5B and C). The typical pattern of cell-marker expression in cEBL was IgM^+, $WC4^-$, and $CD21^+$; In contrast, that of pEBL was IgM^-, $WC4^-$, and $CD21^+$. There was no characteristic pattern in B-SBL except for low CD21 expression although certain samples of B-SBL highly expressed WC4. Comparison of BLV provirus loads and expression of viral protein gp51 between cEBL and pEBL showed no significant difference (data not shown). Taken together, the three types of B-cell lymphoma, cEBL, pEBL, and B-SBL, indicated different patterns of cell-marker expression, which suggested the possibility of the application of marker expression analysis as a tool for the diagnosis of bovine B-cell lymphoma.

Discriminant analysis between B-cell lymphoma and healthy controls

To further characterize three B-cell lymphomas on the basis of the expression levels of the six cell markers, linear discriminant analysis was performed to discriminate

between lymphomas and healthy controls. It was remarkable that the discriminant analysis correctly classified three lymphomas and healthy controls with high sensitivity, specificity and accuracy (>97.6%, Fig. II-6A, upper). Particularly, no classification error occurred between pEBL and controls and between B-SBL and controls. In contrast, a clear discrimination was obtained between cEBL and other types of B-cell lymphoma. Above all, discrimination scores of cEBL and pEBL was overlapping widely with each other, which was indicated by poor sensitivity (87.8%), specificity (61.5%), and accuracy (81.5%; Fig. II-6, lower). In contrast, discrimination between pEBL and B-SBL was clear and showed correct classification in 100% of both samples. Then, multiple discriminant analysis was used to visualize the difference in cell-marker expression among three B-cell lymphomas and healthy controls (Fig. II-6B). This analysis showed independent clustering of marker expression in healthy controls from B-cell lymphomas, indicating that cattle with B-cell lymphoma could be distinguished from healthy cattle by the expression patterns of the cell markers. Furthermore, pEBL and B-SBL were obviously distributed between two separate areas, whereas the boundary between cEBL and these two lymphomas was unclear. Thus, the discriminant model based on cell-marker expression was useful for the discrimination between healthy cattle and cattle with B-cell lymphoma or between pEBL and B-SBL but remained insufficient to correctly classify three types of B-cell lymphomas.

Table II-1 Basic information of the cattle analyzed in this study

Contents		Cattle with Lymphoma (50 heads)	Cattle without Lymphoma (7 heads)
Age	0–1 years old	10	2
	1–2 years old	10	0
	2–3 years old	16	0
	≥3 years old	14	4
	No information	0	1
Breed	Holstein	17	6
	Japanese Black	29	0
	Cross breed	3	0
	No information	1	1
Sex	Male	7	2
	Female	39	5
	No information	4	0
BLV infection	Positive	44	3
	Negative	6	4
Sampling sites	Total	$n = 176$	$n = 21$
	Peripheral blood	41	7
	Lymph node	90	12
	Spleen	16	1
	Thymus	6	1
	Solid tumor in organ	23	0

Table II-2 Classification and diagnosis of clinical samples suspected as bovine leukemia

Diagnosis	Number of Cattle	Cell type	B-cell clonality	BLV provirus loads (copies/50 ng DNA)
Classic EBL (cEBL)	26 (52%)	B-cell	High	≥2,000
Polyclonal EBL (pEBL)	8 (16%)	B-cell	Low*	≥2,000
B-cell-type SBL (B-SBL)	4 (8%)	B-cell	High*	<2,000
T-cell-type SBL (T-SBL)	3 (6%)	T-cell	Low*	Unrestricted
Nontypeable	9 (18%)	Did not correspond to any of the diagnoses		

*These groups include samples that showed unclear results of clonality.

Table II-3 The Relationship between lymphocytosis and tumorigenesis in peripheral blood

	Tumorigenesis in peripheral blood			
Lymphocytosis	Observed	Not observed	Not analyzed	Total
Observed	10	0	0	10
Not observed	2	6	2	10
Not analyzed	2	1	3	6
Total	14	7	5	26

*Tumorigenesis was defined as single cell population and high B-cell clonality.

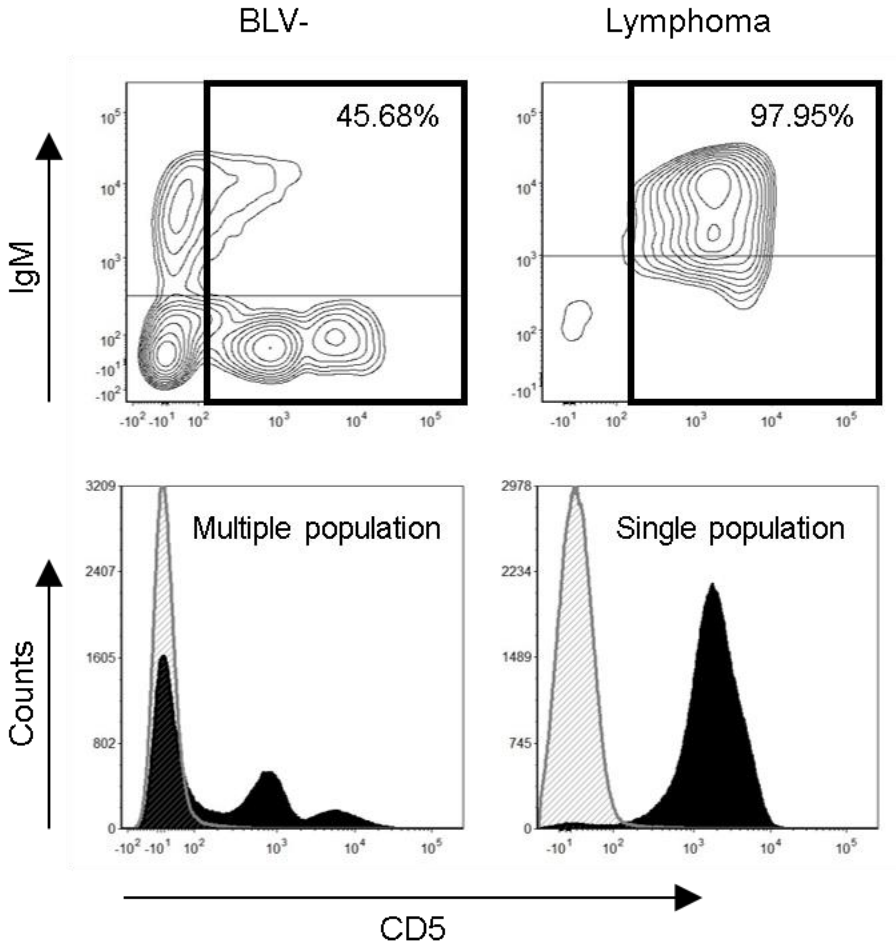

Figure II-1 Flow cytometry analysis to assess cell-marker expression and population diversity. Cell-marker expression of PBMCs from BLV-uninfected cattle or cattle with lymphoma was analyzed by flow cytometry. The results were evaluated as percentages of positive cells and numbers of different cell populations.

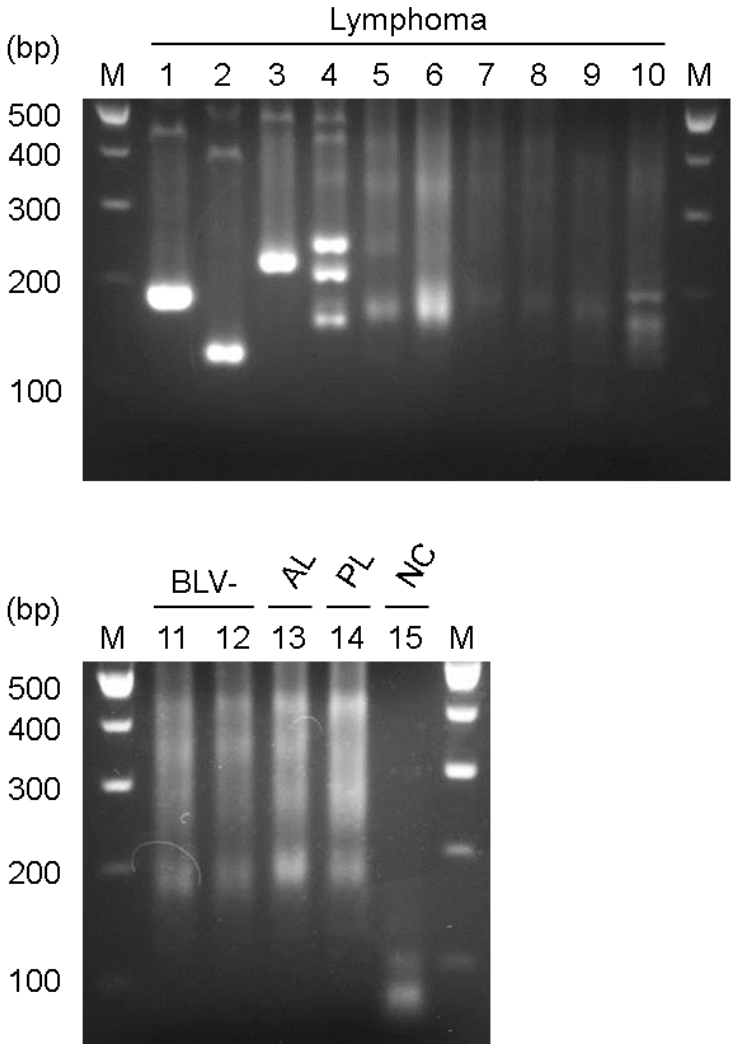

Figure II-2 PCR-based IgH analysis to confirm B-cell clonality. Amplification of the gene encoding the IgH region of interest was performed using DNA extracted from PBMCs and tissue samples of cattle with or without lymphoma. Representative PCR products run on an ethidium bromide-stained 3% TBE agarose gel. Lane: 1, PBMCs; 2, solid tumor in heart; 3, superficial cervical lymph node; 4, PBMCs; 5, solid tumor in heart; 6–8, PBMCs; 9, solid mass in rib bone; 10, solid tumor in thymus; 11–14, PBMCs; 15, DDW; M, 100-bp DNA ladder; AL, aleukemic; PL, persistent lymphocytosis; NC, negative control.

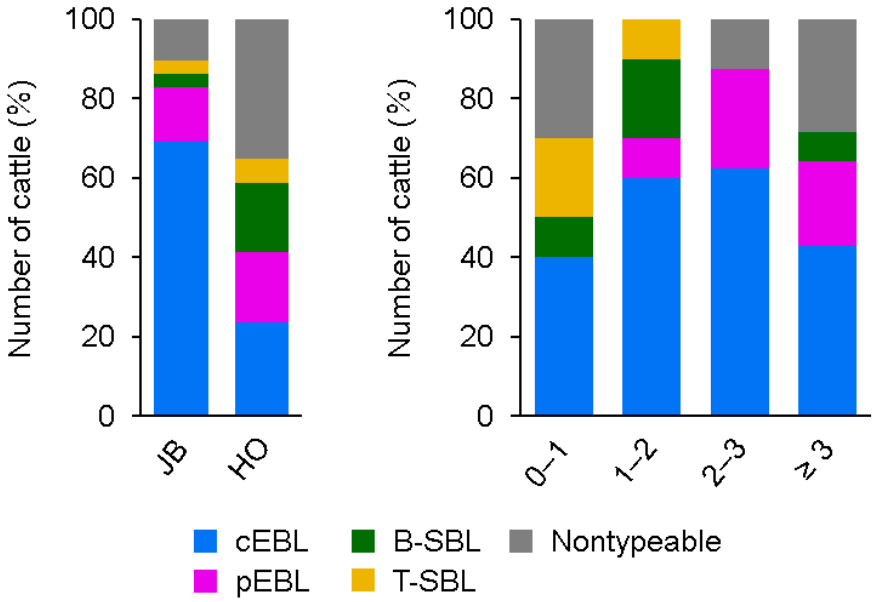

Figure II-3 Breed and age of the cattle with lymphoma. The percentages and numbers of each type of lymphoma were compared between breed (left) and age (right) of the cattle. JB, Japanese Black; HO, Holstein. Numbers on bottom of the right panel indicates age in years.

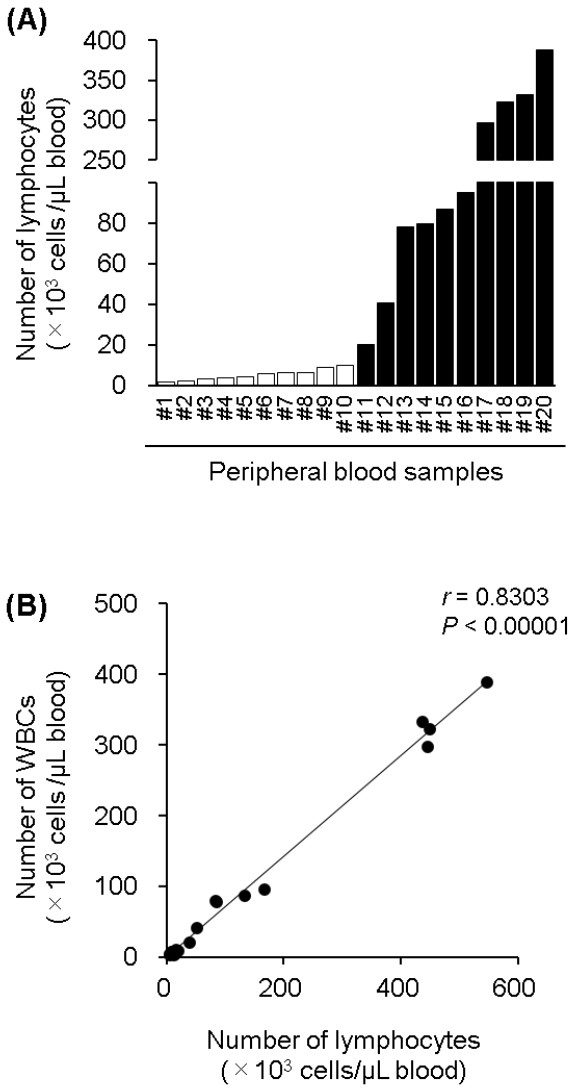

Figure II-4 Lack of lymphocytosis in cEBL cattle. (A) The number of lymphocytes in peripheral blood samples from cEBL cattle is shown (n = 20). Open bar indicates that the lymphocyte count is <10,000 cells per 1 μl of blood. (B) Correlation between the number of whole blood cells and lymphocytes in peripheral blood samples from cEBL cattle (n = 20). P <0.00001, r = 0.8303.

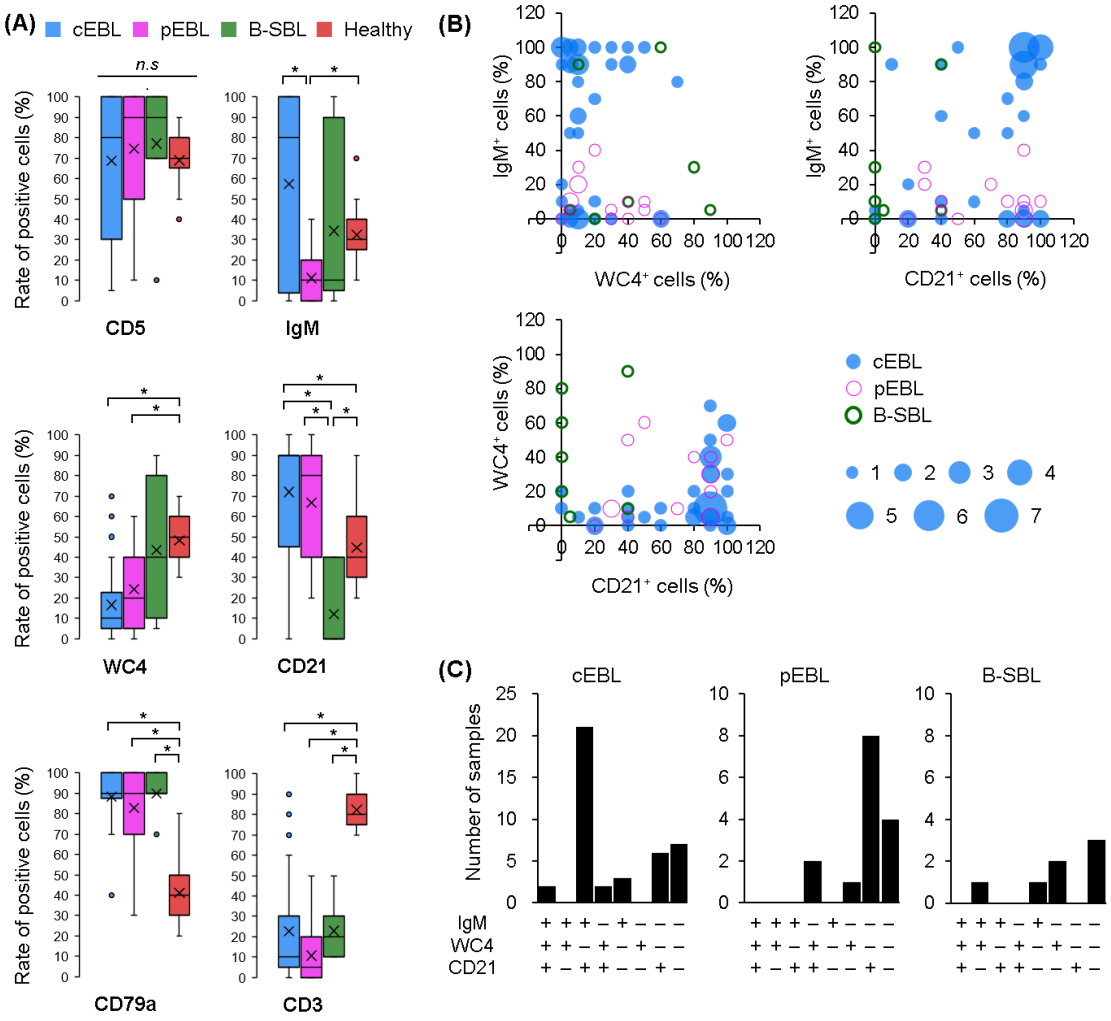

Fig. 5

Fig. 5

Figure II-5 Immunophenotyping based on expression pattern of cell markers between three B-cell lymphomas and healthy controls. (A) The expression levels of six cell markers in samples from cEBL (n = 42), pEBL (n = 15), B-SBL (n = 7), and healthy controls (n = 17) are shown as box-and-whisker plots. Each box indicates median, lower and upper quartiles, and whiskers indicate lower and upper extremes. The x-mark indicates the average, and dots represent outliers that are much greater than normal or much less than normal. *, P <0.05, Kruskal–Wallis test followed by Steel-Dwass test. (B) Dual expression of IgM, WC4, and CD21 in cEBL, pEBL, and B-SBL. Each axis indicates the percentages of positive cells, and the bubble size indicates the number of samples which showed identical expression patterns. (C) Triple expression of IgM, WC4, and CD21 in cEBL, pEBL, and B-SBL. +, cell-marker expression ≥50%; –, cell-marker expression <50%.

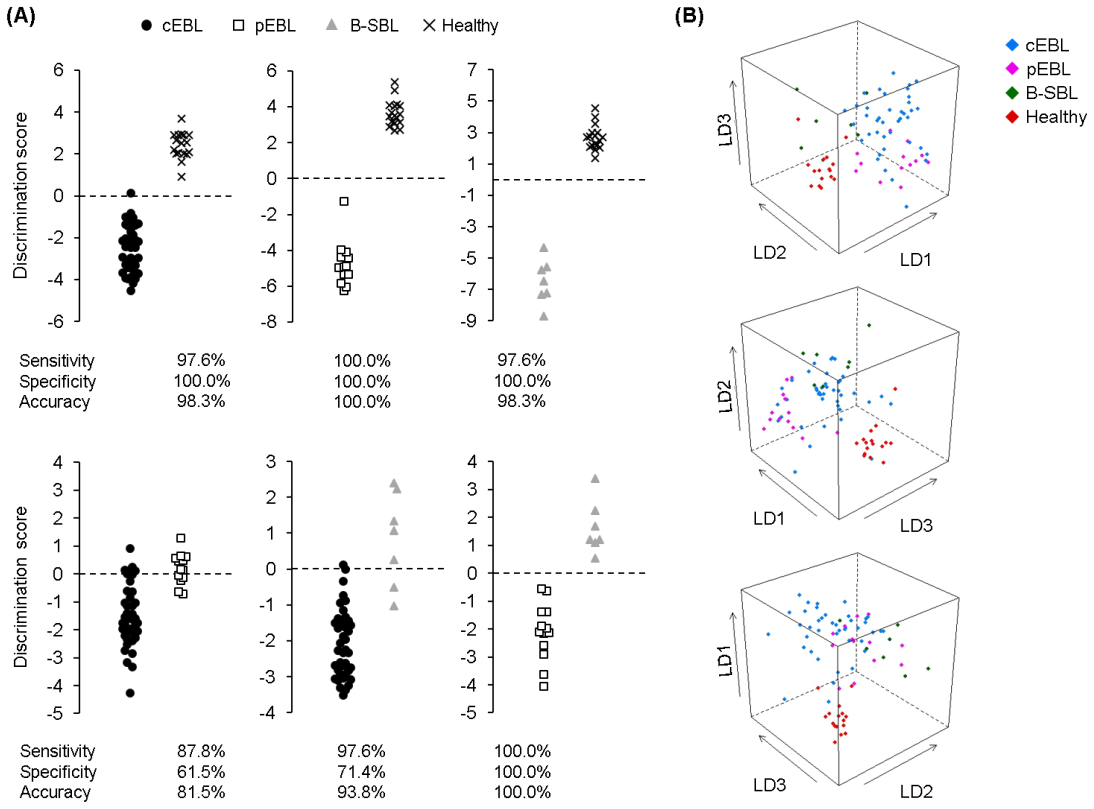

Figure II-6 Discriminant analysis of cEBL, pEBL, B-SBL, and healthy controls. (A) Discriminant scores giving a classification performance of B-cell lymphomas and controls based on linear discriminant analysis with the expression of six cell markers: CD5, IgM, WC4, CD21, CD79a, and CD3. (B) Scatter plots of multiple discriminant analyses to visualize the clustering of cell-marker expression from each B-cell lymphoma and healthy controls. First, second and third linear discriminant are shown as LD1, LD2 and LD3, respectively. cEBL, n = 41; pEBL, n = 13; B-SBL, n = 7; healthy control, n = 17.

DISCUSSION

Diagnosis of EBL requires verification that the samples exhibit neoplastic proliferation of B cells and that BLV infection is associated with tumor development. In this study, cell origin was confirmed by the cell-marker expression, BLV association by the amount of provirus, and tumor maturation by clonal rearrangement of the *IgH* gene. The examination classified the cattle clinically diagnosed as having bovine leukemia into five groups, and revealed a novel type of EBL, pEBL (Table II-2). Furthermore, several atypical EBL cases were found, including early onset of EBL in juvenile cattle and EBL lacking peripheral lymphocytosis (Figs. II-2 and II-3). Immunophenotyping of three B-cell lymphomas, cEBL, pEBL, and B-SBL, made their characteristics clear, which was sufficient to enable the discrimination from healthy phenotypes (Fig. II-6). However, discriminant analysis between cEBL and pEBL or between cEBL and B-SBL did not show clear classification, suggesting the difficulty in the discrimination of bovine B-cell lymphoma only by the analysis of cell-marker expression. Taken together, the combination of the analyses performed in this study, that is, cell-marker expression, numbers of cell population, B-cell clonality, and BLV provirus loads, was useful for the correct diagnosis of the types of bovine leukemia.

BLV spreads within the host through two distinct processes [Gillet *et al*., 2013; Gutiérrez *et al*., 2014]. First, the virus replicates actively and infects a new target during the initial period of the infection (known as the infectious or replicative cycle). Then, the immune response developed in the host limits the infection to new target cells, and thus, cells whose provirus is inserted into genomic regions which can be transcribed, but not promoter regions, were selected. Therefore, the second processes for viral replication depends on the proliferation and expansion of infected lymphocytes (also known as cells at the mitotic cycle). In the experimental infection in cattle, BLV transmission shifted from the infection of new targets to clonal expansion during the 2 months after the inoculation, and negative selection by host immune response eliminated 97% of the clones detected at seroconversion [Gillet *et al*., 2013]. Because of these processes, the analysis of B-cell clonality was an effective method to distinguish lymphomas from the early stage of BLV infection. Furthermore, the results in this study indicated that PCR-based IgH analysis can classify the samples from PL cattle, in which infected cells were probably at the mitotic cycle, into low-clonality (Fig. II-2). Thus, PCR-based IgH analysis is a potentially powerful tool for the simple diagnosis of B-cell

lymphoma.

The possibility that pEBL cattle were just in the PL stage still cannot be denied because of its low B-cell clonality determined in the IgH analysis. However, flow cytometric analysis of the samples from pEBL cattle exhibited a single cell population which looked like mature tumor phenotypes, and a solid mass was found in several organs in the gross pathological analysis (data not shown). Therefore, pEBL phenotypes might be an intermediate state between the PL stage and cEBL stage. In contrast, cEBL lacking peripheral lymphocytosis indicated the possibility of direct development of B-cell lymphoma occurring in cattle in the AL stage. van der Maaten and Miller [1990] have described that, though cattle with persistent lymphocytosis have a high risk of developing tumors, PL stage is not a prerequisite step for the development of lymphoma. In addition, one review on BLV infection described that tumors can occur directly in infected animals without lymphocytosis, whereas that is not clearly delineated prior to this work [Gutiérrez *et al*., 2014]. Although the mechanism for the development of pEBL or non-lymphocytosis EBL has not been elucidated in details, disease progression of BLV infection might be more complicated than previously assumed.

According to a previous study, the average copy number of the BLV gene was 330 copies per 10 ng DNA in whole blood from BLV-infected cattle which did not show lymphoma [Somura *et al*., 2014]. However, the maximum copy number in these cattle was 2,600 copies, which was nearly equal to the average of EBL cattle (2,800 copies). Thus, although the value of 2,000 copies per 50 ng DNA was determined as a set point for BLV association in this study, it appears to be difficult to distinguish B-SBL showing high BLV provirus loads from cEBL. A possible solution is to use an inverse PCR method which identifies the clonality of integration sites of the BLV genome in the host, since cEBL consists of monoclonal expansion of a B-cell clone which hold an identical integration site, whereas integration sites of B-SBL are diverse in each BLV-infected B cells. Because it takes longer time to obtain the results, establishment of simpler methods to analyze integration sites of BLV would be critical for the correct diagnosis of bovine leukemia.

It has been demonstrated that the development of B-cell lymphoma during disease progression of BLV infection takes a long time; thus, EBL generally occurs in adult cattle of >3–5 years old [Gutiérrez *et al*., 2014; Tsutsui *et al*., 2016]. Previously, one report indicated that early onset of EBL at <3 years of age was found in Japan although the diagnosis of EBL has been performed simply by the detection of the *env*

gene [Inoue *et al*., 2013]. The results in this study strongly support this finding because a total of 20 juvenile calves were diagnosed as having cEBL (Fig. II-3). The mechanism of early onset of cEBL remains unclear, but there are several possible related factors, such as mutations in the viral genome, host susceptibility, and infection during the fetal period. First, mutations in the viral genome can affect the incubation period until tumor development because in a previous study the percentage of EBL associated with the L233-Tax protein was significantly higher than that of the P233-Tax in young cattle [Inoue *et al*., 2013]. Moreover, a mutation in an N-linked envelope glycosylation site (N230E) resulted in high provirus loads during the experimental infection in sheep, which led to accelerated pathogenesis and shortening of the incubation period [de Brogniez *et al*., 2015]. Second, it has been reported that certain alleles of the MHC class II *DRB3* gene are involved in susceptibility and resistance to BLV infection [Forletti *et al*., 2013; Miyasaka *et al*., 2013]. Moreover, genomic diversity of the *DRB3* gene varies between cattle breeds, and *BoLA-DRB3*1601* associated with susceptibility to a high BLV provirus load was a frequent allele in Japanese Blacks but was infrequent in Holsteins [Takeshima *et al*., 2003; Miyasaka *et al*., 2011; Miyasaka *et al*. 2012]. Because the results of this study showed strong susceptibility to cEBL onset in Japanese Black, the cattle breed might be a key factor for the understanding of the mechanism for the development of bovine leukemia. Finally, it is possible that early infection during the fetal period can be a cause of early onset EBL. In this study, there were several cEBL cattle of <6 months old; especially, one of them was only 1 month old, which suggested vertical transmission of BLV *in utero* or in the birth canal. An experimental infection with BLV in sheep demonstrated that splenectomized sheep, which lack efficient immune response against viral replication, failed to control progressive accumulation of infected cells and resulted in accelerated onset of leukemia [Florins *et al*., 2009]. Thus, impairment of a BLV-specific immune response, such as immune tolerance induced by mother-to-child transmission, might be involved in the early onset of cEBL in juvenile calves.

The expression levels of the B-cell markers were beneficial for the characterization of B-cell lymphoma. It appears that CD79a is a valid cell-marker to confirm B-cell origin of samples for the diagnosis of cattle with lymphoma although it is difficult to discriminate each type of B-cell lymphoma. In this study, cEBL cattle were divided into two populations on the basis of IgM expression, whereas all pEBL cases were IgM^- phenotypes (Fig. II-5). Moreover, both EBLs were characterized by

high expression of CD21 and low expression of WC4 (known as CD19 homolog). A previous report indicated that IgM^{low} B cells did not express virus protein after *ex vivo* cultivation, whereas IgM^{high} B cells highly expressed virus protein, gp51 [Ikebuchi *et al*., 2014a]. In addition, this report showed that the treatment of anti-WC4 antibody increased the percentages of $gp51^{+}$ cells *in vitro*. In humans, the CD19 molecule is one of the most reliable biomarkers for normal and neoplastic B cells and is involved in the modulation of B-cell receptor (BCR) signaling as a complex with a complement receptor, CD21 [Wang *et al*., 2012]. Thus, there is a possibility that the expression levels of IgM, WC4, and CD21 in the samples of EBL cattle are involved in the modification of BCR signaling, which can affect the expression of viral proteins. Although several B-cell lymphomas in which the expression of CD19 were diminished have been reported in humans [Yang *et al*., 2005; Masir *et al*., 2006], the significance of reduced CD19 expression observed in EBL cattle remains unknown.

EBL causes a large economic loss because it is a lethal disorder of cattle. Currently, no effective vaccine is available to control BLV infection, and thus, potential biomarkers for the prediction of future EBL onset are actively being investigated. However, common definitions for the classification of EBL and for the discrimination from other bovine lymphomas have not been established; thus, the classification of clinical samples has been performed according to the original criteria used in individual papers. Here, novel criteria were identified for the classification of bovine leukemia on the basis of immunophenotypic features, which should be useful for obtaining more reliable clinical information on EBL onset. To elucidate the mechanisms underlying tumor development and to establish effective prediction methods for EBL onset, further analysis is required for clear classification of bovine leukemia.

SUMMARY

Bovine leukemia is classified into two types of lymphomas, EBL and SBL. EBL is caused by the infection with BLV which induces persistent lymphocytosis and B-cell lymphomas in cattle after a long latent period. Although it has been demonstrated that BLV-associated lymphoma occurs predominantly in adult cattle of >3–5 years old, suspicious cases of EBL onset in juvenile cattle were recently reported in Japan. To investigate the current status of bovine leukemia in Japan, immunophenotypic analysis was performed using samples from 50 cattle which were clinically diagnosed as bovine leukemia. On the basis of the results in this study, the samples were classified into five groups, and two different types of EBL were found: cEBL was the phenotype commonly known as EBL, and pEBL exhibited neoplastic proliferation of polyclonal B cells. Moreover, several atypical EBL cases even in cEBL, including early onset of EBL in juvenile cattle were also identified. Comparison of the cell-marker expressions among cEBL, pEBL and B-SBL revealed characteristics of the marker expressions in B-cell leukemia, and these expression patterns could be clearly differentiated from those of healthy phenotypes, whereas it was difficult to discriminate between cEBL, pEBL and B-SBL only by the expression of cell markers. This study identified novel characteristics of bovine leukemia which should contribute to a better understanding of the mechanism underlying tumor development of BLV infection.

CHAPTER III

Establishment of novel biomedicine for cattle targeting PD-ligands

INTRODUCTION

PD-1 is an immunoinhibitory receptor which is expressed on activated T cells and B cells, and induces immune suppression through binding to its ligands, PD-L1 and PD-L2 [Keir *et al*., 2008]. It has been reported that PD-L1 is widely expressed on activated T cells and B cells, dendritic cells, monocytes, and on non-hematopoietic cells such as liver stromal cells, vascular endothelial cells and keratinocytes [Yamazaki *et al*., 2002; Keir *et al*., 2008]. In contrast, PD-L2 expression is comparatively confined to antigen presenting cells, such as dendritic cells and monocytes, but it is also expressed on T cells, B cells, epithelial cells and fibroblasts [Latchman *et al*., 2001; Stanciu *et al*., 2006; Zhong *et al*., 2007; Pinchuk *et al*., 2008; Messal *et al*., 2011].

The blockade of the PD-1/PD-Ls pathway has been proven effective for restoring immune response against cancer and chronic infections, and thus, several antibodies which block human PD-1 and PD-L1 are currently being clinically developed or made commercially available for cancer therapy [Sznol *et al*., 2013; Sheridan, 2014; Sunshine *et al*., 2015]. Furthermore, a clinical trial of PD-L2-Ig is currently underway, and assessed it as a possible third method following antibodies against PD-1 and PD-L1 [Topalian *et al*., 2012b; Sunshine *et al*., 2015]. One clinical study showed that the treatment with anti-PD-L1 antibody led to the inhibition of metastatic lesion growth in 21% and 26% patients with non-small cell lung cancer and melanoma, respectively [Herbst *et al*., 2014], while other studies have indicated that the treatment with anti-PD-1 or anti-PD-L1 antibodies at a dose of 1.0 mg/kg resulted in objective responses (41% and 29%, respectively) in melanoma patients [Brahmer *et al*., 2012; Topalian *et al*., 2012a]. The effects of these antibodies are less understood in the cases of infectious diseases, but anti-PD-L1 antibodies have been shown to enhance T-cell response *in vitro*, such as cell proliferation and the production of interferon-γ (IFN-γ), interleukin-2, tumor necrosis factor-α and Granzyme B, during HIV, HBV, and HTLV infections [Day *et al.*, 2006; Trautmann *et al*., 2006; Boni *et al*., 2007; Kozako *et al*., 2009]. Moreover, a phase II study of Nivolumab against relapsed or refractory adult T-cell leukemia (ATL) caused by HTLV-1 infection is ongoing in Japan [UMIN Center, 2013]. Thus, the blockade of the PD-1 pathway represents a promising immunotherapy against cancer and chronic infections.

On the other hand, studies on PD-1 and PD-L1 in the field of veterinary medicine are still limited. The high expression of PD-L1 was observed in oral

melanoma, osteosarcoma, renal cell carcinoma and hemangiosarcoma in dogs, but there is no report for feline tumors [Maekawa *et al*., 2014; Maekawa *et al*., 2016]. In previous studies, it was demonstrated that the PD-1 pathway is involved in disease progression during several chronic infections of cattle, such as BLV infection, Johne's disease and anaplasmosis [Ikebuchi *et al*., 2010; Ikebuchi *et al*., 2011; Okagawa *et al*., 2015; Okagawa *et al*., 2016]. Furthermore, the antibody blockade of the PD-1/PD-L1 interaction *in vitro* induces the re-activation of the immune response in these diseased cattle [Ikebuchi *et al*., 2010; Ikebuchi *et al*., 2013; Okagawa *et al*., 2015; Okagawa *et al*., 2016]. However, little is known concerning bovine PD-L2 compared to PD-1 and PD-L1, and there are no functional analyses of PD-L2 in domestic animals.

In Chapter I and II, the early detection method for BLV and clear classification of EBL were successfully established. However, they are still insufficient for controlling BLV infection because of the lack of "therapeutic methods". Since BLV belongs to the family *Retroviridae* and is closely related to HTLV-1 [Gillet *et al*., 2007; Aida *et al*., 2013], BLV infection represents a suitable target for the treatment with agents which block the PD-1 pathway. In the case of developing biomedicines for cattle, recombinant protein derived from cattle, such as bovine PD-L2-Ig, is the simplest way with common laboratory technique. Furthermore, since establishment of fully bovine mAbs which block PD-1/PD-Ls interaction requires special technology, for example bovine antibody library using phage display or transgenic mouse genetically engineered for the expression of bovine IgG [Brekke *et al*., 2003], it is a suitable option for the goal of this study to modify the anti-bovine PD-L1 mAb previously established to chimeric antibody. Therefore, in this chapter, two biomedicines for cattle targeting PD-Ls were developed; one is a bovine PD-L2-Ig, and the another is an anti-bovine PD-L1 mAb. In Section I, bovine PD-L2 was identified, and its function to promote immune responses was examined *in vitro* using PD-L2-Ig. In Section II, a rat-bovine chimeric antibody which was specific to bovine PD-L1 (Boch4G12) was established and its ability was examined *in vitro* and *in vivo*. The findings in this study indicated that novel biomedicines targeting PD-Ls are potentially applicable for immune therapies to control BLV infection.

CHAPTER III

SECTION I

Identification and *in vitro* characterization of bovine PD-L2

MATERIALS AND METHODS

Blood samples

Peripheral blood samples were obtained from healthy adult Holstein-Friesian cattle at Hokkaido University. Genomic DNA was extracted from 500 µl blood samples using the Wizard genomic DNA kit (Promega) according to the manufacturer's instructions. Diagnoses of BLV infection in cattle were conducted by nested-PCR amplifying the *BLV LTR* gene at the Hokkaido University Veterinary Teaching Hospital, as described in Chapter I.

Cloning of bovine PD-L2

Bovine PBMCs were purified by using density gradient centrifugation on Percoll (GE Healthcare). Total RNA was extracted from cultivated PBMCs in the presence of concanavalin A (ConA; 5mg/ml; Sigma-Aldrich) using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Subsequently, cDNA synthesis was performed from the total RNA using Moloney murine leukemia virus reverse transcriptase (TaKaRa Bio). Two primers designed from the information of predicted bovine PD-L2 sequences (GenBank accession No: NM001292036), boPD-L2 F 5′-TTG GCT TCT GTG AGG ACT GA-3′ and boPD-L2 R 5′- TCA AAT AGC TCT GTC CAC TTC TC-3′, were used for the amplification of bovine PD-L2. The amplicon was purified using the FastGene Gel/PCR Extraction Kits (Nippon Genetics) and cloned into pGEM-T Easy vectors (Promega). Purified plasmids derived from three different clones were sequenced using the CEQ 8000 DNA Analysis System (Beckman Coulter). To identify the 3′ end of bovine PD-L2, 3′-rapid amplification of cDNA ends (RACE) was conducted using boPD-L2 inner 5′-TTC TCT CCC TCA TCG TTG CT-3′ and 3′-RACE System (Thermo Fisher Scientific). An unrooted neighbor-joining tree was constructed from determined sequences by using MEGA software program. PD-1 binding sites of PD-L2 were predicted from data reported [Lázár-Molnár *et al*., 2008].

Generation of bovine PD-L2-Ig

The extracellular region of bovine PD- L2 was amplified using primers in which recognition sites for restriction enzymes were added, boPD-L2-Ig F 5′-CGC GGC TAG CAT GATGAGGCT GCC ACA G-3′ (containing *Nhe*I restriction site) and boPD-L2-Ig R 5′-CGC GGA TAT CAG ATG AAG GGA TCT TGG GG-3′ (containing *Eco*RV

restriction site). After the digestion with *Nhe*I (TaKaRa Bio) and *Eco*RV (New England Biolabs, Ipswich, MA, USA), the fragment was inserted into a cloning site of a modified pCXN2.1 vector which contained Fc region of rabbit IgG1 at the C-terminus (kindly provided by Dr. T. Yokomizo, Juntendo University, Tokyo, Japan) [Zettlmeissl *et al*., 1990; Niwa *et al*., 1991]. Then, this vector was transfected into Chinese hamster ovary (CHO)-DG44 cells (kindly provided by Dr. Y. Suzuki, Division of Bioresources, Research Center for Zoonosis Control, Hokkaido University) using Lipofectamine LTX (Thermo Fisher Scientific), and the selection of cell clones which stably produced bovine PD-L2-Ig was conducted. Briefly, CHO-DG44 cells which the plasmid vector was inserted in their genome were selected in CD DG44 medium (Thermo Fisher Scientific) supplemented with 4 mM GlutaMAX-I (Thermo Fisher Scientific), 0.18% Pluronic F-68 (Thermo Fisher Scientific) and 800 μg/ml G418 (Wako, Osaka, Japan). After cultivation for 2 weeks, cells were screened for the stable expression of bovine PD-L2-Ig using dot blotting with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG Fc polyclonal antibody (A120-111P; Bethyl Laboratories, Montgomery, TX, USA). Cell populations superior for producing bovine PD-L2-Ig were cloned by limiting dilution, and these cell clones were screened by using the same methods described above.

To obtain bovine PD-L2-Ig, shake culture of CHO-DG44 cells producing PD-L2-Ig in a stable manner was conducted in the presence of G418 for 7 days at 37 °C in 5% CO_2. After the cultivation, the supernatants were concentrated by using Centricon Plus-70 (Merck Millipore, Billerica, MA, USA), and soluble bovine PD-L2-Ig was purified using Ab-Rapid SPiN EX (ProteNova, Tokushima, Tokyo) according to the manufacturer's protocol. The concentration of purified PD-L2-Ig was determined using the Rabbit IgG ELISA Quantitation Kit (Bethyl Laboratories), and total protein in the sample was measured by the BCA protein assay kit (Thermo Fisher Scientific).

Western blotting

To confirm molecular weight of bovine PD-L2-Ig, western blotting was performed as described previously [Ikebuchi *et al*., 2013]. In brief, purified PD-L2-Ig protein was lysed in 2× sodium dodecyl sulfate (SDS) buffer (125 mM Tris-HCl, 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol) and boiled for 5 min. The sample was then separated on 12% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (Merck Millipore). After blocking with 3% skim milk in PBS

containing 0.05% Tween 20 (PBS-T), the membrane was incubated with HRP-conjugated anti-rabbit IgG Fc polyclonal antibody (A120-111P; Bethyl Laboratories) at room temperature for 1 h. Antibody signals on the membrane was visualized with DAB substrate (0.05% 3,3-diaminobenzidine tetrahydrochloride, 0.03% $CoCl_2.6H_2O$, and 0.02% H_2O_2 in PBS-T).

Binding assay to bovine PD-1

To confirm the binding capacity of PD-L2-Ig to bovine PD-1, 50 μg/ml PD-L2-Ig or isotype control rabbit IgG (Beckman Coulter) were incubated with bovine PD-1-expressing CHO-DG44 cells [Ikebuchi *et al.*, 2013] or vector transfected CHO-DG44 cells as mock-transfected controls for 30 min. After staining with phycoerythrin (PE)-conjugated anti rabbit IgG antibody (Beckman Coulter), binding of PD-L2-Ig to bovine PD-1 was detected using FACS Verse (BD Biosciences) and FCS Express 4 (De Novo Software). Then, competitive assay was performed to investigate the specificity of the binding reaction of PD-L2-Ig. Briefly, 5 μg/ml PD-L2-Ig was incubated with soluble bovine PD-1-Fc fusion protein, bovine PD-1-Ig [Ikebuchi *et al.*, 2013], for 30 min at various concentrations (1, 2.5, 5, 10, 15, 20 and 40 μg/ml), after which binding of PD-L2-Ig was analyzed as described above.

Immune activation effects of bovine PD-L2-Ig

Bovine PBMCs were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Cell Culture Technologies) and a mixture of 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific) under the stimulation of 5 μg/ml ConA for 48 h, in the presence of 20 μg/ml PD-L2-Ig or isotype control rabbit IgG (Beckman Coulter). Production of IFN-γ in supernatants was measured by using the ELISA for bovine IFN-γ (Mabtech, Nacka Strand, Sweden) according to the manufacturer's protocol.

To examine the effect of PD-L2-Ig on cell proliferation, PBMCs from BLV-infected cattle were labeled with 2 mM carboxyfluorescein diacetate succinimidyl ester (CFSE; Thermo Fisher Scientific) in PBS at 37 °C for 15 min, and cultured for 3 days in RPMI-1640 containing 5 μg/ml ConA in the presence of 20 μg/ml PD-L2-Ig or isotype control rabbit IgG. Then, cells were harvested and stained with anti-CD3 (MM1A; WSU Monoclonal Antibody Center) which had been pre-labeled with Zenon Alexa Fluor 647 Rabbit IgG Labeling Kits (Thermo Fisher Scientific), and were

analyzed using FACS Verse (BD Biosciences) and FCS Express 4 (De Novo Software).

To assess the synergistic effects of the combinations of PD-L2 with anti-bovine PD-1 antibody, PD-L2-Ig (20 μg/ml) and PD-1 antibody (20 μg/ml) were added to the cultures without ConA. Rabbit IgG and rat IgG (Sigma-Aldrich) were used as isotype controls for PD-L2-Ig and PD-1 antibodies, respectively. After 48 h, IFN-γ production in supernatants harvested was determined as described above.

Statistical analysis

Differences between groups were examined for statistical significance using the Wilcoxon signed rank test, or the Kruskal-Wallis test followed by the Steel-Dwass test A. *P*-value of <0.05 was considered as indicating statistical significance.

RESULTS

Sequence analysis of bovine PD-L2

The putative amino acid sequences of bovine PD-L2 from Holstein–Friesian cattle were determined by sequence analysis (Fig. III-I-1A). Bovine PD-L2 comprises a putative signal peptide of 18 aa, a transmembrane domain of 23 aa and an intracellular domain of 32 aa. Its extracellular domain, which comprises 201 aa, contains four potential N-linked glycosylation sites, and the putative binding sites for PD-1 [Lázár-Molnár *et al*., 2008] were largely conserved in all mammalian PD-L2. Phylogenetic analyses have divided mammalian PD-L2 into two groups. Group 1 includes *Perissodactyla*, *Artiodactyla*, and *Carnivora* and group 2 includes Primates and *Rodentia*, with bovine PD-L2 clustering in the artiodactyl species group (Fig. III-I-1B). Comparative analysis of the PD-L2 sequences between several species showed that bovine PD-L2 has amino acid identities of 91.1% (sheep), 89.7% (pig), 87.9% (horse), 82.1% (dog), 85.3% (chimpanzee), 84.6% (human) and 74.3% (mouse).

Bovine PD-L2-Ig binds to PD-1

Bovine PD-L2-Ig was expressed *in vitro* using the pCXN2.1 vector and protein expression system in CHO-DG44 cells. Purified PD-L2-Ig was confirmed as a protein band of about 82 kDa on 12% polyacrylamide gels (Fig. III-I-2A). According to Rabbit IgG ELISA and BCA protein assay, the purity of PD-L2-Ig was 97.7% (PD-L2-Ig/total protein = 819.6 mg/ml / 838.7 mg/ml). Subsequently, the binding affinity of PD-L2-Ig for bovine PD-1 was confirmed using flow cytometry analysis. As expected, bovine PD-L2-Ig bound to cells expressing bovine PD-1, but not to PD-1$^-$ cells (Fig. III-I-2B). To confirm the binding specificity of PD-L2-Ig, bovine PD-1-Ig was added during the reaction between PD-L2-Ig and PD-1 expressing cells. Soluble bovine PD-1-Ig clearly inhibited the binding of PD-L2-Ig to membrane PD-1 in a dose-dependent manner, suggesting PD-L2-Ig specifically bound to bovine PD-1 (Fig. III-I-2C).

Bovine PD-L2-Ig significantly enhances cell proliferation and IFN-γ production

To investigate the effect of bovine PD-L2 on cell proliferation and cytokine production, PBMCs from healthy or BLV-infected cattle were stimulated with ConA and cultured in the presence of bovine PD-L2-Ig or rabbit IgG as a control. PD-L2-Ig enhanced the production of IFN-γ in PBMCs from both healthy and BLV-infected cattle

(Fig. III-I-3A). In agreement with this result, the proliferation of $CD3^+$ T cells from BLV-infected cattle which were cultured in presence of PD-L2-Ig for 72 h was clearly enhanced compared to that of $CD3^+$ T cells treated with control IgG (Fig. III-I-3B).

Bovine PD-L2-Ig enhances IFN-γ production in combination with PD-1 antibody

To confirm dual effects for cytokine production of PD-L2-Ig and PD-1 antibody rather than individual treatments, PBMCs from healthy cattle were cultured with PD-L2-Ig in the presence or absence of bovine PD-1 antibody. IFN-γ production from PBMCs treated with PD-L2-Ig in the combination with anti-PD-1 antibody was greater than that of treatment with control antibodies or the individual treatment (Fig. III-I-4). Dual treatment significantly enhanced IFN-γ production compared to PD-L2-Ig alone, whereas not significantly greater than that by single treatment with PD-1 antibody.

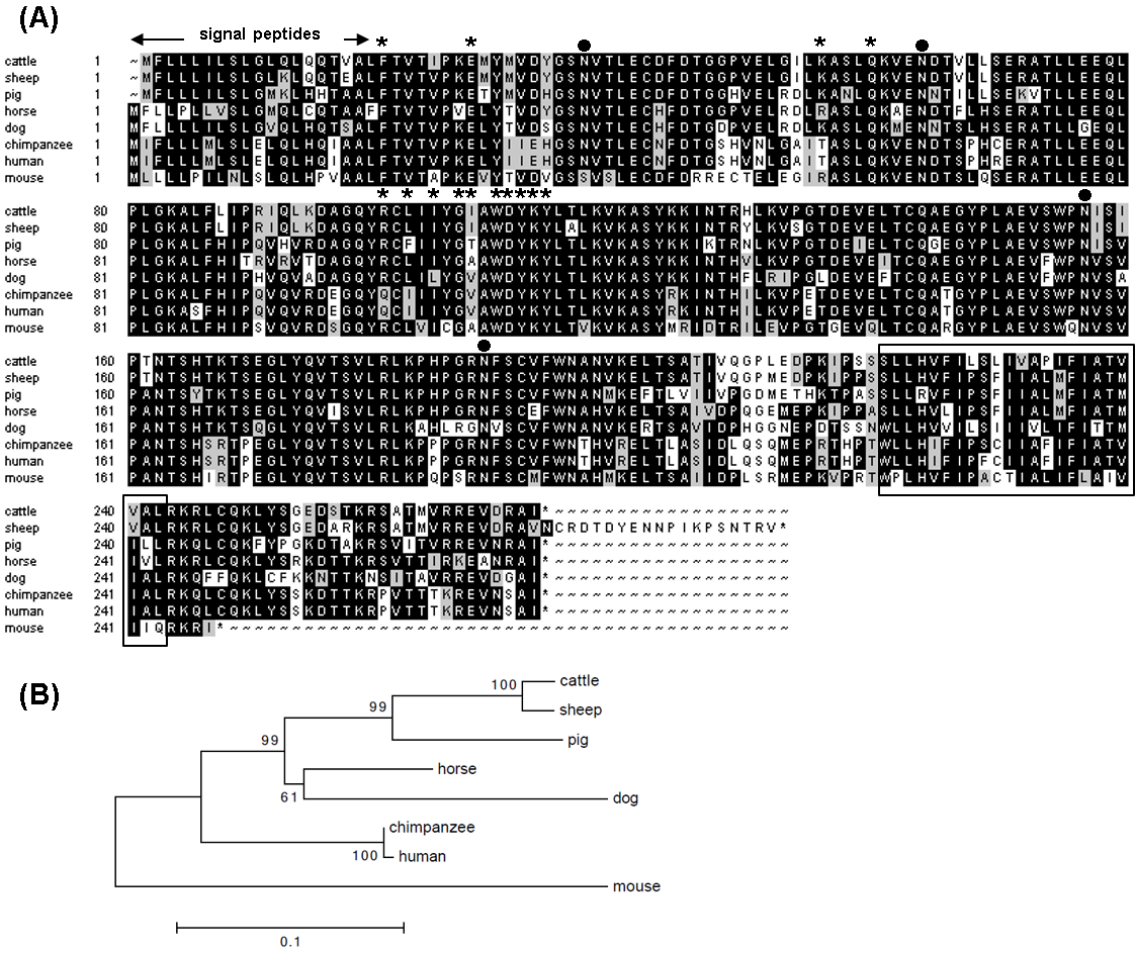

Figure III-I-1 Putative amino acid sequences of bovine PD-L2. (A) Alignment of deduced amino acid sequences of PD-L2 from several animal species. Amino acid residues identical to bovine PD-L2 are boxed in black and conservative substitutions are shown in gray letters. The transmembrane domain is boxed and potential N-glycosylation sites and putative binding sites to PD-1 are marked with black circle and asterisks, respectively. (B) A phylogenetic tree was constructed based on the amino acid sequences of the PD-L2 genes of each species using the neighbor-joining method with MEGA software. Numbers indicate bootstrap percentages (100 replicates), and a scale indicates the divergence time.

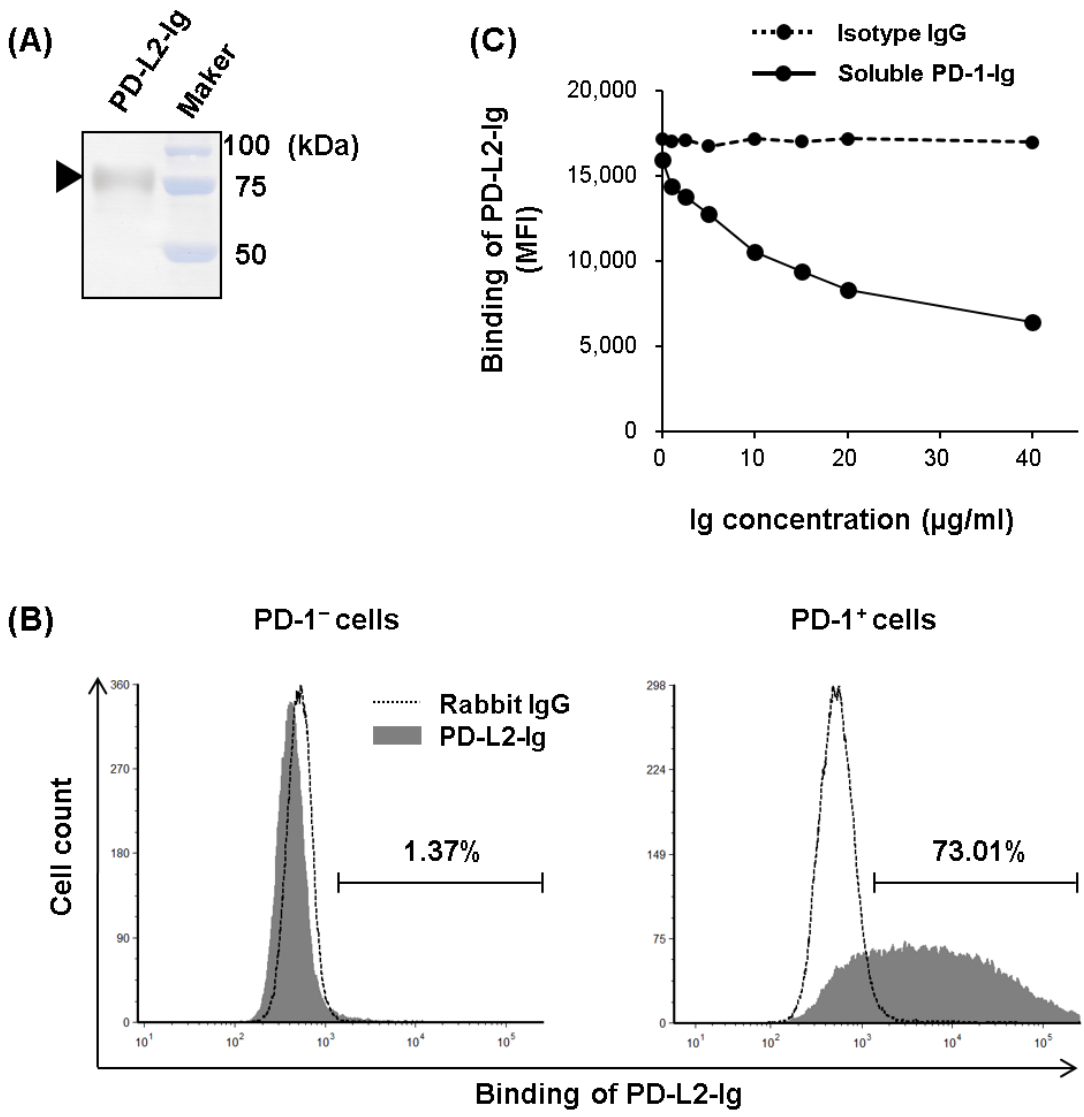

Figure III-I-2 Generation of bovine PD-L2-Ig and binding conformations of bovine PD-L2-Ig and bovine PD-1. Bovine PD-L2-Ig was produced in CHO-DG44 cells which were transfected with pCXN2.1 vector and purified from the culture medium after screening. (A) Purified bovine PD-L2-Ig was confirmed using Western blotting. (B and C) Binding ability of PD-L2-Ig to bovine PD-1 was evaluated by flow cytometry. (B) CHO-DG44 cells expressing bovine PD-1 or mock-transfected cells were incubated with PD-L2-Ig. (C) Soluble bovine PD-1-Ig in serial dilution (1, 2.5, 5, 10, 15, 20 and 40 μg/ml) was reacted with PD-L2-Ig before binding assay to PD-1 on cell membrane.

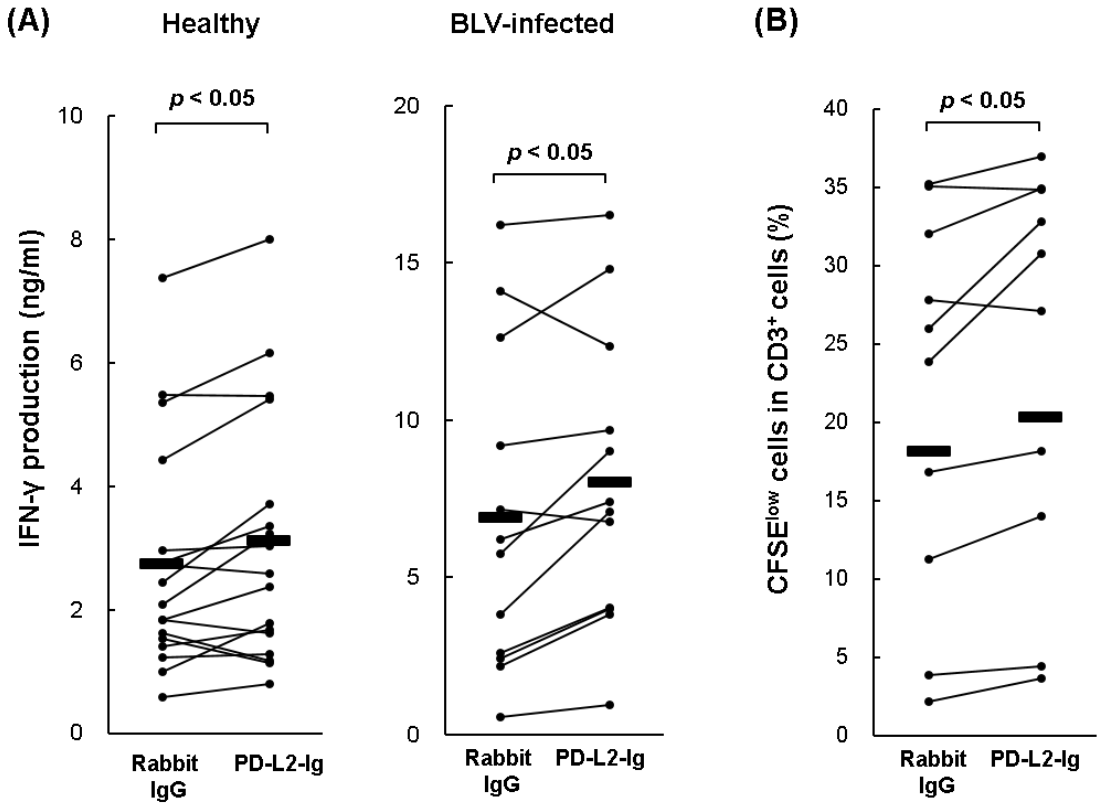

Figure III-I-3 Enhancement of T-cell functions by bovine PD-L2-Ig. (A) Bovine PBMCs from healthy (n = 17) and BLV-infected cattle (n = 12) were stimulated with 5 μg/ml ConA and cultured with 20 μg/ml PD-L2-Ig or rabbit IgG. IFN-γ production was measured by using ELISA. Lines indicate mean values from each group. Wilcoxon signed rank test. (B) PBMCs from BLV-infected cattle (n = 10) were labeled with CFSE and cultured with 20 μg/ml PD-L2-Ig or rabbit IgG in the presence of 5 μg/ml ConA. Proliferation of $CD3^+$ T cells was evaluated by detecting $CFSE^{low}$ population by flow cytometry. Lines indicate mean values from each group. Wilcoxon signed rank test.

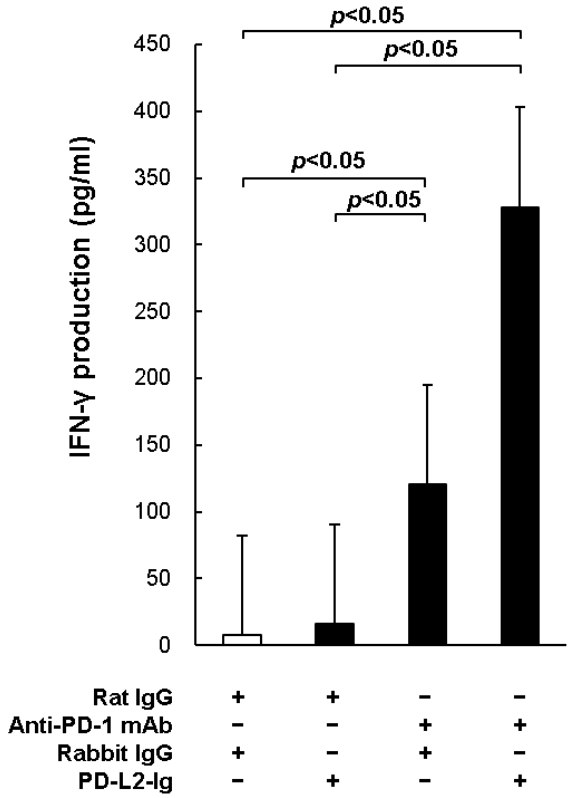

Figure III-I-4 IFN-γ production by dual treatment with bovine PD-L2-Ig and anti-PD-1 antibody. PBMCs from healthy cattle (n = 9) were treated with various combination with PD-L2-Ig, anti-PD-1 antibody, and those IgG controls for 48 h. After the cultivation, IFN-γ production was measured by ELISA. Kruskal-Wallis test followed by Steel-Dwass test.

DISCUSSION

In this study, it was demonstrated that bovine PD-L2- Ig can enhance T-cell proliferation and IFN-γ production, in agreement with the findings in previous studies on human and mouse models. Additionally, IFN-γ production induced by PD-L2-Ig was further enhanced by anti-bovine PD-1 antibody which have been established previously. In the previous studies, it has been shown that the blockade of immune inhibitory pathways, including PD-1/PD-L1, restores T-cell function during BLV infection which was a model of chronic bovine infectious diseases [Ikebuchi *et al*., 2010; Ikebuchi *et al*., 2011; Okagawa *et al*., 2012; Ikebuchi *et al*., 2013; Konnai *et al*., 2013; Ikebuchi *et al*., 2014]. Although the precise mechanisms to enhance cell proliferation and IFN-γ production by bovine PD-L2-Ig remain unknown, PD-L2-Ig can be added to the list of potential immunotherapeutic approaches against chronic diseases in cattle.

Because most of clinical trials targeting the PD-1/PD-Ls pathways have used anti-PD-1 or PD-L1 antibodies, the functions of PD-L2 remain poorly characterized compared to those of PD-1 and PD-L1. In contrast to previous reports, recent studies have shown that PD-L2 is involved in immune activation, such as induction of cytokine production and T cell proliferation [Liu *et al*., 2003; Shin *et al*., 2003; Mkrtichyan *et al*., 2012]. It has been reported in one study that experimental injection of PD-L2-expressing tumor cells promoted tumor rejection mediated by $CD8^+$ T cells *in vivo* [Liu *et al*., 2003]. Another report also demonstrated that PD-L2-Ig enhances anti-tumor immune response by inhibiting the proliferation of regulatory T cells and by decreasing the numbers of PD-1-expressing $CD8^+$ cells within tumors [Mkrtichyan *et al*., 2012]. Currently, GlaxoSmithKline are conducting a clinical trial of AMP-224, as the third method targeting the PD-1/PD-Ls pathway, following anti-PD-1 or PD-L1 antibodies [Topalian *et al*., 2012b; Sunshine *et al*., 2015]. In addition to tumor studies, PD-L2 knockout mice infected with *Trypanosoma cruzi* developed greater parasitemia than wild type mice, suggesting that PD-L2 has a protective role in the immune response against this parasite [Dulgerian *et al*., 2011].

Previous studies demonstrated that increase in the expression of PD-1 and PD-L1 is closely associated with disease progression of BLV-infected cattle [Ikebuchi *et al*., 2010; Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2013]. Although PD-1 expression was not investigated in this study, IFN-γ production was restored in both of healthy and BLV-infected cattle by the blockade of the PD-1/PD-L1 pathway by PD-L2-Ig. The

mechanism of immune activation via PD-L2 remains speculative, but recent reports suggested the presence of an unknown receptor(s) for PD-L2, since PD-L2 stimulates T cells independent of PD-1 expression [Liu *et al*., 2003; Shin *et al*., 2003; Wang *et al*., 2003]. More recently, it has been reported that PD-L2 binds to RGMb, which was originally identified in the nervous system as a co-receptor for bone morphogenetic proteins [Xiao *et al*., 2014]. In this report, the authors commented that understanding the PD-L2/RGMb pathway may provide insights into how to optimally modulate the PD-1 pathway in cancer immunotherapy by minimizing adverse events. However, the expression level and functional role of RGMb in lymphocytic neoplasm are still unknown, even in humans. Moreover, there is no information on RGMb in cattle. Thus, it is difficult to determine whether the enhancement of immune response induced by bovine PD-L2 is directly or indirectly related to PD-1 on cell membrane, and to clarify them will be an aim of future studies.

In conclusion, consistent with recent reports, bovine PD-L2-Ig enhanced T-cell proliferation and IFN-γ production. It has been reported that PD-L2-Ig enhanced the therapeutic efficacy of anti-tumor vaccines and led to complete eradication of established tumors in 60% of mice [Mkrtichyan *et al*., 2012]. Currently, numerous bovine diseases with poor prognoses have prevailed because of the lack of effective treatments and vaccines. Thus, the results of this study may facilitate the design of clinical agents which enhance anti-microbial immunity for the treatment of bovine chronic infections. In humans, several clinical trials have been scheduled or are in progress in order to assess the effects of the combinations of antibodies targeting the PD-1/PD-Ls pathway with cancer vaccines, anti-tumor antibodies and chemotherapies. Therefore, the efficacy of vaccines in the combination with bovine PD-L2-Ig is worth investigating to establish more effective vaccines strategies. Current studies are elucidating the possibility of clinical application of PD-L2-Ig as a novel strategy to control BLV infection in the field.

CHAPTER III

SECTION II

In vitro and *in vivo* antivirus activity of an anti-PD-L1 rat-bovine chimeric antibody against BLV infection

MATERIALS AND METHODS

Blood samples

Bovine blood samples were obtained from several farms and veterinary services or from an experimental farm at Field Science Center for Northern Biosphere, Hokkaido University, and BLV infection was diagnosed at the Hokkaido University Veterinary Teaching Hospital, as described in Chapter I. PBMCs from healthy or BLV-positive cattle were purified by density gradient centrifugation using Percoll (GE Healthcare) and were cultured in RPMI-1640 medium (Sigma-Aldrich) containing 10% heat-inactivated FBS (Thermo Fisher Scientific), 2mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo Fisher Scientific).

Cells

CHO-DG44 cells were kindly provided by Dr. Y. Suzuki, as described in Section I of Chapter III, and cells stably expressing enhanced green fluorescent protein (EGFP) or bovine PD-L1/EGFP were established in a previous study [Ikebuchi *et al*., 2014b]. In brief, a gene encoding the extracellular domain of bovine PD-L1 was cloned into pEGFP-N2 (Clontech, Mountain View, CA, USA). This plasmid or pEGFP-N2 was then transfected into CHO-DG44 cells using Lipofectamine LTX reagent (Thermo Fisher Scientific) to produce PD-L1/EGFP or EGFP cells, respectively. Cells which expressed EGFP or PD-L1/ EGFP in a stable manner were selected using 800 μg/ml G418 (Enzo Life Sciences, Farmingdale, NY, USA) and then cloned by limiting dilution. EGFP and PD-L1/EGFP cells were maintained in CD DG44 medium (Thermo Fisher Scientific) supplemented with 4 mM GlutaMAX-I (Thermo Fisher Scientific) and 0.18% Pluronic F-68 (Thermo Fisher Scientific).

Anti-bovine PD-L1 rat monoclonal antibody

A mAb 4G12 which is specific to bovine PD-L1 was generated as described previously [Ikebuchi *et al*., 2014b]. Briefly, a rat was immunized with soluble PD-L1 recombinant protein emulsified with complete Freund's adjuvant. Lymphocytes from the iliac lymph node were then fused with myeloma cells 24 h after immunization. Antibodies in supernatants produced by the hybridomas were screened using flow cytometry and those which recognized bovine PD-L1/EGFP cells but not EGFP cells were cloned by limiting dilution. Immunization of the rat and cultivation of the

hybridoma were performed at Cell Engineering Corporation (Osaka, Japan). To prepare a large amount of the antibody for the inoculation of cattle, the hybridoma which produced 4G12 was cultured in Hybridoma-SFM (Thermo Fisher Scientific) for 10 days at the Tohoku University Graduate School of Medicine (Sendai, Japan). Following the cultivation, 4G12 was purified using Protein G Sepharose (GE Healthcare), according to the manufacturer's protocol.

Construction of a plasmid vector encoding Boch4G12

Total RNA was extracted from the hybridoma which produced 4G12 using TRIzol reagent (Thermo Fisher Scientific). To identify the sequence encoding the variable region of 4G12, 5′-RACE was performed using the 5′-RACE System (Thermo Fisher Scientific) with primers specific to the rat immunoglobulin gene (5′-ACA AGG ATT GCA TTC CCT TGG-3′ for rat IgG2a, or 5′-CTC ATT CCT GTT GAA GCT CTT GAC GAC-3′ for rat Ig kappa). The cDNA encoding the variable region was amplified from the poly(C)-tailed RNA using a poly(G) primer and other specific primers (5′-CTC AAT TTT CTT GTC CAC CTT GGT GC-3′ for rat IgG2a, or 5′-CTC ATT CCT GTT GAA GCT CTT GAC GAC GGG-3′ for rat Ig kappa). Following the purification of the PCR amplicons using the FastGene Gel/PCR Extraction Kit (Nippon Genetics), they were cloned into pGEM1-T Easy vector (Promega) and sequenced using the CEQ 8000 DNA Analysis System (Beckman Coulter). The design of the primers used in this study has been outlined in a previous report [Bradbury, 2010].

Genes encoding the variable region of 4G12 heavy chain coupled with a bovine IgG1 (GenBank accession No: X62916) and that of light chain coupled with a bovine Ig lambda (GenBank accession No: X62917) were commercially synthesized in Medical and Biological Laboratories (Nagoya, Japan). Codon in the synthesized genes was optimized in CHO cells. To reduce the antibody-dependent cell-mediated cytotoxicity (ADCC) response, a mutation was introduced into the binding sites of Fcγ receptors as previously described [Armour *et al*., 1999; Shields *et al*., 2001; Ikebuchi *et al*., 2014b]. The synthesized genes were cloned into the pDC6 vector (kindly provided by Dr. Y. Suzuki) to produce pDC6-Boch4G12.

Stable expression and purification of Boch4G12

Cells which produced large amounts of Boch4G12 in a stable manner were established using the dihydrofolate reductase (DHFR)/methotrexate (MTX)-gene

amplification system in CHO-DG44 cells which were DHFR-deficient cells. The pDC6-Boch4G12 was transfected into CHO-DG44 cells and selected in CD OptiCHO AGT medium (Thermo Fisher Scientific) supplemented with 4 mM GlutaMAX-I. After cultivation for 3 weeks, cells were screened for the stable expression of Boch4G12 using dot blotting and ELISA with anti-bovine IgG Fc polyclonal antibody (Rockland Immunochemicals, Pottstown, PA, USA). Ten of the most superior cell populations producing Boch4G12 were cloned by limiting dilution, and the top clone was then screened from each population using the same methods as described above. Then, the cell clones were cultured in CD OptiCHO AGT medium containing 60 nM MTX (Enzo Life Sciences) for gene amplification and re-screened. This procedure was repeated twice with CD OptiCHO AGT medium containing 250 nM or 1 μM MTX, respectively.

Large amounts of Boch4G12 for the inoculation of cattle were obtained by shake culture of the cell clones in the medium without MTX at 37˚C and 125 rpm with 5% CO_2 for 14 days. The antibody in the supernatant of the culture medium was then purified using Ab-Capcher ExTra (ProteNova), according to the manufacturer's instructions. The efficiency of antibody purification was verified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing (with 2-mercaptoethanol; Sigma-Aldrich) and non-reducing conditions. The concentration of Boch4G12 was determined using bovine IgG Fc ELISA (Rockland Immunochemicals).

Binding assay to bovine PD-L1

PD-L1/EGFP cells were incubated with 4G12, Boch4G12, rat IgG2a isotype control (R35-95; BD Bioscience), or bovine IgG1 isotype control (Bethyl Laboratories) in serial dilution (0.01–10 μg/ml). Allophycocyanin (APC)-conjugated anti-rat Ig (Beckman Coulter) or APC-conjugated anti-bovine IgG Fc (Jackson ImmunoResearch, West Grove, PA, USA) were used as secondary antibodies. Binding of the antibodies was detected by FACS Verse (BD Biosciences) and FCS Express 4 (De Novo Software).

Blockade of the PD-1/PD-L1 interaction

To confirm the blockade activity of Boch4G12 against the PD-1/PD-L1 interaction, PD-L1/EGFP cells were incubated with 4G12, Boch4G12, rat IgG2a isotype control (R35-95; BD Bioscience), or bovine IgG1 isotype control (Bethyl Laboratories) in serial dilution (0, 0.32, 0.63, 1.25, 2.5, 5, and 10 μg/ml) at 37˚C for 15 min. Biotinylated bovine PD-1-Ig (2 μg/ml) [Ikebuchi *et al.*, 2013] was then added to each

well without washing and incubated at 37˚C for 30 min. After washing twice, the cells were stained by APC-conjugated streptavidin (BioLegend) at room temperature for 30 min and the binding of PD-1-Ig to PD-L1/EGFP was detected by flow cytometry, as described above.

T-cell reactivation assay

PBMCs from healthy cattle were labeled with 0.2 μM CFSE and incubated with anti-CD3 (MM1A; WSU Monoclonal Antibody Center) at 4˚C for 30 min, followed by anti-mouse IgG1 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4˚C for 15 min. $CD3^+$ T cells were sorted from the PBMCs using an autoMACS Pro (Miltenyi Biotec), according to the manufacturer's instructions. To investigate the effects of Boch4G12 on T-cell proliferation, isolated $CD3^+$ T cells were stimulated with 1 μg/ml anti-CD3 (MM1A; WSU Monoclonal Antibody Center) and 1 μg/ml anti-CD28 (CC220; Bio-Rad), and co-cultured with 10 μg/ml mitomycin C-treated EGFP or PD-L1/EGFP cells in RPMI-1640 medium in the presence or absence of 10 μg/ml IgG from bovine serum (Sigma-Aldrich) and Boch4G12. After 3 days, the cells were harvested and stained with anti-CD4 (CC30; Bio-Rad) which was pre-labeled with Zenon Alexa Fluor 647 (Thermo Fisher Scientific), and anti-CD8 (CC63; Bio-Rad) which was pre-labeled using the Lightning-Link peridinin-chlorophyll-protein complex/cyanin (commonly known as PerCp/Cy5.5) Conjugation Kit (Innova Biosciences, Cambridge, UK). Fixable Viability Dye eFluor1780 (Thermo Fisher Scientific) was used for a live/dead staining. The intensity of CFSE fluorescence was analyzed by flow cytometry, as described above.

To investigate the effects of Boch4G12 on IFN-γ production, PBMCs from BLV-infected cattle were stimulated with heat-inactivated culture supernatants of BLV-uninfected fetal lamb kidney (FLK) or BLV-infected fetal lamb kidney (FLK-BLV) cells, and cultured in the presence of 10 μg/ml IgG from rat serum (Sigma-Aldrich), 4G12, IgG from bovine serum (Sigma-Aldrich), or Boch4G12 for 6 days. The concentration of IFN-γ in the culture supernatants was measured using ELISA for bovine IFN-γ (Mabtech), according to the manufacturer's protocol.

Inoculation of the antibodies in BLV-infected cattle

To confirm the *in vivo* effects of 4G12 and Boch4G12, two cattle were introduced into an animal facility at the Animal Research Center, Hokkaido Research

Organization (Shintoku, Hokkaido, Japan; Table III-II-1) from its experimental farm. A BLV-infected cow (#368, Holstein-Friesian, female, 538 kg, 31 months old) was inoculated with 530 mg (1 mg/kg) of the purified 4G12 intravenously; and a healthy calf (#15–11, Holstein-Friesian, male, 267 kg, 7 months old) was experimentally infected with BLV through the administration of BLV-positive leukocytes (1.0×10^4 copies of provirus), and after developed to the AL stage, the cow was inoculated with 260 mg (1 mg/kg) of the purified Boch4G12 intravenously at 13 weeks post-infection. Peripheral blood samples were collected from both cattle before the inoculation and at least once per week after the inoculation. The animal experiments in this study were approved by the Ethics Committee of the Graduate School of Veterinary Medicine, Hokkaido University.

PD-L1 occupancy

The binding of 4G12 to bovine PD-L1 on circulating IgM^+ B cells was investigated to calculate the PD-L1 occupancy following the inoculation. PBMCs isolated from #368 were pre-incubated with 10 μg/ml rat IgG2a isotype control (BD Bioscience) or 4G12, and then incubated with APC-conjugated anti-rat Ig (Beckman Coulter) at room temperature for 20 min. The same secondary antibody pre-incubated with IgG from rat serum (Sigma-Aldrich) at 37˚C for 15 min was used as an unstained control. After washing twice, anti-IgM (IL-A30; Bio-Rad) which was pre-labeled using the Lightning-Link PE/Cy7 Conjugation Kit (Innova Biosciences) was reacted at room temperature for 20 min. The binding of the antibodies was then detected by flow cytometry, as described above. PD-L1 occupancy was estimated as the percentage of the *in vivo* binding (indicated as the number of cells positively stained by rat IgG2a isotype control) occurred at the total available binding sites (indicated as the number of cells positively stained by a saturated concentration of 4G12).

BLV provirus loads

Genomic DNA was extracted from 1 to 5×10^6 PBMCs of the cattle which inoculated the antibodies using the Wizard Genomic DNA Purification Kit (Promega). Measurement of BLV provirus loads was performed as described in Chapter II.

Statistical analysis

Differences between groups were examined for statistical significance using a

paired *t*-test or Wilcoxon signed rank test. For multiple group comparisons, one-way analysis of variance (ANOVA) was performed, followed by Tukey's test or Dunnett's test. A *P*-value less than 0.05 was considered statistically significant.

Table III-II-1 Information on cattle inoculated anti-bovine PD-L1 antibodies

Cattle	#368	#15-11
Age	31 months old	7 months old
Breed	Holstein-Friesian	Holstein-Friesian
Sex	female	male
BLV infection	naturally infected	experimentally infected
Body weight	538 kg	267 kg
Inoculated antibody	4G12	Boch4G12
Inoculation dose	1 mg/kg, *i.v.*	1 mg/kg, *i.v.*

RESULTS

In vivo antivirus activity of the anti-bovine PD-L1 rat antibody

A previous study demonstrated that the anti-bovine PD-L1 antibody 4G12 induced an immune activation *in vitro* [Ikebuchi *et al*., 2014b]. Therefore, to evaluate its antivirus activity *in vivo*, a cow (n = 1) which had been naturally infected with BLV was inoculated with 1 mg/kg 4G12. T-cell proliferation was temporarily increased following the inoculation, but this increase was not BLV-specific (Fig. III-II-1A). Furthermore, the results of PD-L1 occupancy indicated that the inoculated antibody was maintained in the blood only for one week, following that it was dramatically decreased to a level similar to pre-inoculation (Fig. III-II-1B). In addition, no reduction in BLV provirus loads was observed during the test period (Fig. III-II-1C).

Binding activity of the newly-established Boch4G12

It was hypothesized that the inoculated 4G12 did not show antivirus activities because it was rapidly removed as a xenogenous protein. Thus, a more stable type of antibody (e.g., a chimeric antibody) was required to accurately assess the effects of anti-bovine PD-L1 antibodies. Thus, the construction of a plasmid vector encoding Boch4G12, which represented the variable region of the 4G12 heavy chain coupled with a bovine IgG1, and that of the light chain coupled with a bovine Ig lambda, was conducted (Fig. III-II-2A). To avoid cell cytotoxicity, the mutation was introduced into the constant region of Boch4G12, as described previously [Armour *et al*., 1999; Shields *et al*., 2001; Ikebuchi *et al*., 2014b]. Following transfection of the plasmid vector into CHODG44 cell lines, Boch4G12 was purified from the culture supernatant, and then the efficiency of its stable expression and purification was confirmed by using SDS-PAGE, which showed single bands at around 25 kDa representing the light chain and 50 kDa representing the heavy chain under reduced conditions, and 150 kDa representing the whole antibody under non-reduced conditions (Fig. III-II-2B).

To examine the *in vitro* functions of Boch4G12, the binding activity to bovine PD-L1-expressing cells, PD-L1/EGFP cells, was assessed by flow cytometry. As with the original antibody, Boch4G12 bound to bovine PD-L1 in a dose-dependent manner (Fig. III-II-2C). Since it is essential for therapeutic antibodies targeting PD-L1 molecules to interrupt the PD-1/PD-L1 interaction, the blockade activity of Boch4G12 was confirmed using PD-L1/EGFP cells and soluble bovine PD-1-Ig. Compared to

control antibodies which never inhibited the interaction between PD-1-Ig and PD-L1 expressed on the cell membrane, Boch4G12 significantly suppressed the PD-1/PD-L1 interaction in a similar way to 4G12 (Fig. III-II-2D). Thus, a functional chimeric antibody that is specific to bovine PD-L1 was successfully established.

***In vitro* effect of Boch4G12 on T-cell function**

Anti-PD-L1 antibodies exert their function by suppressing the PD-1 signal following PD-L1 blockade. To investigate whether the newly-established Boch4G12 antibody also affected T-cell activation, $CD3^+$ T cells were isolated from PBMCs of healthy cattle and stimulated with the anti-CD3 and anti-CD28 mAbs. Then the cells were cultured with EGFP or PD-L1/ EGFP cells in the presence or absence of Boch4G12 for 3 days. The presence of PD-L1/EGFP cells significantly inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells, and the addition of Boch4G12 suppressed this inhibition in $CD4^+$ T cells, but not $CD8^+$ T cells (Fig. III-II-3A and 3B). These results indicate that the interaction between bovine PD-1 and PD-L1 induces T-cell suppression and that Boch4G12 can restore their proliferation through PD-L1 blockade. Previous studies have shown that antibodies which bind to bovine PD-L1 upregulate IFN-γ production in PBMCs from healthy or BLV-infected cattle *in vitro* [Ikebuchi *et al*., 2011: Ikebuchi *et al*., 2014b]. To confirm this effect, PBMCs were isolated from BLV-infected cattle, stimulated with FLK-BLV supernatant, and then cultured in the presence of 4G12 or Boch4G12. Both antibodies significantly upregulated IFN-γ production in the supernatants, compared to the control IgG (Fig. III-II-3C), indicating that both the original antibody and Boch4G12 can activate T-cell function in BLV-infected cattle by increasing IFN-γ production. Although Boch4G12 looked like being made significant by the one outlier, the effect of Boch4G12 on IFN-γ production was still significantly higher than that of the control IgG even if that one animal was excluded (P <0.02, data not shown).

***In vivo* analysis of the antivirus activities of Boch4G12**

Since Boch4G12 activated T cells *in vitro*, its function *in vivo* was also examined using a BLV-infected calf. A healthy calf (n = 1) was experimentally infected with BLV and then inoculated with 1 mg/kg Boch4G12. Blood samples were collected from this animal to examine T-cell proliferation and BLV provirus loads for evaluating the antivirus activities of this antibody. $CD4^+$ T-cell proliferation was upregulated

following the antibody inoculation, while $CD8^+$ T-cell proliferation was not affected (Fig. III-II-4A). Furthermore, the response against the BLV antigen was significantly higher at days 1, 14, and 70 compared to that before the inoculation. These results indicate that Boch4G12 prevents PD-1 which is expressed on BLV-specific T cells from binding to PD-L1, and reactivates their immune response. BLV provirus loads in PBMCs were also reduced by up to 74.7% after the inoculation, while they were consistently high before the inoculation (Fig. III-II-4B), suggesting that Boch4G12 can induce antivirus activities to decrease provirus loads in BLV-infected cattle. Although the lowest copy number of BLV was observed at day 55, there was no clear association between high T-cell response and low provirus loads at these specific time points. Instead, the reduction of BLV provirus loads occurred following high T-cell response against BLV antigen observed at day 1 and 14 (Fig. III-II-4A and 4B).

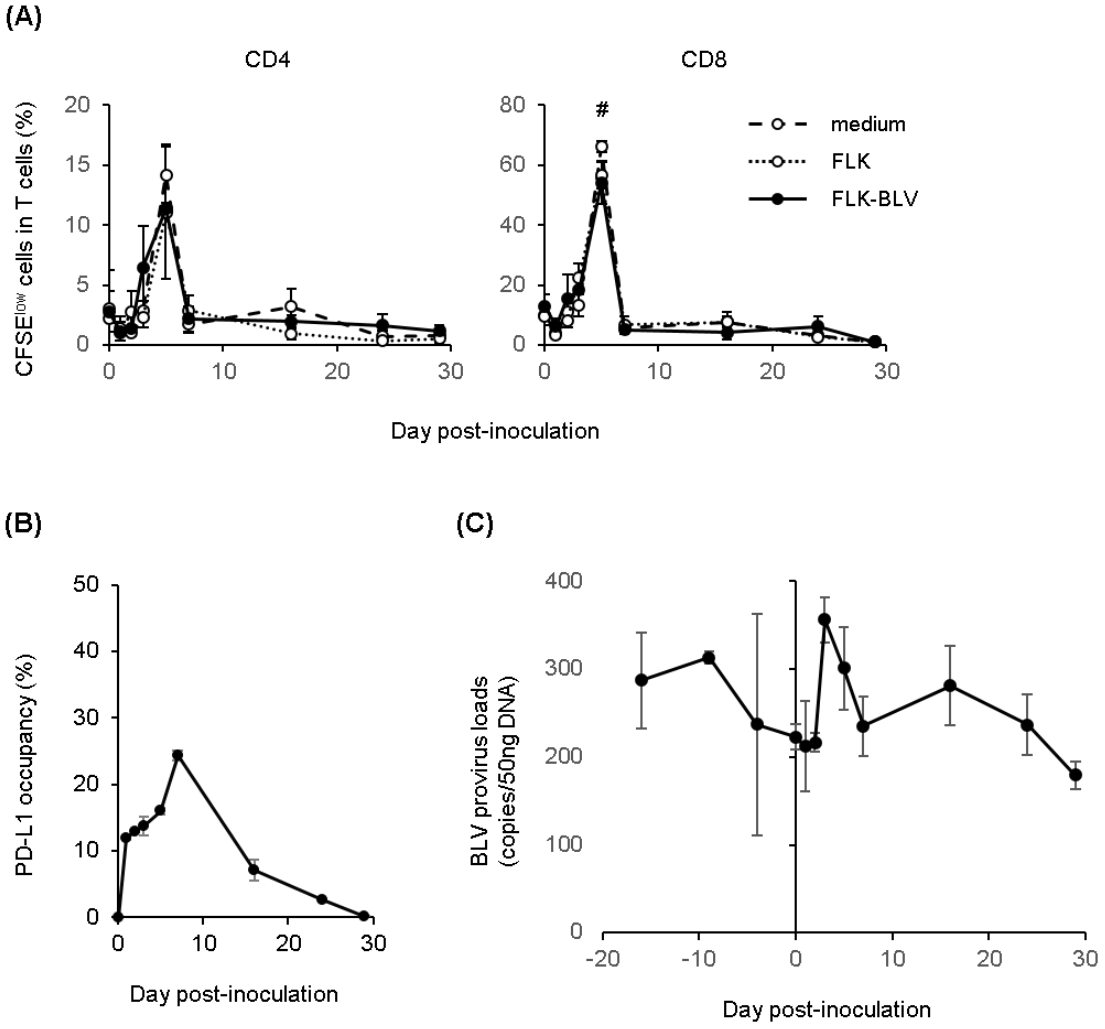

Figure III-II-1 Effects of *in vivo* treatment of a BLV-infected cow with anti-bovine PD-L1 rat monoclonal antibodies. A BLV-infected cow (#368, Holstein-Friesian, female, 538 kg, 31 months old) was inoculated with 530 mg (1 mg/kg) of the purified 4G12 intravenously. (A) The proliferation of $CD4^+$ and $CD8^+$ T cells against BLV antigen. PBMCs isolated from the cow were labeled with CFSE and cultured without stimulation (medium) or with the supernatant of FLK or FLK-BLV cells for 6 days. After the cultivation, the proliferation of T cells was analyzed by flow cytometry. A *P*-value less than 0.05 was considered statistically significant. #, $P < 0.05$ (FLK-BLV, versus day 0; one-way ANOVA followed by Dunnett's test). (B) Changes in PD-L1 occupancy on circulating IgM^+ B cells calculated by the binding of 4G12 to bovine PD-L1. The occupancy was estimated as the percentage of the *in vivo* PD-L1 binding occurred at the total available binding sites. (C) Changes in BLV provirus loads in the cow inoculated with 4G12; the y-axis shows the number of BLV copies included in 50-ngDNA extracts of PBMCs. Data are means ±SEMof at least three replicate experiments.

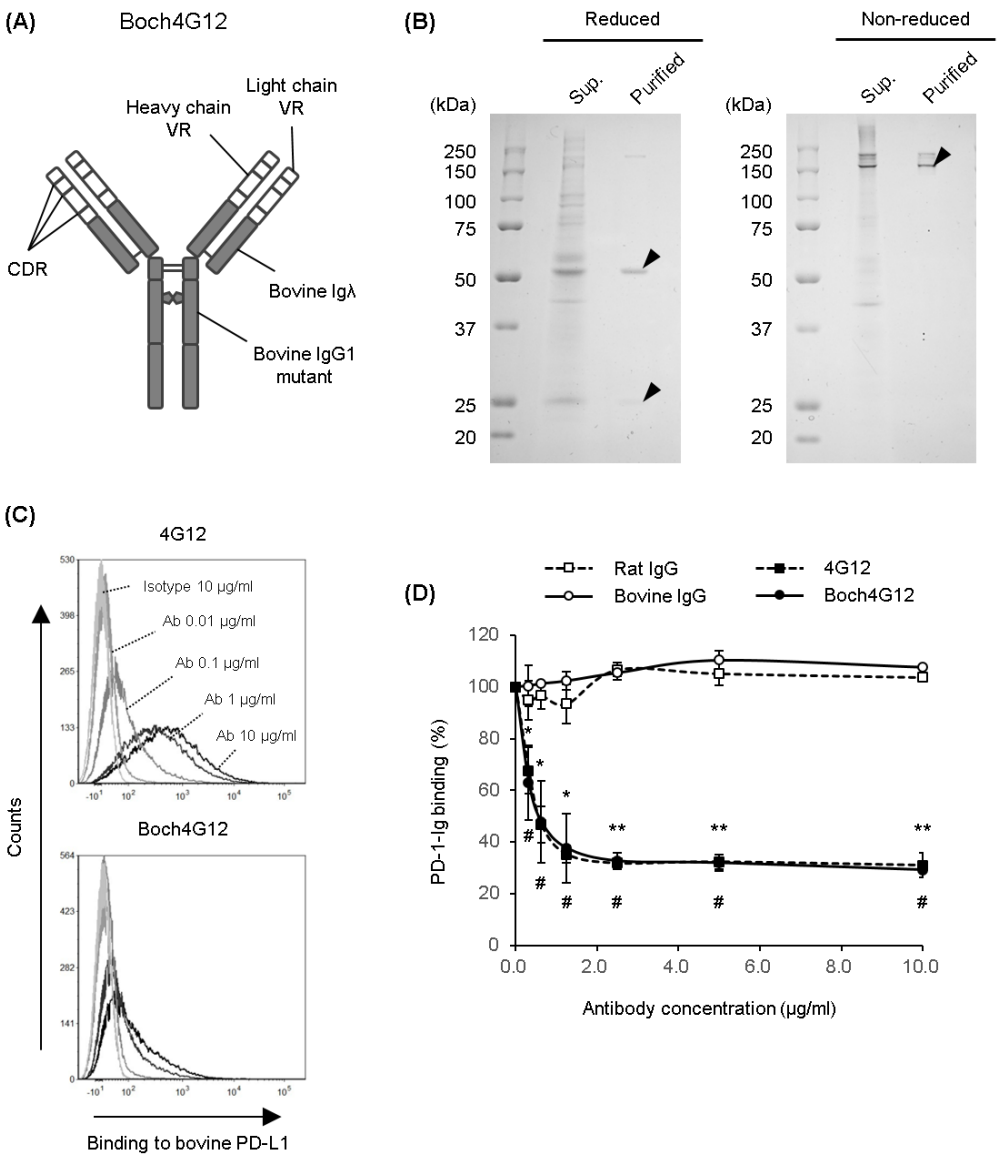

Figure III-II-2 Establishment of Boch4G12 and a binding assay against bovine PD-L1. (A) Diagram of the Boch4G12 structure. White region indicates protein of rat origin and gray indicates protein of bovine origin. VR, variable region; CDR, complementarity-determining region. (B) Confirmation of Boch4G12 expression by SDS-PAGE under reduced and non-reduced conditions. Sup., supernatant before antibody purification; Purified, purified Boch4G12. (C) Binding activity of Boch4G12 against bovine PD-L1. PD-L1/ EGFP cells were stained by 4G12 or Boch4G12 at different concentrations (0.01–10 μg/ml) and the antibody binding was detected using anti-rat IgG or anti-bovine IgG secondary antibodies. (D) The blockade activity of Boch4G12 to interrupt the PD-1/PD-L1 interaction. PD-L1/EGFP cells were pre-incubated with different concentrations of 4G12 or Boch4G12 before being incubated with PD-1-Ig, and the binding of PD-1-Ig was detected by flow cytometry. The y-axis shows the rates of PD-1-Ig binding compared with the non-antibody control. A *P*-value less than 0.05 was considered statistically significant. *, $P < 0.05$, **, $P < 0.01$ (Boch4G12 versus bovine IgG); #, $P < 0.01$ (4G12 versus rat IgG), paired *t*-test. Data are the means ±SEMof three replicate experiments.

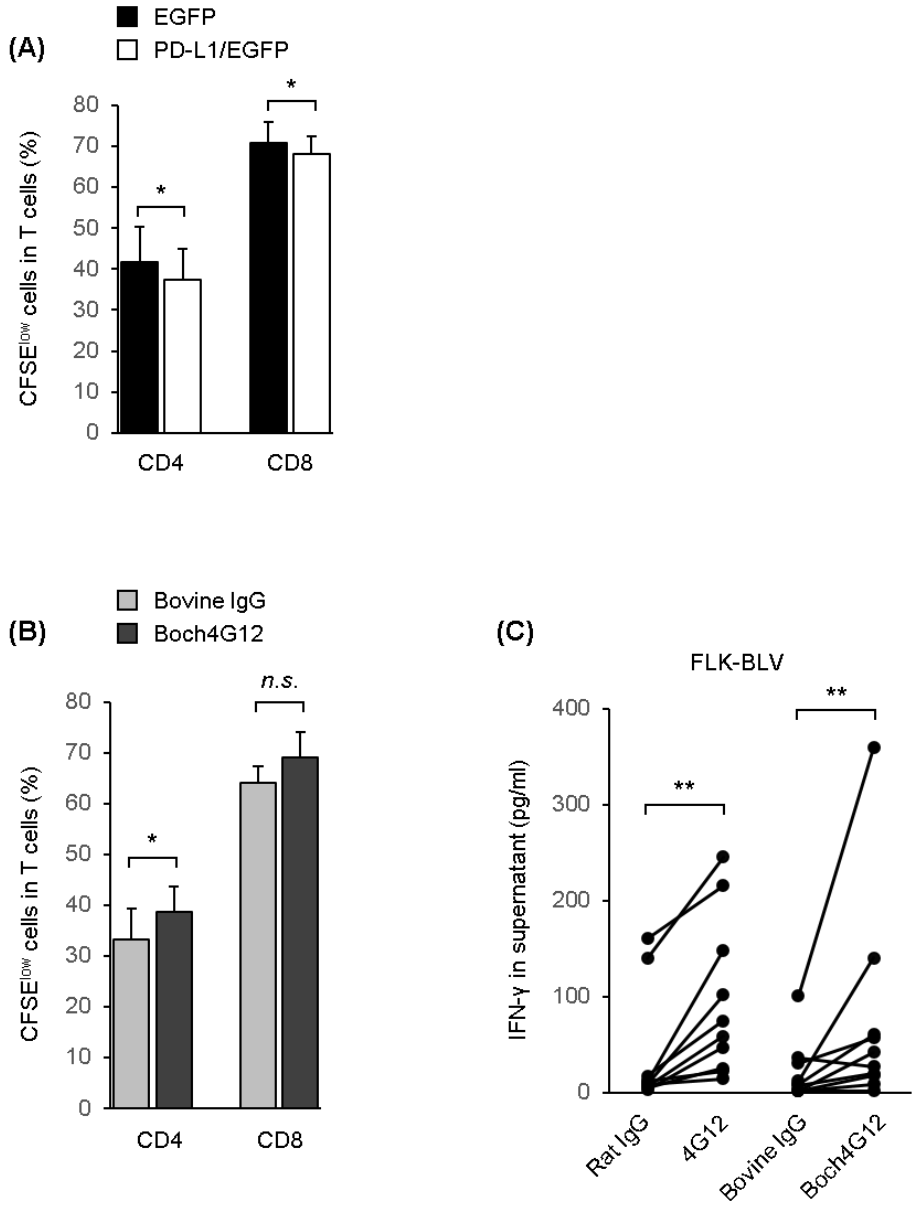

Figure III-II-3 Activation of T-cell proliferation and IFN-γ production by Boch4G12. (A and B) The proliferation of $CD4^+$ and $CD8^+$ cells in purified bovine $CD3^+$ T cells. PBMCs isolated from healthy cattle were labeled with CFSE, stimulated with 1 μg/ml anti-CD3 and anti-CD28 mAbs, and then cultured for 3 days with EGFP cells or PD-L1/EGFP cells (A) without antibodies or (B) PD-L1/EGFP cells in the presence of 10 μg/ml bovine IgG control or Boch4G12. n = 10 and 6, respectively; A P-value less than 0.05 was considered statistically significant. *, P <0.05, paired t-test. (C) IFN-γ production in the culture supernatant of PBMCs from BLV-infected cattle (n = 10, AL:5, PL:5). PBMCs were stimulated with the supernatant of FLK-BLV cells and cultured for 6 days in the presence of 10 μg/ml rat IgG control, 4G12, bovine IgG control or Boch4G12. The concentration of IFN-γ in the culture supernatants was measured using ELISA. A P-value less than 0.05 was considered statistically significant. **, P <0.01, Wilcoxon signed rank test. Data are the means of three replicate experiments.

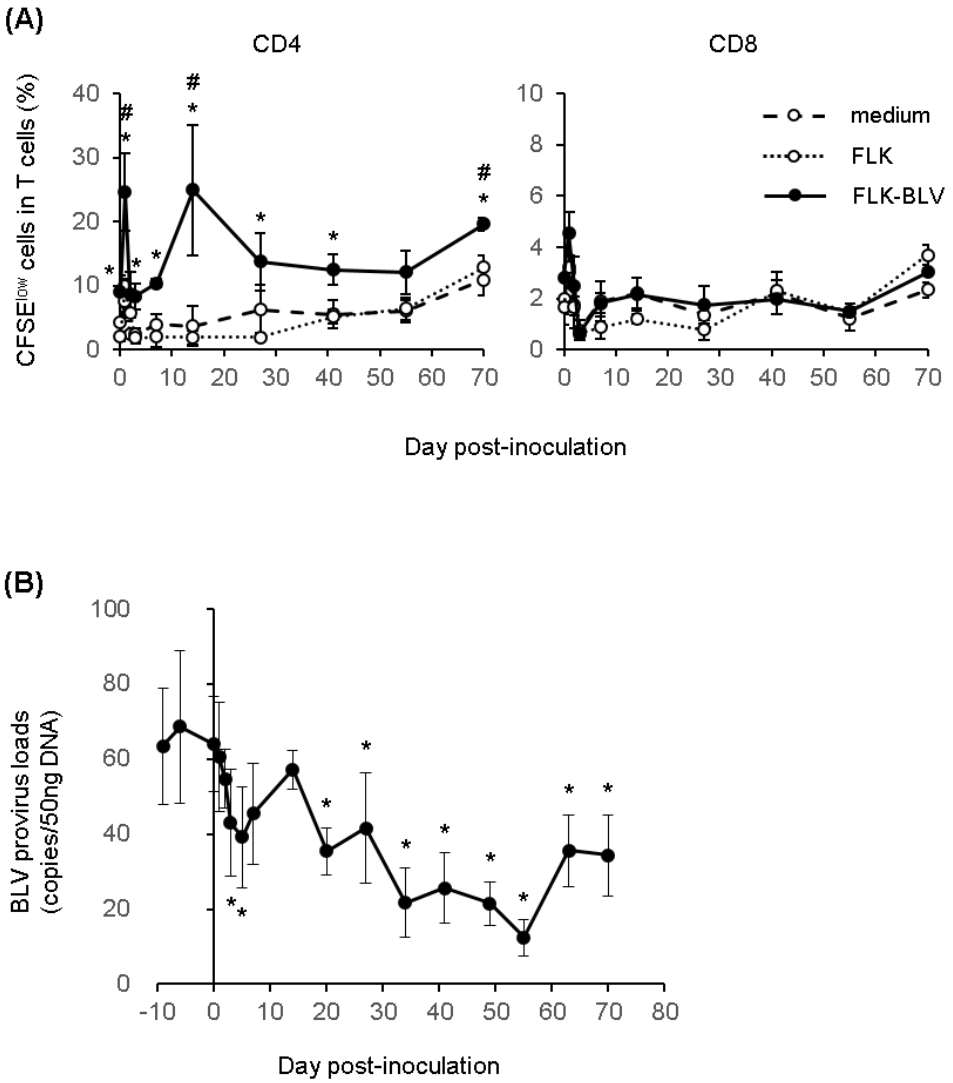

Figure III-II-4 *In vivo* effects of Boch4G12 treatment in a BLV-infected calf. A healthy calf (#15–11, Holstein-Friesian, male, 267 kg, 7 months old) was experimentally infected with BLV through administration of BLV-positive leukocytes and was then inoculated with 260 mg (1 mg/kg) of the purified Boch4G12 intravenously 13 weeks later. (A) The proliferation of $CD4^+$ and $CD8^+$ T cells against BLV antigen. PBMCs isolated from the calf were labeled with CFSE and cultured without stimulation (medium) or with the supernatant of FLK or FLK-BLV cells for 6 days. After the cultivation, the proliferation of T cells was analyzed by flow cytometry. A *P*-value less than 0.05 was considered statistically significant. *, $P < 0.05$ (FLK versus FLK BLV; one-way ANOVA followed by Tukey's test), #, $P<0.05$ (FLK-BLV, versus day 0; one-way ANOVA followed by Dunnett's test). (B) Changes in the BLV provirus loads in the calf inoculated with Boch4G12; the y-axis shows the number of BLV copies included in 50-ng DNA extracts of PBMCs. A *P*-value less than 0.05 was considered statistically significant. *, $P < 0.05$ (versus day 0; one-way ANOVA followed by Dunnett's test). Data are means ±SEMof at least three replicate experiments.

DISCUSSION

In this study, a chimeric antibody, Boch4G12, which is composed of the variable region from anti-bovine PD-L1 rat mAb 4G12 and the constant region from bovine IgG1 was successfully established. Boch4G12 exhibited similar activities to the original antibody in terms of the binding to bovine PD-L1 and interruption of the PD-1/PD-L1 interaction. The inhibition of T-cell function which was induced by PDL1/EGFP cells was restored in the presence of Boch4G12, indicating that this antibody has the potential to reactivate exhausted T cells through PD-L1 blockade. In addition, IFN-γ production was upregulated in PBMCs from BLV-infected cattle when cultured with Boch4G12. A previous study demonstrated that the PD-L1 expression level is increased on IgM^+ B cells in line with the progression of BLV infection [Ikebuchi *et al*., 2011]. Thus, the observed increase in IFN-γ production in the culture supernatant was probably caused by Boch4G12 preventing an interaction between PD-1 on T cells and PD-L1 on infected B cells, reactivating the ability of T cells to respond to the BLV antigen. Finally, a BLV-infected calf which was inoculated with Boch4G12 showed a proliferation of $CD4^+$ T cells in response to the BLV antigen and a reduction in BLV provirus loads, suggesting that Boch4G12 has therapeutic potential for controlling BLV infection.

The clinical effects of antibodies which block PD-1 and PD-L1 have mainly been reported in cancer research to date. However, chronic infections such as HIV, HBV, and HTLV-1 are also potential targets of these therapeutic antibodies since PD-1 expression on virus-specific $CD8^+$ T cells is upregulated in these diseases, and since antibody blockade has been shown to induce T-cell activation *in vitro* [Day *et al.*, 2006; Trautmann *et al.*, 2006; Boni *et al.*, 2007; Kozako *et al.*, 2009; Kozako *et al.*, 2011]. In addition, the treatment of simian immunodeficiency virus-infected macaques with anti-PD-1 antibodies results in the upregulated population of virus-specific $CD8^+$ T cells, a reduction in plasma viral loads, and prolonged survival of the animals [Finnefrock *et al.*, 2009; Velu *et al.*, 2009]. Moreover, a phase II clinical trial of Nivolumab is currently in progress to assess its effects on relapsed or refractory ATL [UMIN Center, 2013]. Therefore, BLV infection was an appropriate target for Boch4G12.

In previous studies, it has been demonstrated that the anti-bovine PD-L1 antibodies increase IFN-γ production when bovine PBMCs are cultured in their presence [Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2014b], and similar results were observed for Boch4G12 (Fig. III-II-3C). Though NK cells are a major source of IFN-γ,

this study did not confirm their role on antivirus activity during the antibody inoculation because compared to T cells, little is known regarding PD-1 expression on NK cells. According to recent reports, PD-1 signaling inhibits the activation cascade induced by not only T-cell receptor but also activating receptors on NK cells [Giuliani *et al.*, 2017], and PD-1 upregulation on NK cells was reported in patients of Kaposi sarcoma and multiple myeloma [Benson *et al.*, 2010; Beldi-Ferchiou *et al.*, 2016]. Moreover, a previous study indicated that NK activity, such as the production of IFN-γ and perforin, CD69 expression and cytotoxicity, was reduced in BLV-infected cattle in line with the provirus loads [Ohira *et al.*, 2016]. Therefore, it will be of great interest to investigate an expression of PD-1 on NK cells and their role in disease development during BLV infection. Then, the *in vitro* activation of T cells induced by Boch4G12 provided a further interest for *in vivo* effects of this antibody. In this study, Boch4G12 were inoculated into a calf which had been experimentally infected with BLV using a similar route to natural infection, since it is difficult to assess antivirus activities to a retrovirus *in vitro*. The results in this study clearly showed that the inoculation of Boch4G12 reduced BLV provirus loads, indicating its antivirus activity *in vivo*, although the number of tested animals was low.

The $CD8^+$ T-cell response plays an important role in the clearance of retroviruses [Buseyne *et al.*, 1993; Hasenkrug *et al.*, 2000]. However, Boch4G12 only induced the proliferation of $CD4^+$ T cells, but not $CD8^+$ T cells (Fig. III-II-4A). This is probably due to the fact that the BLV antigen used in this study (the culture supernatant of FLK-BLV) represents an exogenous antigen and thus is better recognized by $CD4^+$ T cells. Therefore, further analysis using a BLV peptide presented on MHC class I might help to clarify the effect of Boch4G12 inoculation on $CD8^+$ T cells. But this argument is not enough to explain why the addition of Boch4G12 did not show improved proliferation in $CD8^+$ T cells, since alternative modes of antigen presentation, such as cross presentation, could also activate $CD8^+$ T cells. Another possibility is that the blockade of PD-1/PD-L1 interaction on $CD8^+$ T cells was insufficient to overcome its exhausted status because of the presence of other immunoinhibitory molecules such as LAG-3, TIM-3 and CTLA-4. Previously, it has been reported that the expression levels of these molecules, LAG-3 [Konnai *et al.*, 2013], TIM-3 [Okagawa *et al.*, 2012] and CTLA-4 [Suzuki *et al.*, 2015], were upregulated in BLV-infected cattle, and that blockades of the interaction between these receptors and their ligands improved immune response *in vitro*, suggesting their important role during BLV infection. In human and mice, it has

been demonstrated that the number of immunoinhibitory receptors concurrently expressed on the same $CD8^+$ T cells affected the severity of T-cell exhaustion during chronic viral infection [Blackburn *et al*., 2009; Jin *et al*., 2010; Wherry, 2011]. Thus, the upregulation of LAG-3, TIM-3 and CTLA-4 also can substantially affect the dysfunction of immune cells in BLV-infected cattle. The study on *Anaplasma marginale* infection in cattle indicated that the percentages of $CD4^+$ and $CD8^+$ T cells expressing both of PD-1 and LAG-3 were increased during acute phase, suggesting that the PD-1^+ LAG-3^+ T cells contributes to the immune deficiency observed in this infection [Okagawa *et al*., 2016]. The co-expression of PD-1 and other immunoinhibitory receptors on $CD8^+$ T cells and its role on the severity of exhaustion during BLV infection are currently under analysis, which would help to clarify the cause of less improved proliferation in $CD8^+$ T cells observed in this study.

In this study, it was demonstrated that rat mAb 4G12 did not show any antivirus activities in cattle, whereas Boch4G12 decreased BLV provirus loads (Figs. III-II-1 and III-II-4), despite their recognition of same epitope and same methods for purification and inoculation. One possible explanation for this result is the different infection routes used, i.e., natural versus experimental infection. However, the experimental infection conducted in this study was imitated the route of natural infection by the injection of infected lymphocytes from BLV-infected cattle. Previous study on experimental infections of BLV in cattle indicated that, after the infection, the provirus loads were sharply increased to maximal values at 30–68 days post-infection, and then decreased slowly and reached stable states [Gillet *et al*., 2013]. As 7-month-old calf was inoculated with Boch4G12 at 13 weeks post-infection, this calf has developed to the AL stage after the acute infection phase (data not shown). Moreover, the provirus loads in both of the tested animals were categorized as low risk for transmitting BLV to other cattle [Mekata *et al*., 2015], indicating that the difference in background of the animals had little effect on the results of this study. Another possible explanation is that 4G12 was destabilized by anti-drug antibodies; whereas, Boch4G12 was not since the 7-month-old calf had a less developed immune system. However, several reports showed that immune system of calves were developed until 6 months old [Kampen *et al*., 2006; Chase *et al*., 2008], and modified live vaccines for bovine herpesvirus 1 and bovine viral diarrhea virus are often administered in calves of 3 to 4 months old in the clinical field. Thus, it is less possible that Boch4G12 was active because of immature immune response in the 7-month-old calf. The critical difference between the two

antibodies was the constant region derived from bovine IgG1 in Boch4G12, which may have led to a high stability in cattle and have enhanced the long-term effects to induce an antivirus immune response. Indeed, the reduction in provirus loads continued until day 55, suggesting that inoculated Boch4G12 may have persisted in the calf for a longer time. In addition, an ADCC-negative mutation had been introduced into the CH2 domain of Boch4G12 based on the previous reports [Armour *et al*., 1999; Shields *et al*., 2001; Ikebuchi *et al*., 2014b]. No information is currently available on the characteristics of the bovine IgG subclass, but a latest study has demonstrated that this mutation contributed to the reduction in binding ability to bovine FcγRI which is associated with the induction of ADCC, whereas its contribution to the stability of Boch4G12 is still unknown [Okagawa *et al*., 2017]. One shortfall of this study was the failure to evaluate the occupancy of Boch4G12 in the tested animal, since anti-bovine IgG secondary antibodies are highly cross-reactive to BCR on B cells. The detection of circulating Boch4G12 in serum has less meaning, as shown by the fact that in a phase I study of the anti-human PD-1 antibody, the PD-1 occupancy was maintained at a high level after the circulating antibody had become undetectable [Brahmer *et al*., 2010]. Therefore, antibodies that are specific to the variable region of Boch4G12 would enable the future evaluation of PD-L1 occupancy of this antibody after the inoculation.

BLV infection is spreading globally, including through North America, South America, Asia, and Russia [VanLeeuwen *et al*., 2005; Morovati *et al*., 2012; Benavides *et al*., 2013; Murakami *et al*., 2013; Ruzina *et al*., 2013; Rajão *et al*., 2014; Lee *et al*., 2016]. Although most of infected cattle are asymptomatic during BLV infection, several studies have shown an association of BLV infection with decreased milk production [Norby *et al*., 2016; Yang *et al*., 2016]. Currently, there is no effective vaccine and therapeutic method against BLV infection, and hence novel methods are required to control this infection. The finding that the inoculation of Boch4G12 decreased BLV provirus loads indicates that this method could represent a novel approach for avoiding the risks of bovine leukemia, particularly in highly-lactating cows and breeding bulls. In conclusion, an anti-bovine PD-L1 rat-bovine chimeric antibody was successfully established and showed the function to reactivate T cells *in vitro* and *in vivo*. Further experiments using larger numbers of cattle are required to support the efficacy of this antibody for clinical application.

SUMMARY

PD-1, an immunoinhibitory receptor expressed on T cells, is known to be involved in immune evasion in many chronic diseases through its binding to PD-L1 and PD-L2. In previous reports, PD-1 and PD-L1 expression was upregulated in cattle infected with BLV, and an antibody which blocked the PD-1/PD-L1 interaction reactivated T-cell function *in vitro*. However, there is no functional analysis in cattle on PD-L2, a second ligand of PD-1, and furthermore antivirus activities of the blockade agents *in vivo* have not been elucidated. Thus, in this study, two biomedicines for cattle targeting PD-Ls, recombinant bovine PD-L2-Ig and anti-bovine PD-L1 mAb, were developed.

In Section I, PD-L2-Ig was generated based on the cloned cDNA sequence for bovine PD-L2. The binding analysis indicated that bovine PD-L2-Ig specifically bound to bovine PD-1-expressing cells, which was proven by the inhibited binding of PD-L2-Ig to membrane PD-1 after the treatment with soluble PD-1-Ig. T-cell proliferation and IFN-γ production were significantly enhanced in the presence of PD-L2-Ig in bovine PBMCs. Interestingly, IFN-γ production induced by PD-L2-Ig was further enhanced by the combination with anti-bovine PD-1 antibody.

In Section II, BLV-infected cow was inoculated with the anti-bovine PD-L1 rat mAb 4G12, but this mAb showed poor antivirus activities and less persistence in cattle. Then, to improve stability of the antibody in cattle, anti-bovine PD-L1 chimeric mAb Boch4G12 was established. Boch4G12 was able to bind specifically with bovine PD-L1, interrupt the PD-1/PD-L1 interaction, and activate the immune response in both healthy and BLV-infected cattle *in vitro*. To investigate its function *in vivo*, a calf experimentally infected with BLV was inoculated with Boch4G12. Cultivation of PBMCs isolated from the tested calf indicated that the proliferation of $CD4^+$ T cells was increased by Boch4G12 inoculation, and BLV provirus loads were significantly reduced, clearly demonstrating that this treatment induced antivirus activities.

These results indicate that the biomedicines are potentially applicable as therapeutic agents for BLV infection. Further studies will be required to support its efficacy for clinical application.

CONCLUSION

Bovine leukemia virus (BLV) belongs to the *Retroviridae* family, commonly infects bovine B cells, and finally induces fatal B-cell lymphoma called enzootic bovine leukosis (EBL). Although EBL has been eradicated in several European countries, BLV infection is still widely spreading worldwide, with severe negative effects on farm management. Currently, there is no effective methods for therapy or vaccination available against this infection, thus novel methods for controlling BLV are required. In the present study, three strategies were proposed for future contribution to controlling BLV infection. First, the establishment of a novel method detecting BLV was conducted for early detection and eradication of BLV-infected animals for preventing virus spread. A field survey of cattle with lymphoma was a second approach for obtaining immunophenotypic features of lymphoma to understand current status on EBL onset. Finally, the development of bovine biomedicines for the treatment of BLV infection were performed, targeting an immunoinhibitory molecule, programmed death1 (PD-1) and its ligands, PD-ligand 1 (PD-L1) and 2 (PD-L2), which have been demonstrated to be involved in disease progression of BLV infection in previous studies.

CHAPTER I: polymerase chain reaction (PCR) for amplifying the BLV provirus from whole blood, PCR-DB, was established as a simple and rapid method for the diagnosis of BLV infection. The PCR-DB technique exhibited high specificity and reproducibility, although its sensitivity measured by bovine blood containing BLV-infected cell lines was inferior to that of nested PCR. Comparison between PCR-DB and nested PCR using clinical samples indicated that sensitivity and specificity of PCR-DB were 75.5% and 100%, respectively, and that those false-negative results were due to the low levels of BLV provirus. Moreover, the amplification of BLV provirus from tissue samples of cattle with lymphoma was stably succeeded. These results suggest that PCR-DB can be a suitable method for first screening of thousands of cattle, as a viable alternative to conventional methods.

CHAPTER II: immunophenotypic analysis was conducted using samples from cattle with bovine leukemia to investigate their characteristics. The samples were classified into five groups based on their cell-marker expression, B-cell clonality, and BLV provirus loads. During the analysis, several atypical EBL cases were found, such as polyclonal EBL exhibiting neoplastic proliferation of polyclonal B cells and early onset of EBL in juvenile cattle. Comparison of the cell-marker expressions provided the

characteristic patterns of cell-marker expressions in B-cell leukemia, which are useful for the differentiation of cattle with leukemia from healthy phenotypes. Because of these observation, novel characteristics of bovine leukemia was identified, which may contribute to a deeper understanding of the mechanism of tumor development during BLV infection.

CHAPTER III: as a first biomedicine for activating immune response, Fc fusion protein of bovine PD-L2 (PD-L2-Ig) was established on the basis of determined sequence of bovine PD-L2. The binding assay clearly demonstrated that bovine PD-L2-Ig specifically bound to bovine PD-1, since binding intensity of PD-L2-Ig to membrane PD-1 was strongly suppressed by pre-incubation with soluble PD-1. In addition, PD-L2-Ig promoted IFN-γ production and proliferation of $CD3^+$ T cells during the cultivation of lymphocytes isolated from BLV-infected cattle. Interestingly, the combination with anti-bovine PD-1 antibody further enhanced IFN-γ production induced by PD-L2-Ig. As a second agent, anti-bovine PD-L1 rat-bovine chimeric antibody Boch4G12 was developed to evaluated the long-time antivirus activity, with improved stability in cattle since it contains replaced bovine IgG region. Boch4G12 showed abilities to specifically bind bovine PD-L1, and interrupt the PD-1/PD-L1 interaction. Furthermore, activated immune response was observed by *in vitro* culture of bovine T cells with Boch4G12 co-cultured with PD-L1 expressing cells. To investigate its function *in vivo*, a calf experimentally infected with BLV was inoculated with Boch4G12, showing increase in the proliferation of $CD4^+$ T cells against BLV antigen and the reduction in BLV provirus loads. The present study clearly demonstrates that those biomedicines have a potential to be applied for the treatment to control BLV.

In conclusion, this study provides three steps of prevention and treatment for BLV infection. In early stage, the rapid detection should contribute to freeing BLV by separation or elimination of BLV-infected cattle in regular screening. The tumor phenotypic analysis proposes the information for correct classification and diagnosis of cattle with lymphomas, which might be a key of the understanding of the mechanisms for tumor development. The biomedicines described in this study may lead to represent a novel method for avoiding the risks of disease progression for BLV infection, particularly in highly-lactating cows and breeding bulls. Further studies will result in controlling BLV transmission and eradication of EBL from many regions where BLV is highly prevalent.

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SUMMARY IN JAPANESE

和文要旨

牛白血病ウイルス (BLV) はレトロウイルス科に属し、ウシの B 細胞に持続感染して、長い潜伏期間の後に B 細胞性リンパ腫 (地方病型牛白血病: EBL) を引き起こす。ヨーロッパ諸国の一部では、BLV の清浄化を達成し EBL の発生を抑えているが、多くの国々において BLV 感染症の蔓延とそれに伴う畜産業への影響が問題となっている。現在、BLV 感染症に対する有効なワクチンおよび治療法はなく、新たな制御法の開発が強く望まれている。そこで本研究では、BLV 感染症の制御を目的として 3 つの観点から研究を行った。最初に、BLV 感染牛を早期に発見・排除してウイルスの蔓延を防止するため、新たな迅速簡易診断法の樹立を行った。続いて、日本国内で発生している牛白血病発症牛を対象として表現型解析を行い、その免疫学的特徴と現在の発生状況を明らかにした。最後に、これまでの研究から BLV の病態進行への関与が明らかになっている免疫抑制因子、Programmed death-1 (PD-1) およびそのリガンド PD-ligand 1 (PD-L1) または 2 (PD-L2) を標的として、ウシの新規バイオ医薬品を開発して、その有効性を検討した。

第一章 : BLV 感染症の迅速簡易診断法として、DNA 試料の抽出を行わず、末梢血検体から直接ポリメラーゼ連鎖反応 (PCR) 法を行う血液ダイレクト PCR 法(PCR-DB 法) を樹立した。一定量の BLV 感染細胞株を混入させたウシ血液を用いて従来法である nested PCR 法と比較すると、PCR-DB 法は感度こそ従来法に劣るものの高い特異性と再現性を示した。また臨床検体を用いた比較では、PCR-DB 法の感度は 75.5%、特異度は 100%であり、PCR-DB 法で検出されなかった偽陰性検体は全て BLV プロウイルス量が低い感染初期の検体であった。さらに、PCR-DB 法を用いた腫瘍組織検体における BLV 遺伝子の検出に成功し、樹立した PCR-DB 法が多検体のスクリーニングや食肉衛生検査所等での迅速診断に有効であることが示された。

第二章 : 国内で発生している EBL の実態を調査するために、牛白血病発症牛由来の検体を用いて免疫学的な表現型解析を行った。得られた検体からリンパ

球を単離して、細胞表面マーカーの発現、B 細胞クローナリティおよび BLV プロウイルス量を測定して、発症牛を 5 つのグループに分類した。この解析により、これまで明らかでなかった非定型的な EBL、例えばポリクローナルな B 細胞からなるリンパ腫や、若齢牛における EBL の存在が明確に示された。また、5 つのグループのうち B 細胞性リンパ腫である 3 つについて細胞表面マーカーの発現を比較すると、これらの検体では健常牛由来の検体とは異なる特徴的な発現パターンを示すことが分かった。以上から、本研究で示された牛白血病の分類は、国内外における EBL の発生状況の評価、さらには BLV 感染症における腫瘍発生メカニズムの解明に大きく貢献できると考えられる。

第三章：バイオ医薬品として最初に、ウシ PD-L2 の細胞外領域に抗体の Fc 領域を結合させて調製した組み換えタンパク質 (PD-L2-Ig) の免疫賦活効果を検討した。まずウシ PD-L2 の遺伝子配列を同定し、その情報をもとにウシ PD-L2-Ig を作製した。PD-L2-Ig はウシ PD-1 発現細胞に結合性を示し、またこの結合は可溶性 PD-1 によって濃度依存的に阻害されたことから PD-1 特異的であると考えられた。さらに、BLV 感染牛から分離したリンパ球に PD-L2-Ig を添加して培養すると、IFN-γ 産生量や $CD3^+$ T 細胞の増殖が促進され、さらに IFN-γ 産生は抗ウシ PD-1 抗体により増強されることが示された。次に、第二のバイオ医薬品として、ラット由来の抗ウシ PD-L1 モノクローナル抗体を改変して、ウシ生体内での安定性を有し長期的な効果が望めるラット-ウシキメラ抗体 (Boch4G12) を作製した。Boch4G12 は改変前の抗体と同様にウシ PD-1 への結合能を有し、PD-1/PD-L1 結合の阻害能も示し、さらに PD-L1 発現細胞との共培養により低下したウシ T 細胞の応答を再活性化できることを確認した。そして Boch4G12 の生体内での抗ウイルス効果を検討するため、BLV を実験感染させた子牛に Boch4G12 を静脈内投与し、BLV 抗原に対する応答やプロウイルス量を評価した。その結果、抗体投与後から BLV 抗原に応答する $CD4^+$ T 細胞の割合が有意に上昇し、また BLV プロウイルス量が有意に減少することが示された。以上の結果より、本研究で作製したバイオ医薬品は新たな BLV 制御法として応用できると思われる。

本研究では、BLV 感染症の予防あるいは治療を行うための 3 つの戦略を提示した。感染初期の段階では、迅速診断法を用いた定期的なスクリーニングを行うことで、BLV 感染牛を早期に発見・排除して蔓延を防ぐことができる。腫瘍検体の表現型解析の結果は、白血病を疑うウシが発見された場合に正確な分類・診断を行うための基盤情報を提供しただけでなく、今後、BLV による腫瘍発生の分子機構を解明するうえで有用であると考える。またバイオ医薬品の開発は、高泌乳牛や種牛など価値の高い個体に BLV 感染が起こった場合に、病態進行リスクを低減する新たな選択肢としての可能性を示している。今後、さらなる研究により、BLV 蔓延地域におけるウイルス伝播の制御および EBL 根絶の実現が期待される。