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Author(s)	池淵, 良洋
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Studies on Immunoinhibitory Receptor and Viral Antigen Expression in Bovine Leukemia Virus Infection

(ウシ白血病ウイルス感染症における
免疫抑制受容体及びウイルス抗原の発現に関する研究)

Ryoyo Ikebuchi

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ABBREVIATIONS

7-aminoactinomycin D	7-AAD
aleukemic	AL
allophycocyanin	APC
antibody-dependent cell-mediated cytotoxicity	ADCC
B cell lymphoma	BL
B cell receptor	BCR
bovine leukemia virus	BLV
bovine leukemia virus-env-immunoglobulin fusion protein	BLV-env-Ig
bovine leukemia virus-infected	BLV ⁺
bovine leukemia virus-infected cattle with B cell lymphoma	BCBL
bovine leukemia virus-uninfected	BLV ⁻
bovine serum albumin	BSA
carboxyfluorescein diacetate succinimidyl ester	CFSE
Chinese hamster ovary	CHO
chronic lymphocytic leukemia	CLL
concanavalin A	ConA
cytotoxic T lymphocyte antigen 4	CTLA-4
dendritic cells	DCs
enhanced green fluorescent protein	EGFP
enzyme-linked immunosorbent assay	ELISA
Epstein-Barr virus	EBV
fluorescein isothiocyanate	FITC
gene ontology	GO
germinal center	GC
Hanks' balanced salt solution	HBSS
Hanks' balanced salt solution containing 1% bovine serum albumin	HBSS-BSA
horse radish peroxidase	HRP
human immunodeficiency virus	HIV
human T cell leukemia virus-1	HTLV-1
immunoglobulin	Ig
interferon	IFN

interleukin	IL
leukotriene B4 receptor	BLT1
lipopolysaccharide	LPS
long terminal repeat	LTR
lymph node	LN
mean fluorescence index	MFI
methotrexate	MTX
monoclonal antibody	mAb
mouse sarcoma virus-transformed feline cell line	CC81
peripheral blood mononuclear cells	PBMCs
persistent lymphocytosis	PL
phorbol 12-myristate acetate	PMA
phosphate-buffered saline	PBS
phosphate-buffered saline containing 0.05% Tween 20	PBS-T
phosphatidylinositol 3-kinase	PI3-kinase
phycoerythrin	PE
pokeweed mitogen	PWM
programmed death-1	PD-1
programmed death-1-immunoglobulin fusion protein	PD-1-Ig
programmed death-ligand 1	PD-L1
programmed death-ligand 1-immunoglobulin fusion protein	PD-L1-Ig
programmed death-ligand 2	PD-L2
sodium dodecyl sulfate-polyacrylamide gel electrophoresis	SDS-PAGE
soluble form of programmed death-1	sPD-1
surface IgM	sIgM

PREFACE

Cell-mediated immune response is essential for the clearance of pathogens including viruses and tumors from hosts. However, many types of viruses and tumors can escape from the immune system by various mechanisms. For example, *Trypanosoma brucei* is able to change the antigenic character of glycoprotein surface coat (antigen variation) [Pays et al., 2004], latent genes in Epstein-Barr virus (EBV)-infected B cells work for cellular survival and viral latency (latent infection) [Kalla et al., 2012] and high frequencies of circulating regulatory T cells were associated with the suppression of effector functions of CD4⁺ and CD8⁺ T cells in chronic hepatitis C virus infection (immunosuppression) [Manigold et al., 2007]. In addition to above, “T cell exhaustion” also plays a pivotal role in the immune escape. In chronic viral infections or leukemia, such as human immunodeficiency virus (HIV) infection and human T cell leukemia virus type 1 (HTLV-1) lymphoma, although pathogen-specific immune cells are present in hosts, they appear to be exhausted: they have no ability to produce sufficient cytokines to undergo cell proliferation and to activate cytotoxic T lymphocyte activity, so that these cells can not effectively eliminate pathogens [Barber et al., 2006; Wherry et al., 2007; Zajac et al., 1998].

Recent studies have shown that cell surface immunoinhibitory receptors, such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4) and lymphocyte activation gene 3, are involved in the mechanisms for the induction of T cell exhaustion [Wherry, 2011]. The inhibitory signals causing the T cell exhaustion are initiated by binding of specific ligands to the inhibitory receptors on T cells, for instance, PD-ligand 1 (PD-L1) or -ligand 2 (PD-L2) to PD-1 [Zhu et al., 2011]. Those inhibitory signals also play a key role in the induction of self-tolerance and prevention of autoimmunity within a healthy individual. For example, activated effector T cells can transiently express the inhibitory receptors [Chen, 2004], and PD-L1 can be expressed in cells at sites of immune privilege including the placenta and the eye [Francisco et al., 2010]. In chronic infection and malignancy, however, the inhibitory receptors and their ligands are highly and constitutively expressed, resulting in the establishment of the immune escape of the pathogens.

It is also known that blockade of the inhibitory pathway by antibodies specific for either the receptors or the ligands restores T cell function. Among them, the treatment

of anti-PD-1 or anti-PD-L1 antibody in chronic infection and tumors were investigated in many models, significantly enhanced T cell response, and improved clinical conditions [Hori et al., 2006; Nomi et al., 2007; Salama et al., 2003; Trautmann et al., 2006]. For example, the PD-L1 blockade increases cytolytic reactions in HTLV-1-specific CD8⁺ T cells which express PD-1 at high level [Kozako et al., 2009]. On the basis of those reports, clinical trials were conducted in patients with cancer who were administered anti-PD-1 antibody [Berger et al., 2008; Brahmer et al., 2010].

Bovine leukemia virus (BLV) belongs to the members of *Deltaretrovirus*, *Orthoretrovirinae*, *Retroviridae*, and is genetically related to HTLV-1 [Sagata et al., 1985]. While HTLV-1 infects CD4⁺ T cells, BLV infects B cells in cattle, and induces polyclonal expansion of B cells [Mirsky et al., 1996; Schwartz et al., 1994]. Epidemic of BLV infection is resulted in the extensive economic losses in the dairy beef industries [Gillet et al., 2007], because there is no immunization against this untreatable disease in cattle.

The majority of BLV-infected (BLV⁺) cattle are clinically inapparent, and are referred to as asymptomatic or aleukemic (AL). A small fraction of the latently infected individuals develop the disease characterized by persistent lymphocytosis (PL) and B cell lymphoma (BL) at 5-10 years after the infection. BLV infection is established by the transmission of infected lymphocytes which contain provirus genome in host genome. Although the population of BLV⁺ lymphocytes is expanded in line with the disease progression, no expression of viral antigens or particles are observed in lymphocytes in which proviruses are integrated into the host genome in the case of BLV and HTLV-1 infection [Asquith et al., 2005; Gillet et al., 2007]. Meanwhile, *ex vivo* cultivation induces viral antigen expression in a part of BLV⁺ B cells [Gillet et al., 2007]. Due to these complicated observations, the mechanisms of viral transmission to uninfected lymphocytes and the disease progression in cattle still remain unexplained.

Recent works have revealed that cytokine production plays a critical role in the progression of BLV-infection [Kabeya et al., 2001; Konnai et al., 2003; Usui et al., 2007]. In cattle at the PL or BL stage, T cell dysfunction including impaired cell proliferation and cytokine production characterized by the down-regulation of Th1 cytokines, accelerates the disease progression. Although those host immunoregulatory factors are clearly involved in the pathogenesis of the infection, the exact mechanisms of the immunosuppression are not yet known.

The identification of the mechanisms of BLV-induced immunosuppression and BLV proliferation in infected lymphocytes are essential for the development of novel vaccines and therapies against BLV infection. Since the PD-1/PD-L1 pathway is involved in the immunosuppression induced in many types of chronic infection [Keir et al., 2008], the role of the PD-1/PD-L1 pathway on immunosuppression in BLV⁺ cattle is worthy to be investigated. Thus, in Chapter I, PD-L1 expression was analyzed in BLV⁺ cattle, and subsequently the PD-L1 function was investigated by using bovine PD-L1-expressing cells and bovine lymphocytes in Chapter II. In Chapter III, PD-1 expression was analyzed in BLV⁺ cattle using anti-PD-1 monoclonal antibody (mAb), and anti-PD-1 mAb treatment was tested *in vitro* for the candidate of the new therapy against BLV infection. Moreover, to understand the detailed kinetics of BLV antigen expression, the cellular functions of BLV antigen-expressing and -silencing B cells were investigated in Chapter IV.

CHAPTER I

Analysis of PD-L1 Expression in Bovine Leukemia Virus-Infected Cattle

INTRODUCTION

An immunoinhibitory receptor, PD-1, is expressed on the membrane of activated T cells (but not resting T cells) and B cells [Agata et al., 1996], while its ligand, PD-L1, is expressed on the membrane of T and B cells, dendritic cells (DCs), macrophages [Yamazaki et al., 2002], and a wide range of non-hematopoietic cells, such as trophoblasts in the placenta and endothelial cells [Holets et al., 2009; Liang et al., 2003]. PD-L1 is often expressed in macrophages and DCs after the exposure to inflammatory cytokines such as interleukin (IL)-4 and interferon (IFN)- γ [Yamazaki et al., 2002]. IFN- γ treatment especially and strongly upregulates PD-L1 expression in a wide range of cells, multiple myeloma cells, lung cancer cells and epithelial cells [Lee et al., 2006; Liu et al., 2007; Stanciu et al., 2006]. Respiratory syncytial virus infection and Toll-like receptor ligands also augmented PD-L1 expression, indicating that inflammatory environment induces upregulation of PD-L1 expression as a possible mechanism to avoid the excessive immune reaction [Liu et al., 2007; Stanciu et al., 2006].

Recent studies showed that the PD-1/PD-L1 pathway was involved in immune dysfunction in several chronic infections [Blank et al., 2007; Keir et al., 2008]. High PD-1 expression is observed in lymphocytes, specifically virus-specific CD8⁺ T cells, while PD-L1 is also upregulated on myeloid DCs in hepatitis B virus and HIV infection [Chen et al., 2007; Trabattoni et al., 2003; Trautmann et al., 2006; Wang et al., 2008]. In HTLV-1 infection, neoplastic CD4⁺CD25⁺ T cells expressed PD-L1 in freshly isolated lymphocytes [Shimauchi et al., 2007], and high PD-L1 expression was also observed in HTLV-1-infected T cells after *ex vivo* cultivation [Kozako et al., 2009]. This expression pattern of PD-1 and PD-L1 appears negatively to regulate the activation and function of pathogen-specific T cells, resulted in failure to eliminate the infecting virus. In addition to infectious diseases, PD-L1 is also expressed on the membrane of tumor cells in many types of malignancy such as lung cancer, non-Hodgkin lymphoma, ovarian cancer, melanoma and pancreatic cancer, and is involved in immune evasion of neoplasm [Andorsky et al., 2011; Dong et al., 2002; Nomi et al., 2007].

Anti-PD-L1 antibody treatment appears to reverse the exhausted immune reaction [Keir et al., 2008; Urbani et al., 2006]. Blockade of the PD-1/PD-L1 pathway by antibodies specific to PD-L1 has been shown to enhance proliferation and IFN- γ

production of lymphocytes in response to HIV peptides [Rosignoli et al., 2009], and inhibit apoptosis of CD8⁺ effector T cells and tumor growth in mice model [Dong et al., 2002]. Moreover, the technique of MHC-tetramer revealed that the immune reactivation by anti-PD-L1 treatment was mainly occurred in pathogen-specific T cells [Day et al., 2006; Kozako et al., 2009]. A clinical trial reported that PD-L1 expression on the membrane of tumor cells may affect the ability to respond to the blockade of the PD-1/PD-L1 pathway [Brahmer et al., 2010], suggesting that the affirmation of PD-L1 expression is essential to obtain the best effect of the antibody treatment.

Although many reports showed T cell exhaustion by the PD-1/PD-L1 pathway in human and mouse models, no functional analysis of these immunoinhibitory receptors has been reported on cattle and bovine diseases. To determine the contribution of the PD-1/PD-L1 pathway to immune dysfunction caused by several diseases of domestic animals, such as BLV infection, bovine *PD-1* gene has been cloned and it was shown that the expression profiles of *PD-1* mRNA in CD4⁺ and CD8⁺ T cells are closely associated with BLV-induced lymphoma [Ikebuchi et al., 2010]. However, the dynamics of PD-L1 in disease progression during BLV infection remain unknown. In this chapter, in an attempt to determine whether PD-L1 expression is associated with BLV-induced immunosuppression, the expression levels of bovine PD-L1 were measured in BLV⁺ cattle at different disease stages.

MATERIALS AND METHODS

Cell preparation and subset isolation

Bovine peripheral blood mononuclear cells (PBMCs) were purified from heparinized venous blood of healthy Holstein-Friesian and Japanese Black by density gradient centrifugation on Percoll (GE Healthcare). This study was conducted in accordance with guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval number: 11-0059). Samples were collected after informed consents were obtained from clinical veterinarians and farmers. CD4⁺ T cell, CD8⁺ T cell, CD5⁺ cell and CD14⁺ monocyte populations were isolated from PBMCs using the BD IMag Cell Separation System (BD Biosciences) and the following antibodies: anti-CD4 (CACT138A; VMRD), anti-CD8 (IL-A51; a gift from International Livestock Research Institute), anti-CD5 (CACT105A; VMRD) and anti-CD14 (CAM36A; VMRD). The purity of each cell population was confirmed by EPICS XL flow cytometry system (Beckman Coulter) with the EPICS EXPO32 ADC software (Beckman Coulter). Highly purified cells (> 90%) were used for the analysis of the PD-L1 expression.

Expression analysis of bovine *PD-L1* mRNA by quantitative real-time PCR

To investigate the expression levels of *PD-L1* mRNA, RNA samples were extracted from purified CD4⁺, CD8⁺, CD5⁺, CD14⁺ cells and PBMCs incubated for 18 h in the presence of either anti-CD3 (0.2 µg/ml, MM1A, VMRD) or concanavalin A (ConA, 5 µg/ml) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Residual DNA was removed from the RNA samples by the treatment with Deoxyribonuclease I (Invitrogen). cDNA was synthesized from the RNA samples by using Moloney murine leukemia virus reverse transcriptase (Takara Bio) following the manufacturer's instructions. Quantitative RT real-time PCR was performed using the LightCycler 480 system II (Roche Diagnostics). The cDNA template was mixed with 10 µl of SYBR Premix DimerEraser (Takara Bio) and 0.6 µl each of primers (10 pmol/µl) in a total volume of 20 µl. Primers used were 5'-GGG GGT TTA CTG TTG CTT GA-3' and 5'-GCC ACCT CAG GAC TTG GTG AT-3' for bovine PD-L1, and 5'-CGC ACC ACT GGC ATT GTC AT-3' and 5'-TCC AAG GCG ACG TAG CAG AG-3' for β-actin. Each amplification procedure was done in

triplicate, and the results of *PD-L1* mRNA expression are presented as a ratio obtained by dividing the concentration of the *PD-L1* mRNA by that of the β -actin mRNA.

BLV-infection diagnosis

Genomic DNA was extracted from whole blood using the Wizard Genomic DNA Purification kit (Promega). The concentration of purified genomic DNA was measured at OD of 260 nm, and the DNA samples were stored at 4°C until use. BLV infection was tested by nested-PCR to amplify the BLV long terminal repeat (LTR) 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 initial PCR, and 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 [Ikebuchi et al., 2010].

The provirus load was further confirmed by real-time PCR using a LightCycler 480 system II, with SYBR Premix DimerEraser and following primers, BLV-LTR256 and BLV-LTR453 for BLV, and 5'-ACA CAA CTG TGT TCA CTA GC-3' and 5'-CAA CTT CAT CCA CGT TCA CC-3' for bovine β -globin, as described previously [Tajima et al., 2003]. One hundred percent provirus load corresponded to each PBMC having one copy of BLV provirus.

Furthermore, the virus titers were quantified based on the number of syncytia formed by isolated PBMCs co-cultured with a mouse sarcoma virus-transformed feline cell line (CC81). The syncytium formation assay was conducted according to the procedure described previously [Konnai et al., 2003]. The 1×10^5 CC81 cell line was grown with 5×10^5 PBMCs from BLV⁺ animals for 72 h in 24-well plate (Corning) with RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Cell Culture Technologies) and mixture of 2 mmol L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin (Life Technologies) (complete RPMI 1640 medium). The confluent cells were then fixed in methanol for 30 min and stained with 10% Giemsa solution (Merck Millipore) for 30 min. All samples were tested in triplicate and the data are presented as the mean numbers of syncytia.

To investigate the degree of immunosuppression in cattle, *IFN- γ* mRNA was quantified by quantitative RT real-time PCR as described above. Primers used were 5'-ATA ACC AGG TCA TTC AAA GG-3' and 5'-ATT CTG ACT TCT CTT CCG CT-3'.

BLV⁺ cattle with BL (BCBL) were diagnosed clinically, and confirmed by

microscopic and histological examinations. Other BLV⁺ animals were classified as AL or PL based on the number of leukocytes (10,000 cells/ μ l blood) as described previously [Konnai et al., 2006].

Flow cytometric analysis

To analyze the cells expressing PD-L1, single- and dual-color flow cytometric analysis was performed using the following antibodies: anti-CD5 (CACT105A), anti-IgM (BIG73A; VMRD) and rabbit anti-human PD-L1 (H-130; Santa Cruz Biotechnology) as described previously [Konnai et al., 2005]. Purified PBMC (1×10^7 cells/ml) were incubated with the optimal concentration of each antibody for 40 min at 4°C. Then, the cells were washed with phosphate-buffered saline (PBS, pH7.2) containing EDTA (0.5 mg/ml) and stained with either fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Santa Cruz) and phycoerythrin (PE)-conjugated goat anti-mouse IgG (Beckman Coulter). Rabbit IgG isotype control (Beckman Coulter) and mouse normal serum were used as isotype controls. Fluorescence of the cells was measured and analyzed as described above.

Analysis of PD-L1 expression in BLV-gp51 positive cells

To confirm PD-L1 expression in BLV⁺ cells, intracellular staining was performed. PBMCs isolated from BLV⁺ or BLV-uninfected (BLV⁻) cattle were cultivated for 18 h, and cells were stained with anti-human PD-L1 and anti-bovine IgM as described above. After surface staining, cells were fixed and permeabilized by FOXP3 Fix/Perm kit (BioLegend) as manufacturers protocol. Then, cells were stained with anti-gp51 (BLV1; VMRD) pre-labeled with Zenon PE (Life Technologies), washed and immediately analyzed.

Statistics

Spearman rank-correlation and one-way ANOVA with Tukey's post test were performed using GraphPad Prism version 5.0 (GraphPad Software). *P*-values of less than 0.05 were considered to be statistically significant.

RESULTS

***PD-L1* mRNA expression in various bovine PBMC-derived cell types**

In order to investigate the expression levels of bovine *PD-L1* mRNA, real-time PCR was used to quantify the expression of bovine *PD-L1* mRNAs in PBMCs. Firstly, the expression levels of bovine *PD-L1* mRNA were evaluated in several cell types among PBMCs from healthy cattle (Figure I-1a). The expression of *PD-L1* mRNA in CD14⁺ cells, which are representative of monocytes, was higher than that in CD5⁺ cells (the T and B-1 cell fractions), CD4⁺ T cells and CD8⁺ T cells. The *PD-L1* mRNA expression was also observed in the negative fraction, which was considered to contain natural killer cells and conventional B cells among others. To determine the effect of T cell stimulation on the expression of bovine *PD-L1* mRNA, PBMCs from healthy animals were cultured in the presence of anti-CD3 mAb or ConA, a lectin which induces T cell proliferation. As shown in Figure I-1b, the treatment of the antibody or ConA enhanced the expression of bovine *PD-L1* mRNA in PBMCs relative to PBMCs without any stimulation. There were no notable differences in the expression levels of bovine *PD-L1* mRNA before and after cell cultivation without stimulation.

PD-L1⁺ cells were increased in lymphocytes isolated from PL cattle and BCBL

To evaluate PD-L1 expression in BLV⁺ animals, the percentages of PD-L1⁺ cells were determined in PBMCs freshly isolated from BLV⁺ cattle at different disease stages by flow cytometric analysis. Typical cases of PD-L1 in BLV⁺ cattle at different disease stages are shown in Figure I-2a. The mean percentage of PD-L1⁺ cells in PBMCs isolated from cattle at PL was significantly higher than those of uninfected cattle and AL cattle (Figure I-2b). Similarly, the mean percentage of PD-L1⁺ cells was significantly increased in BCBL compared to BLV⁻ cattle. No difference in the mean percentage of PD-L1⁺ cells was observed between BLV⁻ and AL cattle, as well as between PL cattle and BCBL. In the case of retrovirus infections such as HIV and HTLV-1, the upregulation of the PD-L1 expression has been observed in target cells for virus infection or infected cells [Kozako et al., 2009; Wang et al., 2008]. BLV is characterized as a B cell tropic virus and induces aberrant B cell proliferation (especially CD5⁺ or IgM⁺ B cell) during disease progression. As an attempt to evaluate the expression of PD-L1 on B cells, the proportion of the cells expressing

PD-L1 was measured among IgM⁺ cells and CD5⁺ cells. As shown in Figures I-2c and I-2d, the percentages of PD-L1⁺ and CD5⁺ or IgM⁺ cells in PBMCs were clearly increased in cattle at PL stage compared to BLV⁻ and AL cattle. The proportions of PD-L1⁺ and CD5⁺ or IgM⁺ cells isolated from BCBL were elevated compared to those of AL and BLV⁻ cattle, although the differences were not statistically significant.

PD-L1 expression in BLV antigen-expressing B cells

To activate the expression of BLV protein, such as gp51, and to permit the detection of BLV⁺ B cells, PBMCs were cultivated overnight before cell staining. BLV-gp51 expression was not detected in freshly isolated B cells, whereas cultivation of PBMCs for overnight resulted in reactivation of gp51 expression (Figure I-3a) in line with previous findings [Gupta et al., 1982, 1984; Tajima et al., 2005]. The PD-L1 expression was detected in gp51⁺ B cells (Figure I-3b). There is no difference in the percentages of PD-L1 expression between gp51⁺ and gp51⁻ B cells in BLV⁺ cattle.

Correlation between the number of leukocytes, provirus loads or virus titer and PD-L1 expression

To determine if the increased proportion of PD-L1⁺ cells among PBMCs correlated to the changes in the number of leukocytes, provirus loads and virus titer in BLV⁺ cattle, statistical analysis was performed based on the Spearman correlation coefficients (Figure I-4). As expected, the significant positive correlation was detected between the proportions of PD-L1⁺ cells and the number of leukocytes (Figure I-4a), provirus loads (Figure I-4b) and virus titer (Figure I-4c) in BLV⁺ cattle. These results suggest that increase in the number of PD-L1⁺ cells could influence the expansion of BLV⁺ cells during the disease progression.

Correlation between IFN- γ expression levels and PD-L1 expression

It has been known that IFN- γ , a key cytokine for virus clearance, is downregulated during BLV infection [Konnai et al., 2003]. This phenomenon is characterized as immunosuppression that facilitates disease progression during BLV infection through an unknown mechanism. Thus, the correlation between the percentages of PD-L1⁺ cells and IFN- γ expression was studied in PBMCs from BLV infected animals. As observed in the previous study [Konnai et al., 2003], increased IFN- γ levels correlated significantly with reduced provirus load in BLV⁺ animals (Figure I-5a). Interestingly,

lower proportions of circulating PD-L1⁺ cells correlated strongly with increased IFN- γ levels (Figure I-5b). These results prompted the notion that decreased levels of IFN- γ during the virus proliferation and the disease progression could be due to the increase in the number of PD-L1⁺ cells.

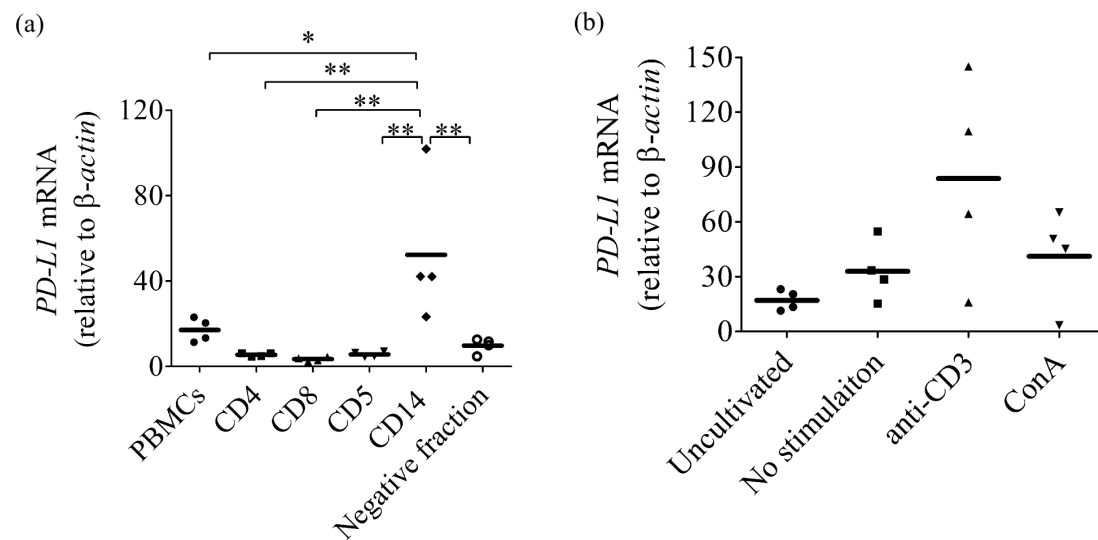


Figure I-1. Analysis of bovine *PD-L1* mRNA expression by real-time PCR.

(a) *PD-L1* mRNA expressions were determined in total PBMCs from healthy Holstein cattle, subpopulations of CD4⁺, CD8⁺, CD5⁺, CD14⁺ cells and negative fraction for these subpopulations ($n = 4$). (b) *PD-L1* mRNA expression in PBMCs incubated with or without either anti-CD3 antibody or ConA ($n = 4$). The level of the *PD-L1* mRNA expression was shown as the ratios obtained by dividing concentrations of the PCR products from *PD-L1* mRNA by those from β -actin mRNA. Statistical comparisons were made using the one-way ANOVA with Tukey's post test. Differences between groups were considered statistically significant at probability values of $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

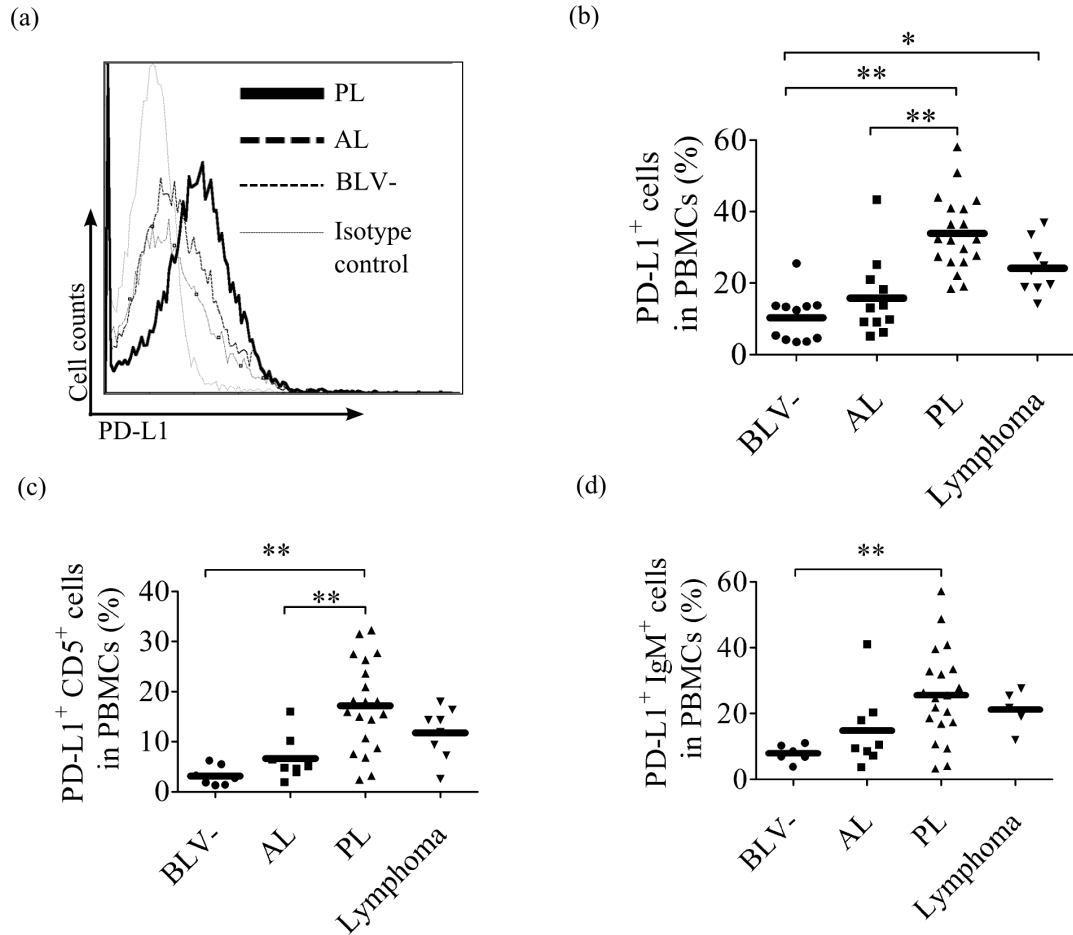


Figure I-2. Flow cytometric analysis of bovine PD-L1 expression in BLV⁺ cattle at different disease stages.

(a) Representative histogram of PD-L1 expression in BLV⁺ cattle at different disease stages determined by flow cytometric analysis. Bold line indicates PD-L1 expression in PL cattle. (b, c, d) Flow cytometric analysis of the PD-L1 expression on PBMCs (b), CD5⁺ (c), and IgM⁺ (d) cells during the BLV-induced disease progression. PBMCs from BLV⁻ ($n = 11$ in b, 5 in c and d) and BLV⁺ cattle with AL ($n = 11$ in b, 8 in c and d), PL ($n = 19$) and BL ($n = 9$ in b, 8 in c, 5 in d) were analyzed. Each of line indicates the mean percentages in each group. Statistical comparisons were made using the one-way ANOVA with Tukey's post test. Differences between groups were considered statistically significant at probability values of $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

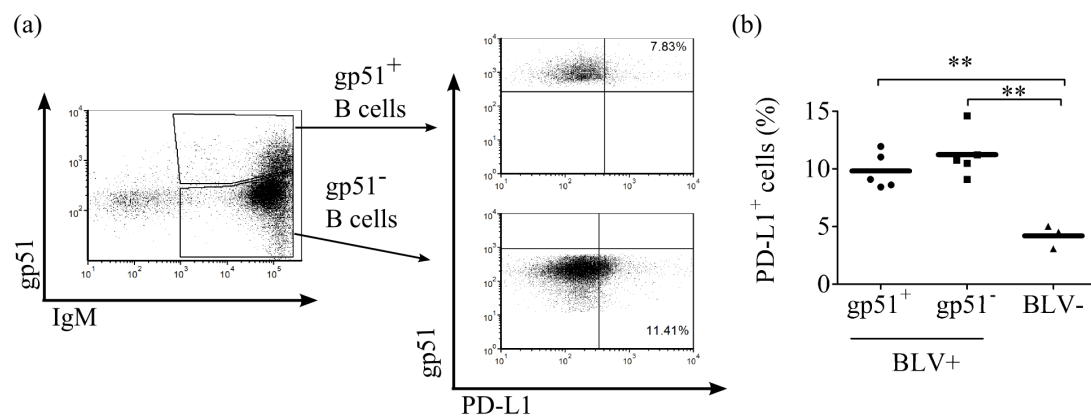


Figure I-3. PD-L1 expression in BLV antigen-expressing B cells.

(a) An example of expression of BLV-gp51 and PD-L1 in cultivated lymphocytes. Values in the quadrant indicate the percentage of PD-L1⁺ cells in lymphocytes. (b) Percentages of PD-L1-expressing gp51⁺ and gp51⁻ ($n = 5$) IgM⁺ B cells from BLV⁺ and BLV⁻ cattle ($n = 9$). Statistical comparisons were made using the one-way ANOVA with Tukey's post test. Differences between groups were considered statistically significant at probability values of $P < 0.05$ (** $P < 0.01$).

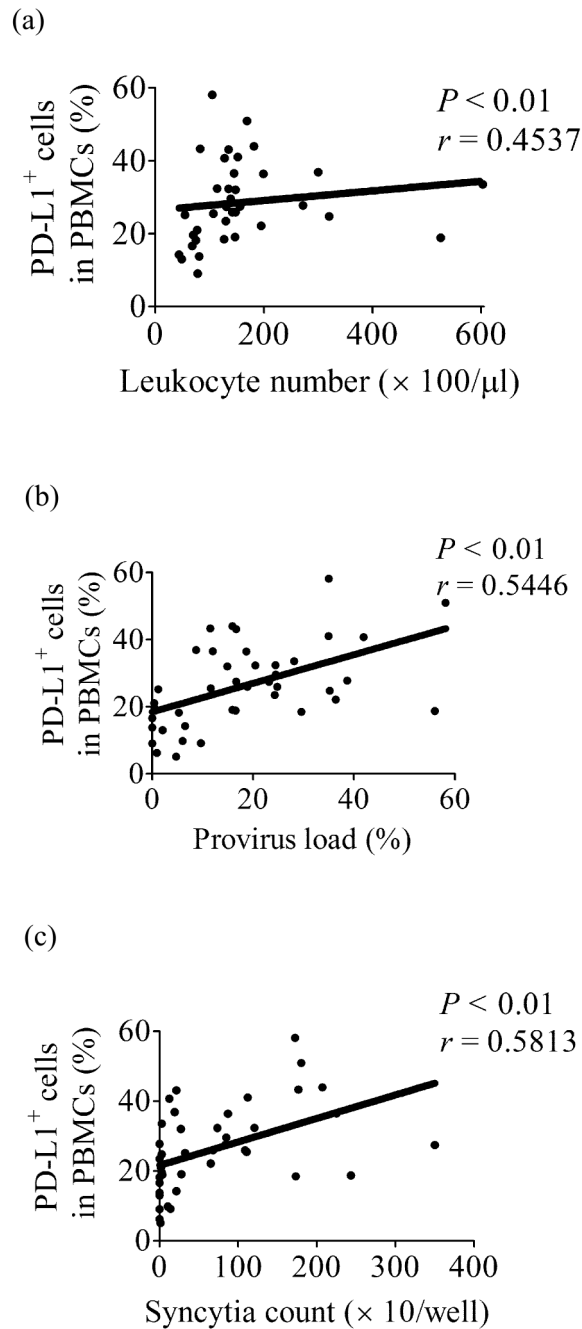


Figure I-4. Correlation between markers of disease progression and PD-L1 expression in BLV⁺ cattle.

Positive correlation between the leukocyte number (a: $n = 36$; AL: 8 PL: 20 BCBL: 8), provirus load (b: $n = 41$; AL: 12 PL: 20 BCBL: 9) or virus titer (c: $n = 39$; AL: 12 PL: 19 BCBL: 8) and the percentages of PD-L1⁺ cells in PBMCs corresponding to Figure I-2b. The provirus load and virus titer were determined using quantitative real-time PCR and syncytium formation assay, respectively. Correlation statistics were analyzed using the Spearman correlation.

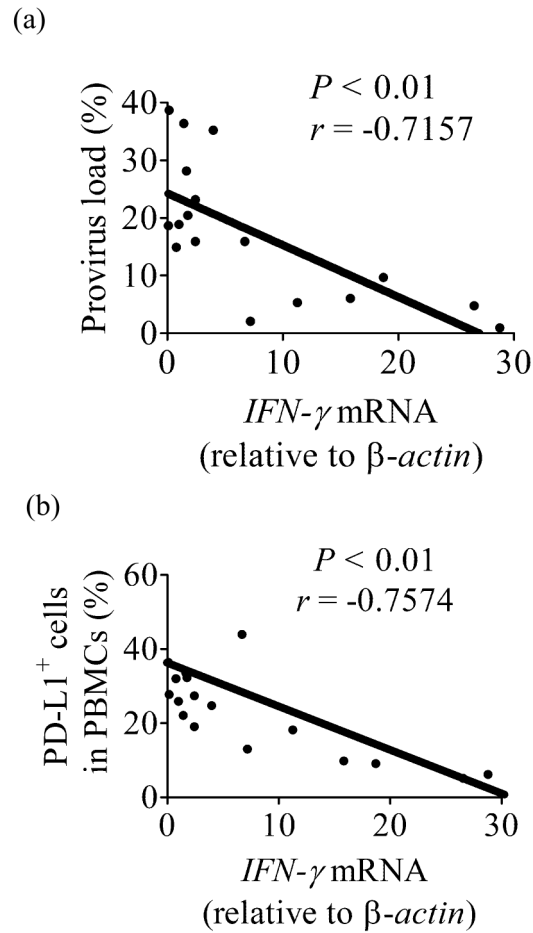


Figure I-5. Correlation between PD-L1 and $IFN-\gamma$ expression in BLV⁺ cattle. Inverse correlation between $IFN-\gamma$ mRNA expression and provirus load (a), and percentages of PD-L1⁺ cells in PBMCs (b) ($n = 17$; AL: 6 PL: 8 BCBL: 3). The level of $IFN-\gamma$ mRNA expression is shown as the ratios obtained by dividing the concentrations of the PCR products from $IFN-\gamma$ mRNA by those from β -actin mRNA. Correlation statistics were analyzed using the Spearman correlation.

DISCUSSION

In domestic animals including cattle, there are still many intractable diseases with poor-prognosis due to the lack of effective treatment and vaccination. This may be attributed to lack of a better understanding of immunological mechanisms leading to immune evasion. Recent studies have indicated the involvement of a major inhibitory network of the PD-1/PD-L1 pathways in immune dysfunction in several human diseases. However, there are only a few reports available on the role of the PD-1/PD-L1 pathways in domestic animals. In this study, the correlation between BLV infection and PD-L1 expression was demonstrated.

In this chapter, PD-L1 expression on PBMCs, especially B cells proliferating abnormally was found to be upregulated in cattle with advanced disease, consistent with previous reports showing the upregulation of PD-L1 expression in retrovirus infections. For example, in HTLV-1 infection, the upregulation of PD-L1 on CD4⁺ and CD25⁺ T cells, as well as the upregulation of PD-1 on CD8⁺ T cells, especially HTLV-1-specific T cells together with anti-virus T cell dysfunctions have been reported [Kozako et al., 2009; Shimauchi et al., 2007]. BLV, which is related to HTLV-1, causes chronic infections of B cells that may lead to leukemia and lymphoma in cattle through yet to be elucidated mechanisms of disease progression. Nevertheless, there has been evidence of dysfunction of cellular immunity that has been associated with the progression of the disease [Konnai et al., 2003]. Thus, the findings obtained in this study underscore earlier reports suggesting that the PD-1/PD-L1 pathway in retrovirus infection is associated with T cell dysfunction during disease progression.

To determine whether PD-L1 expression was specifically upregulated on BLV⁺ B cells compared to BLV⁻ B cells, PD-L1 expression on BLV-gp51⁺ B cells among PBMCs after *ex vivo* cultivation was measured. The overnight cultivation was needed for the analysis because BLV-gp51 was found not to be expressed on freshly isolated PBMCs from infected cattle, even at the late stage of the disease. Despite its clear correlation with the provirus load and virus titer, PD-L1 expression level in gp51⁺ B cells was not different from that in gp51⁻ B cells. The reason for this observation might be that only gp51 staining is not able to discriminate BLV⁺ B cells from BLV⁻ B cells, although there is the possibility that the PD-L1 expression was augmented in whole B cells in cattle at the PL stage. Previous reports supposed that BLV⁺ B cells

were composed of BLV-antigen-expressing and silencing B cells [Gillet et al., 2007], so it is difficult to investigate PD-L1 expression in accurate BLV⁺ B cells, not BLV antigen-expressing B cells, by current staining technique. Further analyses on the expression of BLV antigen in B cells are described in Chapter IV.

The mechanism of PD-L1 upregulation during BLV-induced disease progression remains unknown. In HIV models, several hypotheses on what elevates PD-1 and PD-L1 expressions, such as cytokine microenvironment [Kinter et al., 2008], virus-derived proteins [Muthumani et al., 2008], influx of microbial products [Said et al., 2010] and chronic antigen presentation [Streeck et al., 2008], have been proposed. These hypotheses might contribute to understanding the mechanism of PD-L1 upregulation during BLV infection, considering that changes in cytokine microenvironment [Kabeya et al., 2001; Konnai et al., 2003; Pyeon et al., 1996, 1998] and aberrant modulation of host molecules by a BLV transcription factor, Tax [Twizere et al., 2003] have been previously reported in BLV⁺ animals. Nevertheless, further elucidation of the mechanism for the elevation of PD-L1 expression is warranted to fully understand the cell signaling pathways involved in the modulation of host immune responses.

In conclusion, PD-L1 aberrantly expressed on B cells in BLV⁺ cattle, with the elevated proportion of PD-L1⁺ cells correlating with immune evasion during the course of the disease. Thus, PD-1-induced T cell dysfunction through its binding to the PD-L1 on B cells might contribute to disease progression.

SUMMARY

The immunoinhibitory receptor PD-1 and its ligand, PD-L1 are involved in immune evasion mechanisms for several pathogens causing chronic infections. Blockade of the PD-1/PD-L1 pathway restores anti-virus immune responses, with concomitant reduction in viral load. In a previous report, it was shown that, in BLV infection, the expression of bovine PD-1 was closely associated with disease progression. However, the functions of bovine PD-L1 are still unknown. To investigate the role of PD-L1 in BLV infection, PD-L1 expression was examined in BLV⁺ cattle in comparison with uninfected cattle. The proportion of PD-L1-positive cells, especially among B cells, was upregulated in cattle with the late stage of the disease compared to cattle with the early stage. The proportion of PD-L1⁺ cells correlated positively with prediction markers for the progression of the disease such as leukocyte number, virus load and virus titer, while it inversely correlated with the degree of IFN- γ expression. These data suggest that PD-L1 expression was upregulated in line with disease progression and immunosuppression in cattle during chronic BLV infection. Therefore, PD-L1 would be a potential target for developing immunotherapies against BLV infection.

CHAPTER II

The Influence of PD-L1 Blockade by PD-1-Immunoglobulin Fusion Protein and Anti-PD-L1 Monoclonal Antibody in PD-L1-Expressing Cell Lines and Bovine Lymphocytes

INTRODUCTION

Immunoglobulin (Ig)-fusion protein is one of the protein engineering techniques to increase or decrease in serum half-life [Jazayeri et al., 2008]. It is also suitable for the detection of the binding partner with “orphan receptor” whose ligand is unknown [Arase et al., 2002] and stimulation or block the binding receptor. The PD-1/PD-L1 pathway was also investigated using soluble PD-1-Ig fusion protein (PD-1-Ig). PD-1-Ig treatment blocked the pathway and overcame PD-L1- or PD-L2-induced immunosuppression [Geng et al., 2006; He et al., 2004; Wan et al., 2006]. However, the opposite phenomenon was also reported that DCs exposed to PD-1-Ig were changed into a suppressive phenotype, resulting in the inhibition of antigen-specific T cell proliferation [Kuipers et al., 2006]. Moreover, PD-1-Ig treatment promotes programmed cell death of activated CD4⁺ T cells and B cells [Dong et al., 2003; Kim et al., 2008]. These reports suggest that the binding partners of PD-1-Ig, PD-L1 or PD-L2, have unknown functions.

PD-L1 was once thought of as just a ligand for PD-1 because the intracellular region of mouse, human and bovine PD-L1 is only about 30 amino acids in length and there is no known functional domain. However, PD-L1-induced signaling is now thought to be certainly present, but to have unknown functions due to accumulating and conflicting reports on PD-L1 as the activation or inhibitory receptors aside from PD-1. For example, PD-L1-deficient T cells express lower Bcl-xl, which is an anti-apoptosis gene, than wild-type cells and are more sensitive to killing by cytotoxic T cells *in vivo*, indicating that PD-L1 was essential for survival of activated T cells [Pulko et al., 2011]. It has been also reported that PD-L1 promotes stable immunological synapse formation [Zinselmeyer et al., 2013]. These two reports suggest that PD-L1 works as a receptor for the immune activation. In contrast, cross-linking of PD-L1 induced apoptosis of EBV-transformed B cells, indicating that PD-L1 could act as a receptor which induces apoptosis-resistant signals [Kim et al., 2008].

The ligation of PD-L1 by CD80, which is also a binding partner with CD28 and CTLA-4, complicates the understanding of the function of PD-L1 as the receptor. PD-L1 has been demonstrated to interact with CD80 on T cells isolated from *Cd28*^{-/-} *Ctla4*^{-/-} or *Pdcd1*^{-/-} mouse and inhibit proliferation and cytokine production in PD-L1⁺ T cells [Butte et al., 2007, 2008]. Meanwhile, a number of studies have demonstrated

that PD-L1 is able to stimulate T cell function [Dong et al., 1999; Subudhi et al., 2004; Wan et al., 2006]. For example, T cells are activated by PD-L1 expressed on NK cells via the independent mechanisms from PD-1 [Saudemont et al., 2005].

In Chapter I, augmented PD-L1 expression was confirmed in BLV⁺ cattle with PL stage. The PD-L1 blockade is accepted as appropriate strategy to improve immune responses against tumors and chronic infections [Keir et al., 2008], therefore, in this chapter, to investigate the effect of PD-L1 blockade on immune reaction and cell death, the PD-L1-expressing cell lines and lymphocytes isolated from BLV⁺ cattle were treated with PD-1-Ig or anti-PD-L1 mAb.

MATERIALS AND METHODS

Expression of recombinant soluble bovine PD-L1-Ig fusion protein (PD-L1-Ig)

cDNA encoding the extracellular domain fragment of bovine PD-L1 without a signal sequence was amplified by PCR and inserted into the cloning site of a modified pCAGGS (provided by Dr. J. Miyazaki, Osaka University; [Niwa et al., 1991]) that contained a mouse CD150 signal sequence at the N terminus and the Fc fragment of rabbit IgG at the C terminus [Arase et al., 2002]. PD-L1-Ig was expressed in Chinese hamster ovary (CHO)-DG44 cells (provided by Dr. Y. Suzuki, Hokkaido University) transfected stably by Lipofectamine LTX (Life Technologies), and purified from the media with Ab-Capcher ExTra (ProteNova) according to the manufacturer's protocol. The purification of PD-L1-Ig was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the quantity of that was determined by Rabbit IgG ELISA Quantitation Set (Bethyl Laboratories).

Stable expression of bovine PD-L1 on CHO-DG44 cells

A part of bovine *PD-L1* gene encoding the extracellular domain was cloned into pEGFP-N2 (Clontech). The plasmid that contained enhanced green fluorescent protein (EGFP) at the C terminus was transfected into CHO-DG44 cells using Lipofectamine LTX, and the cells were selected by the medium containing G418 (800 µg/ml; Enzo Life Sciences) for 10 days, and were cloned by limiting dilution. The stable cell lines were screened for fluorescence using FACS Verse (BD Biosciences), and the three cell lines showing the brightest fluorescence were used for screening of anti-bovine PD-L1 mAbs. The PD-L1 expression on the cell membrane was checked by LSM 700 (Carl Zeiss).

Generation of anti-bovine PD-L1 mAb

A rat was immunized with 170 µg of PD-L1-Ig emulsified with complete Freund's adjuvant. On the 24th day of the immunization, lymphocytes isolated from the iliac lymph node (LN) were fused with myeloma cells. The supernatants from hybridomas were screened by flow cytometry using the three cell lines stably expressing PD-L1 with EGFP. The hybridomas were also screened using Cos-7 cells that were transiently transfected with bovine PD-L1 encoding pCMV-Tag-1 (Agilent

Technologies). Hybridomas producing antibodies that reacted with PD-L1 but not EGFP-expressing cell lines were cloned by limiting dilution. The immunization of rats and the cultivation of hybridomas were performed at Cell Engineering Corporation.

Expression of recombinant soluble bovine PD-1-Ig

Gene coupled with the extracellular domain of bovine PD-1 and Fc region of bovine IgG1 (Figure II-1) was commercially synthesized according to preferential codon usage of mammalian cells in Medical and Biological Laboratories and inserted into pDN11 (provided by Dr. Y. Suzuki). To reduce antibody-dependent cell-mediated cytotoxicity (ADCC) response to PD-1-Ig treatment, the mutation was introduced into the binding sites for Fc γ receptors as described in references (Figure II-1) [Armour et al., 1999; Shields et al., 2001].

CHO-DG44 cells were transfected with pDN11 encoding PD-1-Ig and selected in CD OptiCHO AGT (Life technologies) supplemented with 800 μ g/ml G418. After 3 weeks, the cells were screened for production capacity of PD-1-Ig by dot blotting and enzyme-linked immunosorbent assay (ELISA) with anti-bovine IgG Fc (Rockland). The expression of PD-1-Ig was also confirmed by SDS-PAGE and Western blotting as described below. Ten cell lines producing high amount of PD-1-Ig were cloned by limiting dilution and screened again. Then, gene amplification was performed by the medium containing 60 nM methotrexate (MTX; Enzo Life Sciences) and screened again. PD-1-Ig was produced by shake cultivation of the top three cell lines producing the highest amount of PD-1-Ig in the medium without G418 and MTX and purified with Ab-Capcher ExTra according to the manufacturer's protocol. To confirm the interaction between PD-1-Ig and PD-L1, PD-L1-expressing CHO-DG44 cells were stained with PD-1-Ig, biotin-conjugated anti-bovine IgG Fc (Rockland) and allophycocyanin (APC)-conjugated streptavidin (BioLegend).

Western blotting

Supernatants containing PD-1-Ig were mixed with 2 $\times$ SDS buffer (125 mM Tris-Hcl pH 6.8, 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol) and boiled for 10 min. Samples were separated on 12% SDS-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane (Merck Millipore). After blocked with 3% skim milk in PBS containing 0.05% Tween 20 (PBS-T), the membranes were incubated at room temperature for 1 h with horse radish peroxidase (HRP)-conjugated anti-bovine

IgG Fc (Rockland). After washing, the membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) to visualize signals, and analyzed with a Fluor-S Multi Imager (Bio-Rad Laboratories).

Plasmids for PD-L1 expression on the cell membrane

Schematic representation of PD-L1 proteins was described in Figure II-2. DNA fragments encoding whole PD-L1 and the C-terminal deletions of PD-L1 were amplified by forward primer (5'-CTA GCT AGC ACC ATG AGG ATA TAT AGT GTC TTA AC-3') containing a restriction site for *NheI* and following reverse primers containing a restriction site for *XhoI*; 5'-CAA TCT CGA GTT ACG TCT CCT CAA ACT GTG TAG-3' for whole PD-L1; 5'-CAA TCT CGA GTT ACT TTG AAT TCA TAT CTC GGG-3' for PD-L1-ΔC279; 5'-CAA TCT CGA GTT ATT CTA CAT CCA TCA TTC TCA C-3' for PD-L1-ΔC269; 5'-CAA TCT CGA GTT ACA GAC AGA AGA TGA CTG C-3' for PD-L1-ΔC259, and were cloned into pCIneo (Promega). Construction of PD-L1-EGFP was described above. pcDNA3 (Invitrogen) encoding leukotriene B4 receptor (BLT1) tagged with Flag at N-terminal was also provided by Dr. T. Yokomizo [Hase et al., 2008] and used for negative control.

Lymphocytes preparation and B cell depletion

PBMCs were isolated from BLV⁺ cattle by density gradient centrifugation as described in Chapter I. For B cell depletion, PBMCs were stained with anti-IgM (BIG73A) and anti-mouse IgG1 MicroBeads (Miltenyi Biotec) and depleted with autoMACS Pro (Miltenyi Biotec) according to the manufacturer's protocol.

Cell apoptosis assays

Cos-7 and Hela cells were grown in complete RPMI 1640 medium. The cells were transfected with the above plasmids by Lipofectamine 2000 (Life Technologies), and PD-1-Ig (50 µg/ml) or anti-PD-L1 mAbs (4G12 and 5A2; 20 µg/ml) with goat anti-rat IgG Fc (12 µg/ml; Thermo Scientific) were added into medium after 2 days of transfection. Bovine IgG (Sigma-Aldrich) or rat IgG (Sigma-Aldrich) was used as negative control for PD-1-Ig or anti-PD-L1 mAbs. One day later, the cells were harvested, and PD-L1 or BLT1 expression was confirmed by EGFP fluorescence for PD-L1-EGFP, staining with anti-DYKDDDDK antibody (2H8; Trans Genic) [Sasaki et al., 2012] and PE-conjugated anti-mouse IgG for BLT1-Flag and staining with

anti-PD-L1 mAb (4G12) and anti-rat IgG PE (Beckman Coulter) for other plasmids. After surface staining, the cells were stained with Annexin-V-APC (BD Biosciences) and 7-aminoactinomycin D (7-AAD) (BD Biosciences) following the manufacturer's instructions.

PBMCs were cultivated with bovine IgG or PD-1-Ig (50 µg/ml) for 2 days. The cells were stained with following antibodies: anti-CD4 (CACT138A) pre-labeled with Zenon PE; anti-CD8 (CACT80C; VMRD) pre-labeled with Zenon Alexa Fluor 647 (Life Technologies); anti-IgM (BIG73A) pre-labeled with Lightning-Link PE-Cy7 (Innova Biosciences). To investigate the effect of PD-1-Ig on cell death in PD-L1⁺ cells, the cells were stained with anti-PD-L1 (4G12) with APC-conjugated anti-rat IgM+IgG (Beckman Coulter) and anti-IgM with PE-conjugated anti-mouse IgG. After washing, the cells were stained with Annexin-V-FITC (BD Biosciences) and 7-AAD, and analyzed by flow cytometry.

IFN-γ and IL-10 ELISA

PBMCs or B cell-depleted PBMCs were cultivated with bovine IgG or PD-1-Ig (50 µg/ml) in the presence or absence of phorbol 12-myristate acetate (PMA; Sigma-Aldrich) and ionomycin (20 ng/ml and 1 µg/ml; Sigma-Aldrich). After 2 days, the supernatants was collected and frozen for further analysis. The production of IFN-γ was measured by ELISA for bovine IFN-γ (Mabtech) according to the manufacturer's protocol. Results were calculated based on a standard curve ranging from 7.8 pg/ml to 500 pg/ml. Sandwich ELISA of IL-10 was performed with two antibodies: anti-IL-10 (CC318; AbD Serotec) as capture antibody and biotin-conjugated anti-IL-10 (CC320; AbD Serotec) as detective antibody. Briefly, 96 well plates were coated overnight with CC318 diluted with PBS. After washing with PBS and blocking by PBS-T containing 0.1% bovine serum albumin (BSA; Sigma-Aldrich), samples were incubated in the wells for 2 h. Following washing, diluted detective antibodies (CC320) were added to the wells and incubated for 1 h. After further washing, Neutra-Avidin-HRP was added and incubated for 1 h. Finally, plates were washed and incubated with TMB One Component Substrate (Bethyl Laboratories), and absorbance was measured by MTP-650FA (Corona Electric). Standard curve was constructed using plasma which was separated from a cattle-derived blood stimulated with 10 µg/ml ConA and 10 µg/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 48 h. One (64) arbitrary unit is defined in terms of amount of IL-10 in 15.625 (1000) µl of the

stimulated plasma. Reported values represent the mean of duplicate samples.

Proliferation assay

PBMCs were cultivated with bovine IgG or PD-1-Ig (50 µg/ml) in the presence of PMA and ionomycin for 3 days as described above. [³H]-Thymidine (0.5 µCi/well: ICN Biochemicals) was added to the culture, and the cells were cultured for additional 6 h, then harvested onto glass filters by using a 96well-cell harvester (PerkinElmer) and incorporated radioactivity was measured using liquid scintillation counter (Aloka). All samples were tested in triplicate, and the data were presented as mean stimulation index.

Statistical analysis

Two-way ANOVA and Wilcoxon matched-pairs test were performed using GraphPad Prism version 5.0. *P*-values of less than 0.05 were considered to be statistically significant.

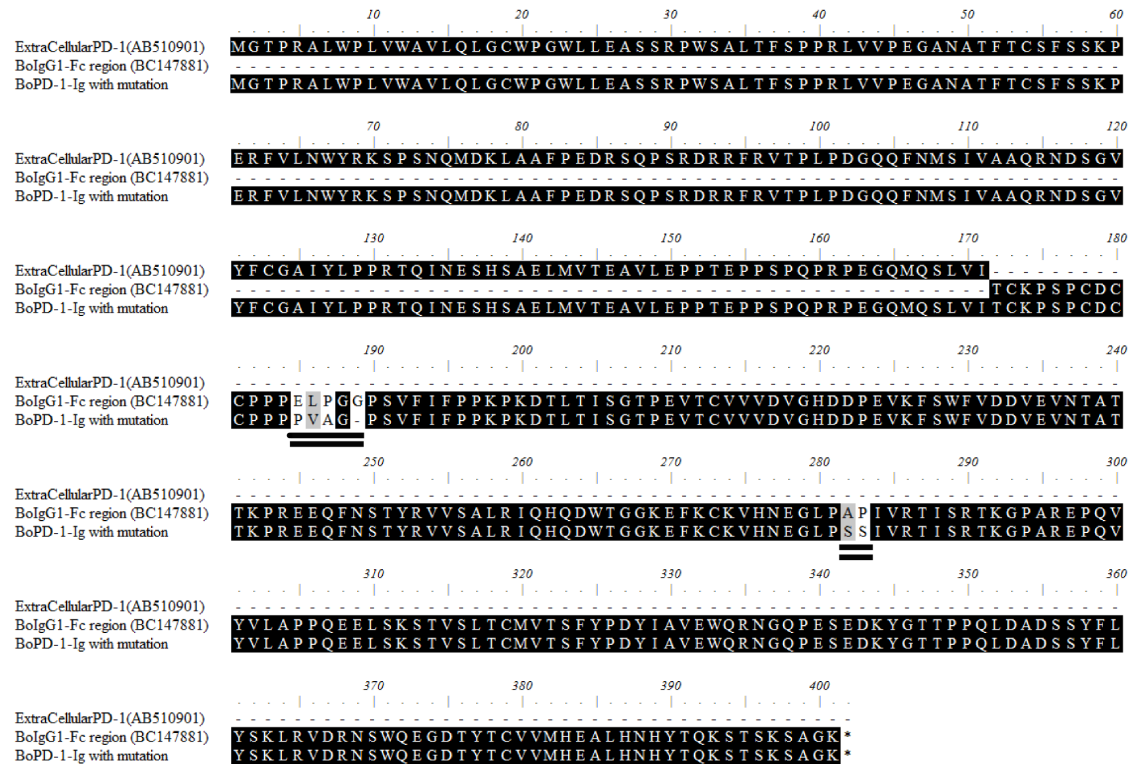


Figure II-1. Amino acid sequences of the extracellular region of bovine PD-1, bovine IgG1-Fc region and bovine PD-1-Ig. GenBank accession numbers are described in each title. Double lines indicate mutation sites for the reduction of ADCC response.

RESULTS

Establishment of bovine PD-1-Ig and anti-bovine PD-L1 mAbs

PD-1-Ig expression was confirmed by Western blotting with anti-bovine IgG Fc. PD-1-Ig was produced in the supernatant from PD-1-Ig-expressing CHO-DG44 cells and was detected at about 67 kDa (Figure II-3a). To confirm the binding of PD-1-Ig and anti-PD-L1 mAbs with PD-L1 on the cell membrane, PD-L1-EGFP expressed in CHO-DG44 cells were stained with PD-1-Ig and anti-PD-L1 mAbs. Both PD-1-Ig and three types of anti-PD-L1 mAbs recognized PD-L1-EGFP-expressing cells (Figure II-3b, c). PD-1-Ig bound EGFP-expressing cells slightly and independently of PD-L1 expression, indicating the non-specific binding.

PD-1-Ig treatment increased cell death in PD-L1-expressing cell lines

To investigate influence of PD-L1 cross-linking by PD-1-Ig on cell viability of PD-L1-expressing cells, Cos-7 cells were transiently transfected with the plasmids encoding bovine PD-L1 and 50 µg/ml of PD-1-Ig were added. PD-1-Ig was confirmed not to bind to untransfected Cos-7 and Hela cells at this concentration in advance (data not shown). Annexin-V and 7-AAD staining revealed that dead cells (Annexin-V⁺ 7-AAD⁺) were increased and live cells (Annexin-V⁻ 7-AAD⁻) were decreased only in PD-L1^{high} cells, not PD-L1⁻ cells when the cells were incubated with PD-1-Ig as compared with bovine IgG (Figure II-4a, b). There was no induction of cell death by the PD-1-Ig treatment in BLT1^{high}, BLT1^{low} and BLT1⁻ cells (Figure II-4c), indicating that PD-1-Ig treatment specifically induces the apoptosis of PD-L1-expressing cells through signaling involved in the cell viability.

PD-1-Ig-induced cell death was not dependent on intracellular region of PD-L1

There is no known signaling motif in the intracellular region of PD-L1 [Keir et al., 2008]. To confirm whether the intracellular region of PD-L1 is involved in the PD-L1-mediated cell death, Cos-7 cells transfected with the plasmids encoding the C-terminal deletion mutants of PD-L1 were treated with PD-1-Ig. As expected, the stimulation by PD-1-Ig increased the proportions of dead cells in PD-L1^{high} cells when PD-L1-ΔC279, PD-L1-ΔC269 and PD-L1-ΔC259 were transfected (Figure II-5a, b, c). Moreover, the percentages of dead cells in PD-L1-EGFP^{high} cells were augmented

(Figure II-5d), indicating that PD-L1-mediated cell death occurred via mechanism independent of the intracellular region of PD-L1. The cell mortality in EGFP-expressing cells was not affected by the stimulation with PD-1-Ig. The percentage of live PD-L1^{high} cells was decreased by PD-1-Ig treatment among the cells transfected with all plasmids other than control plasmids (data not shown). When HeLa cells were transfected with PD-L1-ΔC259 and treated with PD-1-Ig, the proportions of dead cells in PD-L1-expressing cells were increased in the same as the case of Cos-7 cells (Figure II-5e). Meanwhile, PD-L1 cross-linking by one type of anti-PD-L1 mAb, 4G12, not 5A2, also induced the augmentation of the proportion of dead cells in the PD-L1-EGFP-expressing cells (Figure II-5f), suggesting that the binding with PD-1 was not essential for the PD-L1-mediated cell death.

PD-1-Ig treatment reduced cytokine production and cell proliferation in bovine lymphocytes

To investigate the effect of PD-L1 blockade on the immune function of lymphocytes from BLV⁺ cattle, the response of cell proliferation and cytokine production in PBMCs cultivated with PD-1-Ig or anti-PD-L1 mAbs were measured. The IFN-γ production was enhanced by PD-L1 blockade using three types of anti-PD-L1 mAbs, supporting the results of many previous reports (Figure II-6a) [Keir et al., 2008]. However, unexpectedly, the cell proliferation response to PMA/ionomycin was decreased by PD-1-Ig treatment (Figure II-6b). PD-L1 blockade by PD-1-Ig also reduced the production of IFN-γ and IL-10 in PBMCs treated with PD-1-Ig in the presence or absence of PMA/ionomycin as compared with those treated with bovine IgG (Figure II-6c, d).

PD-1-Ig treatment increased B cell death in bovine lymphocytes

To investigate the mechanism of PD-1-Ig-induced immunosuppression, annexin-V and 7-AAD staining were performed in PBMCs. PD-1-Ig treatment led to decreased frequency of live cells (data not shown) and increased frequency of dead cells in IgM⁺ B cells, but not in CD4⁺ and CD8⁺ T cells among lymphocytes isolated from BLV⁺ cattle (Figure II-7a). The reduction of IFN-γ production by PD-1-Ig treatment was diminished by B cell depletion from PBMCs (Figure II-7b), although IL-10 was not (Figure II-7c), indicating that B cell-death was one of the mechanisms of PD-1-Ig-mediated immunosuppression. Since PD-L1 expression was mainly observed

in B cells among lymphocytes isolated from BLV⁺ cattle as described in Chapter I, it was hypothesized that PD-1-Ig bound PD-L1⁺ B cells and induced PD-L1-mediated cell death in B cells along with the observation in Figure II-4 and 5, resulting in the immunosuppression. Expectedly, the percentages of dead PD-L1⁺ B cells were augmented by PD-1-Ig treatment (Figure II-7d) and those of the live cells were decreased (data not shown). However, those of dead PD-L1⁻ B cells were also upregulated (Figure II-7d).

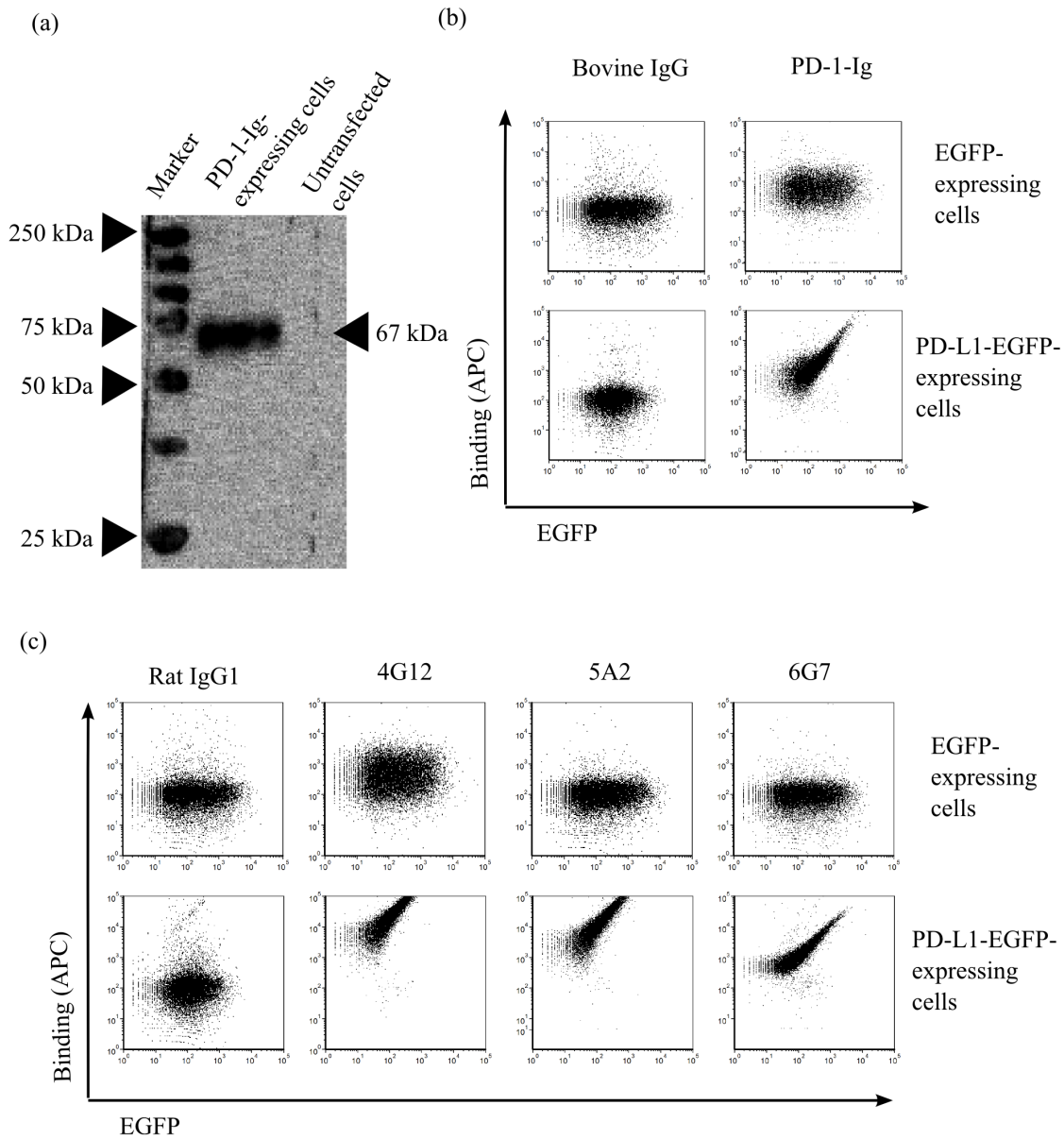


Figure II-3. Establishment of PD-1-Ig and anti-bovine PD-L1 mAbs.

(a) Western blot analysis of PD-1-Ig. Anti-bovine IgG Fc antibody recognized PD-1-Ig at about 67 kDa from supernatant of PD-1-Ig-expressing CHO-DG44 cells. (b, c) PD-1-Ig (b) and three types of anti-PD-L1 mAbs (c) recognized PD-L1-EGFP-expressing CHO-DG44 cells, not EGFP-expressing CHO-DG44 cells. Bovine IgG (for PD-1-Ig; b), rat IgG1 (for 5A2; c), rat IgG2a (for 4G12, data not shown; c) and rat IgM (for 6G7, data not shown; c) were used as negative control.

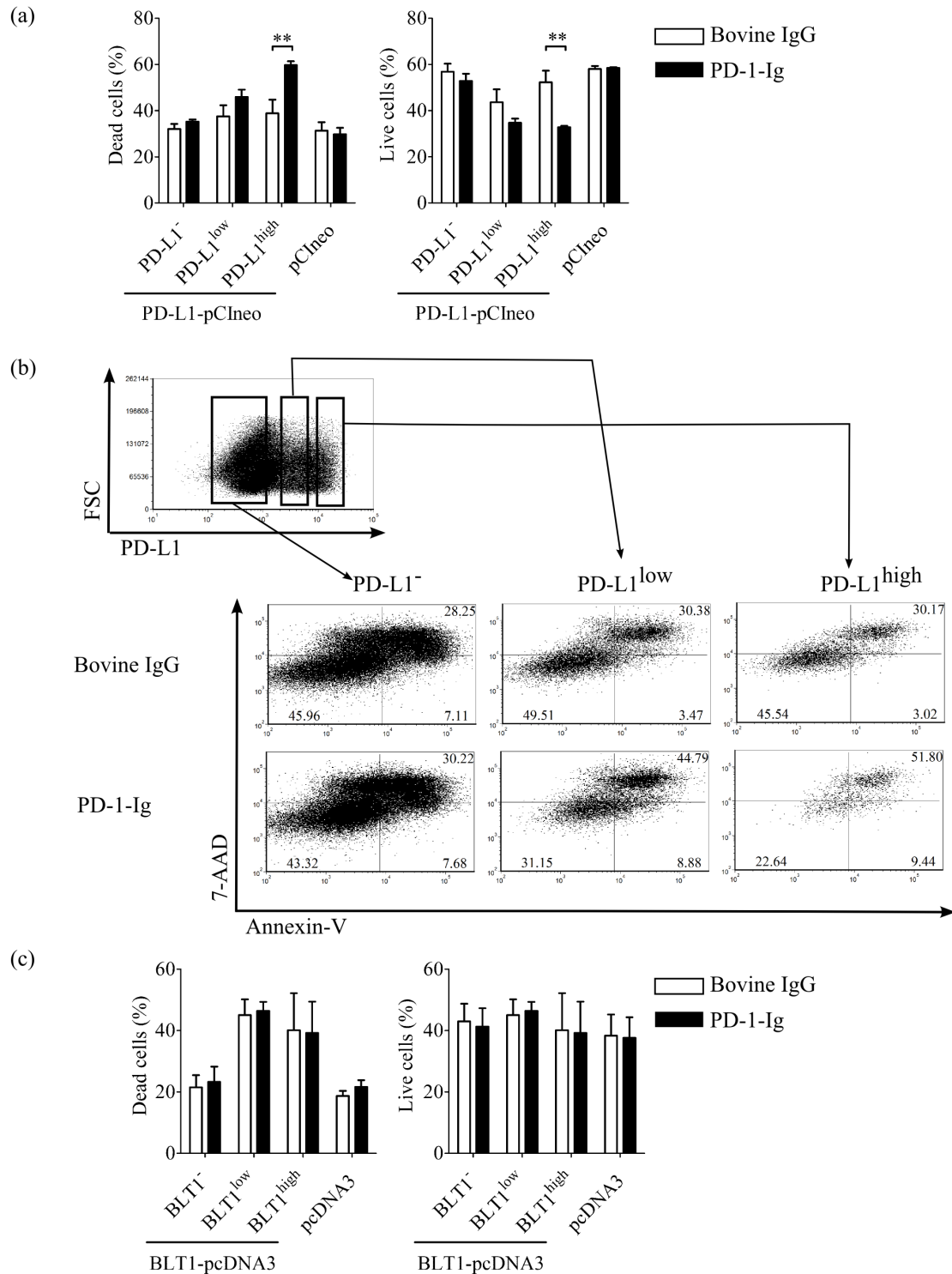


Figure II-4. Cell death in PD-L1-expressing cells treated with PD-1-Ig.

(a, c) Percentages of dead cells (Annexin-V⁺ 7-AAD⁺) and live cells (Annexin-V⁻ 7-AAD⁻) among Cos-7 cells transfected with pCIneo encoding PD-L1 (a) or pcDNA3 encoding BLT1 (c) and treated with bovine IgG or PD-1-Ig (50 μ g/ml). pCIneo or pcDNA3 was used as transfection control. PD-L1^{-/low/high} cells were distinguished by staining with anti-PD-L1 mAb. (b) Gating strategy and representative dot plots of PD-L1-expressing Cos-7 cells stained with

Annexin-V and 7-AAD. Values in the quadrant indicate the percentage of the cells. Mean values $\pm$ SEM from three independent experiments are shown. Statistical comparisons between bovine IgG and PD-1-Ig were made using the two-way ANOVA. Differences were considered statistically significant at $P < 0.05$ (** $P < 0.01$).

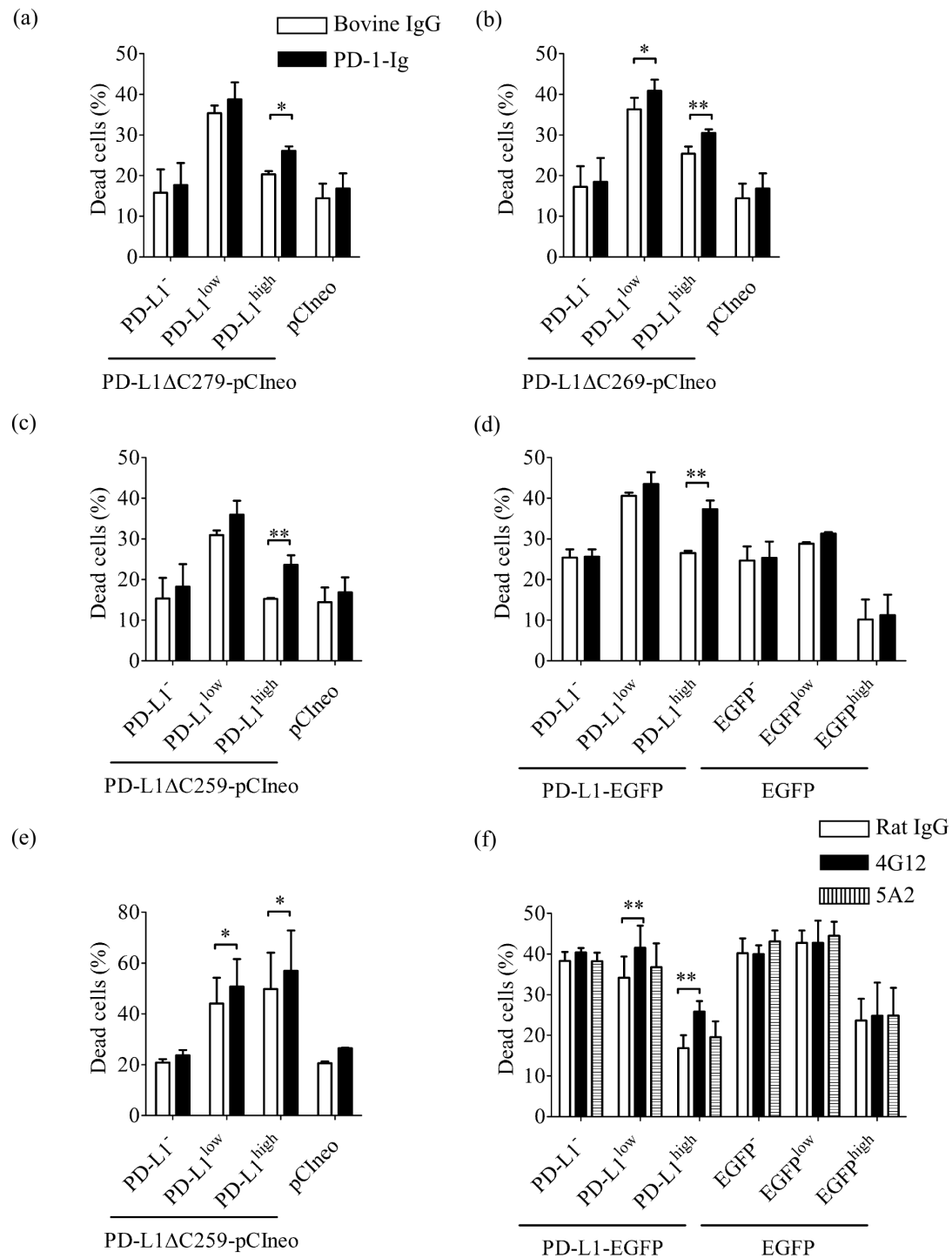


Figure II-5. Cell death in cells transfected with plasmids encoding C-terminal deletion mutants of PD-L1 and treated with PD-1-Ig.

(a, b, c, d) Percentages of dead cells in Cos-7 cells transfected with pCIneo encoding PD-L1-ΔC279 (a), PD-L1-ΔC269 (b), PD-L1-ΔC259 (c) and PD-L1-EGFP (d) after stimulation by bovine IgG or PD-1-Ig (50 μg/ml). pCIneo or pEGFP-N2 was used as transfection control. PD-L1^{-/low/high} cells were distinguished by staining with anti-PD-L1 mAb or EGFP fluorescence. (e) Same as Figure II-5c except that HeLa cells were transfected. (f) Same as Figure II-5d

except that Cos-7 cells were incubated with two types of anti-PD-L1 mAbs (20 µg/ml; 4G12 and 5A2) and anti-rat IgG Fc (12 µg/ml). Mean values $\pm$ SEM from three independent experiments are shown. Statistical comparisons between bovine IgG and PD-1-Ig were made using the two-way ANOVA. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

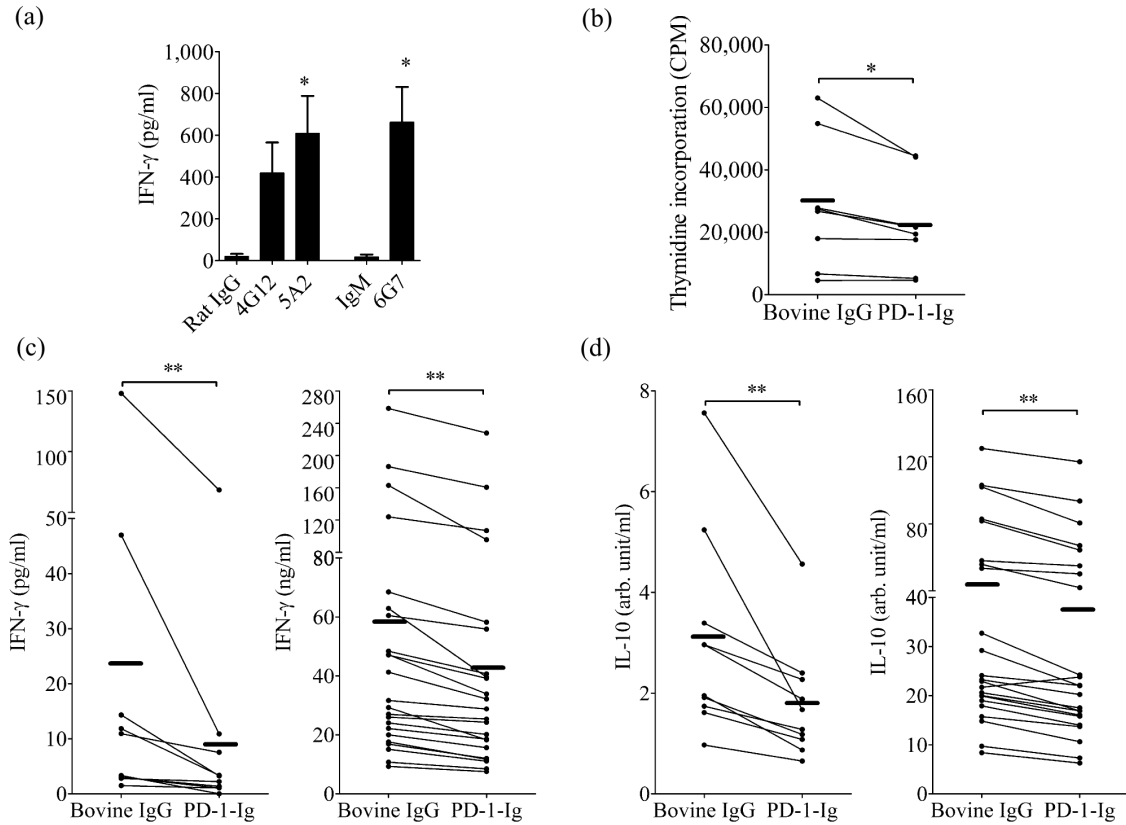


Figure II-6. Effect of PD-L1 blockade by anti-PD-L1 mAbs and PD-1-Ig treatment on immune reaction in bovine lymphocytes.

(a) PBMCs isolated from BLV⁺ cattle were cultured with rat IgG control, rat IgM control or three types of anti-PD-L1 mAbs (4G12, 5A2, 6G7). IFN- γ production was measured by ELISA ($n = 8$). (b, c, d) PBMCs were cultured with bovine IgG control or PD-1-Ig (50 μ g/ml) in the presence or absence of PMA/ionomycin. Proliferation responses in the presence of PMA/ionomycin were measured by thymidine incorporation (b; $n = 8$). IFN- γ (c) and IL-10 (d) production were measured by ELISA (PMA/ionomycin in right; $n = 23$, no stimulant in left; $n = 10$). Each of line indicates the mean values in each group. Statistical comparison between rat IgG, 4G12 and 5A2 was made using the one-way ANOVA with Tukey's post test. Those between rat IgM and 6G7 or bovine IgG and PD-1-Ig were made using the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

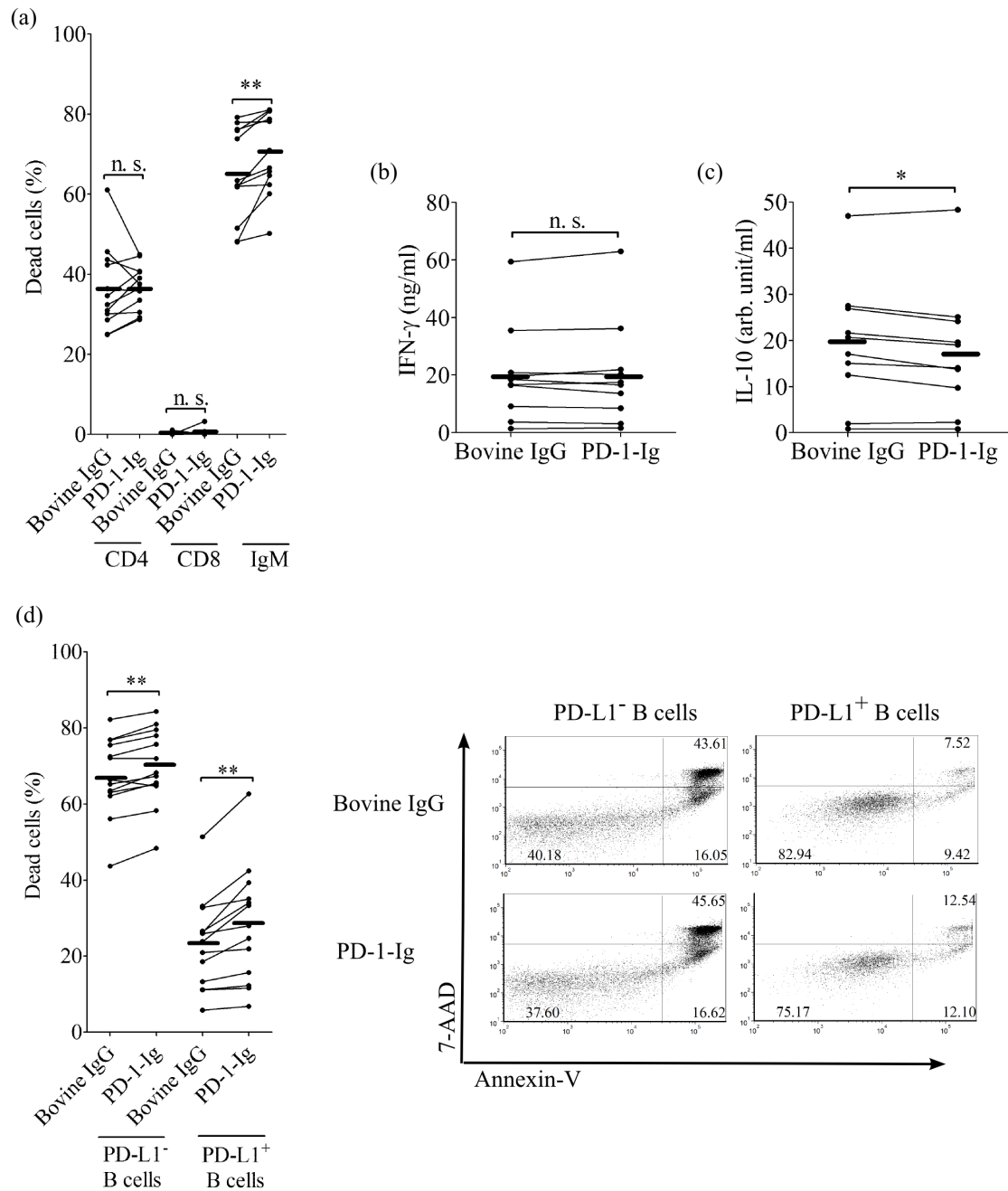


Figure II-7. Effect of PD-1-Ig treatment on B cell survival in bovine lymphocytes.

(a) Percentages of dead cells in CD4⁺ T cells, CD8⁺ T cells and IgM⁺ B cells among PBMCs treated with bovine IgG or PD-1-Ig ($n = 12$). (b, c) IFN- γ (b) and IL-10 (c) productions in IgM⁺ B cell-depleted PBMCs were measured by ELISA ($n = 10$). (d) Proportions of dead cells in PD-L1⁺ or PD-L1⁻ B cells among PBMCs isolated from cattle were analyzed ($n = 13$). Each of line indicates the mean values in each group. Statistical comparisons between bovine IgG and PD-1-Ig were made using the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

DISCUSSION

The blockade of the PD-1/PD-L1 pathway by anti-PD-L1 mAb is widely recognized as appropriate strategy to enhance the immune reaction and more effective than that by anti-PD-1 mAb because some types of anti-PD-L1 mAbs can inhibit the interaction of not only PD-1 and PD-L1, also PD-L1 and CD80 [Keir et al., 2008]. Otherwise, some classes of anti-PD-L1 mAbs interfere with PD-L1 binding to CD80, not with PD-L1 binding to PD-1. The treatment of this anti-PD-L1 mAb accelerated the symptoms of autoimmune-mediated diabetes in mouse model [Paterson et al., 2011], indicating that the PD-L1/CD80 pathway, as well as the PD-1/PD-L1 pathway, is involved in immunoinhibition. Moreover, Ghiotto et al. [2010] reported that other type of anti-PD-L1 mAb which is not able to block the interaction of PD-1 with PD-L1 increased the binding of PD-1 with PD-L1. For these reports, it is important to carefully select clones producing the appropriate anti-PD-L1 mAb for immune reactivation. In this study, three types of anti-PD-L1 mAbs that enhance IFN- γ production in bovine lymphocytes were obtained. Among them, 5A2 may be a suitable antibody for immune activation because PD-L1 cross-linking by 5A2 and anti-rat IgG antibody did not induce PD-L1-mediated cell death in PD-L1-expressing Cos-7 cells. Although it remains to be determined whether they block the PD-1/PD-L1 or PD-L1/CD80 pathway, these antibodies might be useful tools for investigating PD-L1 function in bovine immune system.

In this study, forced expression of PD-L1 in Cos-7 cells revealed that PD-L1^{high} cells were more sensitive to PD-L1-mediated cell death than PD-L1^{low} cells. These data indicated that the high expression level of PD-L1 is essential for the induction of PD-L1-mediated cell death. If the PD-L1-mediated cell death often occurs in physiological condition *in vivo*, anti-PD-L1 mAb treatment could offer two benefits, the blockade of the PD-1/PD-L1 pathway and the eradication of PD-L1^{high} tumor cells or virus-infected cells. Actually, the reduction of PD-1⁺ CD8⁺ T cells by the treatment of PD-L2-Ig fusion protein *in vivo* tipped the balance in favor of non-exhausted PD-1^{low} CD8⁺ T cells [Mkrtichyan et al., 2012]. Although there is a danger that PD-L1-mediated cell death might be induced in PD-L1⁺ antigen presenting cells required for T cell activation, the strategy that PD-L1⁺ lymphoma cells are eradicated by PD-L1-mediated cell death may deserve the consideration for clinical application.

Meanwhile, lymphocytes obtained from BLV⁺ cattle incubated with PD-1-Ig

downregulated immune reaction in this study. It was also confirmed that the treatment of polyclonal anti-human PD-L1 antibody inhibited the IFN- γ production in bovine lymphocytes (data not shown). These observations were thought to be attributed to PD-L1-mediated cell death in bovine lymphocytes, due to the facts that augmented PD-L1 expression in B cells isolated from BLV⁺ cattle (Chapter I) and no downregulation of IFN- γ in B cell-depleted PBMCs treated with PD-1-Ig. However, dead cells among PD-L1⁻ B cells were also increased in response to PD-1-Ig treatment. While increase in the number of apoptotic PD-L1⁺ B cells might affect apoptosis in PD-L1⁻ B cells sequentially, it still remains to investigate the mechanism of PD-1-Ig-induced cell death in bovine lymphocytes.

This study and the previous report suggested the presence of PD-L1-induced inhibitory signaling into PD-L1⁺ cells [Kim et al., 2008]. However, PD-L1 on cancer cells has been shown to act as an anti-apoptotic receptor, clarified with PD-1-Ig treatment [Azuma et al., 2008]. The reason why a consensus on the existence of PD-L1-induced signaling does not reach is that the known signaling motifs or molecules are not present in the intracellular region of PD-L1. In this study, it was observed that the intracellular region of PD-L1 was not involved in PD-L1-mediated cell death in Cos-7 and Hela cells. These data suggest that the extracellular region of PD-L1, not complex of PD-1 with PD-L1, may bind some molecules or receptors which induce the inhibitory or anti-apoptosis signaling. Further investigation using the immunoprecipitation technique would be required to identify the unknown molecules interacting with PD-L1.

There are few reports showing PD-L1-mediated cell death in PD-L1-expressing cells “*in vivo*”, because the many interactions of B7 family members, such as PD-1 and PD-L1/PD-L2, CTLA-4 and CD80/CD86, CD28 and CD80/CD86 and PD-L1 and CD80 pathways are involved in both inhibition and activation of immune responses. Moreover, PD-L1 expression is upregulated in inflammatory environment enriched in IFN- γ and other cytokines. Those cytokines enhance the resistance against apoptosis in the cells, and thereby, PD-L1-mediated cell death *in vivo* may be rarely observed. This study revealed that PD-L1-mediated cell death by PD-1-Ig is able to be induced without intracellular region of PD-L1 and occurs in only PD-L1^{high} cells. These data are informative to clarify the *in vivo* mechanism of PD-L1-mediated cell death.

SUMMARY

PD-L1 blockade is accepted as the novel strategies for the reactivation of exhausted T cells. Meanwhile, mechanism of PD-L1-mediated inhibitory signaling after PD-L1 stimulation by PD-1-Ig or PD-L1 cross-linking by anti-PD-L1 mAb is still unknown, although it might induce cell death of PD-L1⁺ cells required for regular immune reaction. In this study, PD-L1 blockade by the PD-1-Ig or anti-PD-L1 mAb treatment was tested in PD-L1-expressing cell lines and bovine lymphocytes to investigate whether the treatment induces immune reactivation or PD-L1-mediated cell death. PD-1-Ig treatment and PD-L1 cross-linking by anti-PD-L1 mAb with anti-rat IgG secondary antibody mainly increased the number of dead cells in PD-L1^{high} cells prepared from Cos-7 cells in which bovine PD-L1 was enforced to be expressed. The PD-L1-mediated cell death also occurred in Cos-7 and Hela cells transfected with plasmids encoding C-terminal deletions of PD-L1. In bovine lymphocytes, anti-PD-L1 mAb treatment upregulated IFN- γ production, whereas PD-1-Ig treatment decreased the cytokine production and cell proliferation. The IFN- γ production in B cell-depleted PBMCs was not reduced by PD-1-Ig treatment and the percentages of dead cells in PD-L1⁺ B cells were increased by PD-1-Ig treatment, indicating that PD-1-Ig-induced immunosuppression could be caused by PD-L1-mediated B cell death. This is the first report indicating that PD-L1-mediated cell death can be induced by PD-L1 lacking intracellular region and in only the cells expressing PD-L1 at high level. This study provides the novel information for understanding the signaling through PD-L1.

CHAPTER III

Analysis of PD-1 Expression and the Influence of PD-1 Blockade on Immune Reaction and Viral Expression in Bovine Leukemia Virus-Infected Cattle

INTRODUCTION

Immunoinhibition is considered as one of the reasons responsible for the refractory nature of several types of tumors and chronic infections [Dunn et al., 2004; Keir et al., 2008]. One of them, BLV, is known to induce immunosuppression and BL in cattle [Kabeya et al., 2001]. During the chronic infection, the suppression of both CD4⁺ T cell proliferation and cytotoxic immune response against BLV antigens is correlated to disease progression [Kabeya et al., 2001; Orlik et al., 1996]. To develop strategies to effectively control BLV infection, the mechanism responsible for this immunosuppression needs to be clarified.

PD-1 has been recognized as being at the heart of peripheral immune tolerance and pathogen-specific immunoinhibition [Keir et al., 2008]. In various types of chronic infections such as HIV and HTLV-1 infection, and tumors, such as ovarian cancer and pancreatic cancer, PD-1 and PD-L1 play an important role in inhibiting chronically activated T cells specific for pathogens, resulting in the induction of T cell exhaustion [Elrefaei et al., 2008; Kozako et al., 2009; Matsuzaki et al., 2010; Nomi et al., 2007; Rosignoli et al., 2009]. Pathogen-specific T cells express higher PD-1 than total T cells [Day et al., 2006; Kozako et al., 2009], so that the treatment with mAb specific for PD-1 reactivates pathogen-specific immune responses such as proliferation, cytokine production and cytotoxic capabilities of exhausted T cells *ex vivo* [Kozako et al., 2009; Rosignoli et al., 2009] and *in vivo* [Finnerfrock et al., 2010; Velu et al., 2009], and have been tested in clinical trials with cancer patients [Berger et al., 2008; Brahmer et al., 2010].

In the field of veterinary medicine, the PD-1/PD-L1 pathway is also investigated in pig [Jeon et al., 2007; Peng et al., 2010], chicken [Matsuyama-Kato et al., 2012] and cat [Folkl et al., 2010], and found to contribute to pathogenesis and immune evasion of chronic infectious diseases. In Chapter I, it was also shown that the expression of PD-L1 in B cells, which were target cells for BLV infection, was upregulated in BLV⁺ cattle as the disease progressed. In previous reports, anti- “human” PD-1 or PD-L1 “polyclonal” antibodies were used to analyze their expression and to block the PD-1/PD-L1 pathway [Folkl et al., 2010; Ikebuchi et al., 2011]. Under some experimental conditions, anti-PD-1 polyclonal antibody induced IL-10 production by monocytes, resulting in the inhibition of CD4⁺ T cell function [Said et al., 2010].

However, at the present time, mAbs specific for animal PD-1 which can reactivate exhausted immune reaction are not available, although they are essential for further investigation of veterinary immunology and development of new therapy for refractory diseases, such as BLV infection.

In this chapter, anti-bovine PD-1 mAbs were established and their functional capabilities were assessed using PBMCs from BLV⁺ and BLV⁻ cattle *in vitro*. The upregulation of PD-1 expression was found in CD4⁺ and CD8⁺ T cells isolated from BCBL. The treatment with an anti-PD-1 mAb upregulated IFN- γ production and reduced both B cell activation and BLV-gp51 expression in PBMCs isolated from BLV⁺ cattle. These data suggest that anti-PD-1 mAb can be applicable for antibody drug to control BLV infection.

MATERIALS AND METHODS

Construction and expression of recombinant soluble bovine PD-1-Ig

Soluble PD-1-Ig was expressed in a mammalian cell expression system. The cDNA encoding the extracellular domain of bovine PD-1 was amplified by PCR and inserted into the cloning site of a modified pCAGGS that contained a mouse CD150 signal sequence at the N terminus and the Fc fragment of bovine IgG1 at the C terminus as described in Chapter II. PD-1-Ig was produced in Cos-7 cells transfected transiently by Lipofectamine 2000, and purified from the media with Protein G Sepharose 4 Fast Flow (GE Healthcare) according to the manufacturer's protocol. The expression and purification of PD-1-Ig were confirmed by SDS-PAGE and ELISA using anti-bovine IgG Fc.

Generation and screening of mAb specific for bovine PD-1

To obtain mAbs specific for bovine PD-1, a rat was immunized with about 66 µg of PD-1-Ig and complete Freund's adjuvant. Nineteen days after, 100 µg of PD-1-Ig was shot into the rat as a boost. On the 24th day of the first immunization, lymphocytes isolated from the iliac LN were fused with myeloma cells and cloned, and the supernatants from hybridomas were screened by ELISA for the reactivity of culture supernatant with PD-1-Ig. Clones that produced antibodies specific for bovine IgG1 Fc were excluded by ELISA using bovine IgG as an antigen. The immunization of rats and ELISA were performed at Cell Engineering Corporation. The hybridomas were also screened by flow cytometry using Cos-7 cells that were transfected with bovine PD-1 encoding pCMV-Tag-1. Hybridomas producing antibodies which reacted with PD-1 were cloned by limiting dilution.

Western blotting

To test the reactivity of anti-bovine PD-1 mAbs, Western blot analysis was performed using CHO-DG44 cells that stably express bovine PD-1. CHO-DG44 cells were transfected with pCMV-Tag-1 encoding bovine PD-1 using Lipofectamine LTX (Life Technologies). Transfectants were selected in CD-DG44 medium (Life Technologies) containing G418 (Wako; 800 µg/ml) for 2 weeks. Cells were then incubated with anti-bovine PD-1 mAb (produced from the hybridoma 5D2), followed

by the incubation with anti-rat IgG MicroBeads (Miltenyi Biotec). CHO-DG44 cells highly expressing PD-1 were sorted by auto MACS Pro, cultivated for a week, and then re-sorted. CHO cells transfected with non-coding pCMV-Tag-1 were selected in the same way and used as a negative control. Cells were lysed in 2× SDS buffer and Western blotting was performed as described in Chapter II except that the membranes were incubated for 2 h with anti-PD-1 mAbs (2C12 and 3G2: 3 µg/ml, 2H7 and 5D2: 1 µg/ml), followed by washing and incubation with HRP-conjugated anti-rat IgG (MP Biomedicals). The membrane was also probed with anti-actin (C4; Merck Millipore) and anti-myc tag antibodies (Abcam; goat polyclonal antibody) as a loading control and a positive control.

Samples from cattle and BLV diagnosis

Blood and mesenteric LN samples from Japanese black and Holstein-Friesian were investigated. In this chapter, blood samples of 95 cattle altogether (BLV⁺: 53, BLV⁻: 42) bred in several farms were obtained. They were collected in about ten installments. Peripheral blood was collected from cattle into tubes containing sodium heparin (Ajinomoto). All of the cattle from which the blood samples were obtained had been diagnosed with BLV infection by nested PCR as described in Chapter I between 2008 and 2012. LN samples were provided by meat inspection center and veterinary hospitals in Japan, and diagnosed by nested-real time PCR. The first amplification was conducted by KOD FX neo (Toyobo) using primers; BLV-LTR 1 and BLV-LTR 533. Less than 20,000 LN cells were added in PCR mixture as templates. The second amplification was performed using SYBR Premix DimerEraser using primers; BLV-LTR 256 and BLV-LTR 453.

To diagnose BL, phenotypic analysis of PBMCs and/or tumor-bearing LN (mesenteric, iliac, gastric or superficial cervical LN) cells from BCBL which had been diagnosed clinically were performed by flow cytometry. Double staining were conducted using anti-bovine IgM (BIG73A) pre-labeled with Zenon Alexa Fluor 488 (Life Technologies) and following antibodies; anti-WC4 (CC55; CD19-like; AbD Serotec) [Naessens et al., 1997], anti-B-B7 (GB25A; CD21-like; VMRD) and anti-CD5 (CACT105A). Alexa Fluor 647-conjugated anti-mouse IgG (Life Technologies) was used for the antibodies other than anti-IgM as a secondary antibody. The samples in which more than 85% of cells were B cells (IgM⁺ CD5⁺, IgM⁻ CD19-like⁺ or IgM⁻ CD21-like⁺) were diagnosed as BL induced by BLV.

Cell preparation from blood and LNs

PBMCs were purified from the blood samples by density gradient centrifugation on Percoll as described in Chapter I. LN tissues were minced in PBS and passed through a cell strainer (40 μ m; BD Biosciences) to obtain single cell suspension.

PD-1 expression analysis

To block nonspecific staining, $5\text{--}20 \times 10^5$ PBMCs were incubated in PBS containing 10% goat serum (Sigma-Aldrich) at room temperature for 15 min. Cells were then washed and stained for PD-1, CD4, CD8, IgM and CD25 for 30 min at room temperature. The following antibody conjugates were used in Figure III-2: PD-1:5D2; CD4:CACT138A pre-labeled with Zenon Alexa Fluor 488; CD8:CACT80C pre-labeled with Lightning-Link PerCP-Cy5.5 (Innova Biosciences); IgM:BIG73A pre-labeled with Zenon PE; CD25:CACT116A pre-labeled with Lightning-Link PE-Cy7. In Figure III-4, the cells were stained with the following antibodies: PD-1, CD4 pre-labeled with Zenon Alexa Fluor 488, CD8 pre-labeled with Lightning-Link PerCP-Cy5.5, and IgM pre-labeled with Lightning-Link PE-Cy7. After washing with PBS containing 10% goat serum, cells were incubated with APC-conjugated anti-rat IgM+IgG for 30 min at room temperature. Cells were then washed and immediately analyzed by FACS Verse and FCS Express 4 (De Novo Software).

To measure the upregulation of PD-1 expression in stimulated bovine lymphocytes isolated from BLV⁻ cattle, PBMCs were cultivated for 24, 48, and 72 h at 37°C with 5% CO₂ in complete RPMI 1640 medium in the presence of pokeweed mitogen (PWM, 5 μ g/ml, Sigma-Aldrich) or PMA and ionomycin (20 ng/ml and 1 μ g/ml).

PD-1 blockade assay

To determine the effect on the immune activation by anti-PD-1 mAb treatment, bovine PBMCs were cultured with 20 μ g/ml of anti-PD-1 mAb or rat IgG in the presence or absence of PMA/ionomycin, or in the presence of 10 μ g/ml BLV-gp51 peptide mixture (15 mer). The peptide mixture covering the entire length of gp51 and overlapping by 11 amino acids, were synthesized in Hokkaido System Science. Flag peptide (DYKDDDDK) was used as a negative control for gp51 peptide mixture. After 2 days, cells were collected for the detection of apoptosis and expression analysis of gp51, WC4 and CD80. The supernatants were harvested and stored at -20°C for

IFN- γ and IL-10 ELISA. For real-time PCR or cell proliferation assay, cultivated cells were collected after 1 day or 5 days. In a portion of experiments, isolated B cells from PBMCs were cultured with anti-PD-1 mAb. PBMCs were incubated with anti-bovine IgM, while IgM⁺ B cells were isolated by autoMACS Pro and anti-mouse IgG1 MicroBeads.

IFN- γ and IL-10 ELISA

IFN- γ and IL-10 ELISA were performed as described in Chapter II.

Cell proliferation assay

To investigate the effect of PD-1 blockade on the proliferation of PBMCs, carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay was performed. In summary, PBMCs were incubated with 2 μ M CFSE (Life technologies) diluted with PBS at 37°C for 15 min, washed with complete RPMI 1640 medium three times and cultured for 5 days. Then, cells were stained with anti-IgM pre-labeled with Lightning-Link PE-Cy7 and analyzed immediately by flow cytometry. Percentages of CFSE^{low} IgM⁻ lymphocytes were measured for evaluation of the proliferation of lymphocytes other than B cells.

Expression analysis of BLV-gp51, CD19-like and CD80

To analyze the effect of PD-1 blockade on B cell function, cultivated PBMCs were stained with anti-IgM pre-labeled with Zenon Alexa Fluor 488 and anti-WC4 or anti-CD80 (AbD Serotec) or anti-BLV-gp51. Alexa Fluor 647-conjugated anti-mouse IgG was used for anti-WC4 and anti-CD80 as a secondary antibody. BLV-gp51 staining of either PBMCs or isolated B cells was performed as described in Chapter I. Appropriate isotype controls were used for each sample.

Real-time PCR

Total RNA was extracted from cultivated PBMCs by RNeasy Plus Mini Kit (QIAGEN), and cDNA synthesis and quantitative real-time PCR were performed as described in Chapter I. Primers used for the amplification of *BAFF* cDNA were 5'-CCA AGC TGG AGG AAG GAG ATG AAC TC-3' and 5'-CTC CAT CTC GGG ATA TCT TAG CAT C-3'. The amount of *BAFF* mRNA expression was divided by the expression of *GAPDH* and β -*actin* mRNA as internal control genes. Each

amplification procedure was done in duplicate, and the results were indicated as relative change to control (no antibody treatment).

Detection of apoptosis

To detect apoptotic B cells, cultivated PBMCs were stained with anti-IgM as a first antibody and Alexa Fluor 647-conjugated anti-mouse IgG as a secondary antibody. Following washing, cells were incubated with Annexin V-FITC (Beckman Coulter) for 15 min and added 7-AAD. The results are presented as percentages of apoptotic cells (FITC⁺ 7-AAD⁺) in total IgM⁺ B cells.

Statistical analysis

Spearman rank-correlation, one-way ANOVA with Tukey's post test, two-way ANOVA and Wilcoxon matched pairs test were performed using GraphPad Prism version 5.0. *P* values < 0.05 were considered statistically significant.

RESULTS

Anti-PD-1 mAb reacts with PD-1-expressing cells

Supernatants containing antibody from 576 hybridoma colonies were screened for their binding to bovine IgG and PD-1-Ig by ELISA. Hybridomas that produced mAbs recognizing bovine IgG were excluded. Four hybridomas (2C12, 2H7, 3G2, and 5D2) were cloned and confirmed as clones producing mAb that reacted with Cos-7 expressing PD-1 but not with cells transfected with the control vector (Table III-1 and Figure III-1a). Control antibody (Rat IgG) did not react with both of the cells. The degree of fluorescent signals of 2H7 and 5D2 were apparently different from those of 2C12 and 3G2. Anti-PD-1 mAbs, except for 2C12, were able to recognize heat-denatured PD-1 at approximately 68kDa by Western blotting, showing that 2C12 could recognize a conformational epitope of bovine PD-1 (Figure III-1b).

Anti-PD-1 mAb reacts with bovine lymphocytes

To confirm that anti-PD-1 mAb can recognize bovine PD-1 naturally expressed on bovine lymphocytes, surface PD-1 expression was examined on CD4⁺ and CD8⁺ T cells as well as IgM⁺ B cells freshly isolated or stimulated by mitogens, such as PWM and PMA/ionomycin *in vitro*. An example of the gating strategy is shown in Figure III-2a. IgM⁺ B cells were first gated and IgM⁻ cells further analyzed for CD4 and CD8 expression. Of four anti-PD-1 mAbs, the strongest fluorescence was observed in PBMCs stained with 5D2 (data not shown); therefore, cells were stained with 5D2 for subsequent expression analyses of bovine PD-1. PD-1 was expressed mainly on CD4⁺ T cells from freshly isolated PBMCs, whereas CD8⁺ T cells and B cells barely expressed PD-1 (Figure III-2b).

When PBMCs were cultivated in the presence of mitogen, PD-1 expression in lymphocytes was elevated, whereas *in vitro* incubation without any stimulant did not affect PD-1 expression in any population of lymphocytes (Figure III-2b, c). PMA/ionomycin stimulation more quickly induced PD-1 expression than no stimulant, which strongly enhanced in more than 90% of CD4⁺ and CD8⁺ T cells and half of B cells at 72 h (Figure III-2b). Although PWM stimulates both T and B cells [Janossy et al., 1976] like PMA/ionomycin, on one level or another, subsets of CD4⁺ and CD8⁺ T cells stimulated by PWM expressed less PD-1 antigen than PBMCs treated with

PMA/ionomycin at the same point in time, and the rate of the upregulation in PWM stimulation was also slower than that in PMA/ionomycin stimulation. Furthermore, upregulation of PD-1 expression on B cells was not induced by PWM stimulation. In the case of any stimulation, CD4⁺ T cells were prone to more quickly upregulate PD-1 expression than CD8⁺ T cells and B cells.

Previous report revealed activated lymphocytes expressed PD-1 antigen in mice [Agata et al., 1996], bovine PD-1 expression on stimulated lymphocytes detected by one of classical activation marker, CD25, was evaluated. At 48h after PMA/ionomycin stimulation, CD25 expression was strongly upregulated in either CD4⁺ and CD8⁺ T cells and IgM⁺ B cells, and almost all T cells and subset of B cells expressed PD-1 (Figure III-2d). Otherwise, under PWM stimulation, CD25 expression varied widely among the subsets. Moreover, CD4⁺ CD25⁻ T cells expressed PD-1, but CD8⁺ CD25⁻ T cells barely did. PD-1 expression was examined in PBMCs isolated from three healthy cattle and similar results were obtained in each case.

Anti-PD-1 mAbs activate IFN- γ production

To clarify the function of the mAb that inhibit the PD-1/PD-L1 pathway and the inhibitory signal from PD-1, PD-1 blockade assay was performed. PBMCs isolated from BLV⁻ cattle were cultured for 48 h in the presence of each anti-PD-1 mAb or control antibody, and IFN- γ production was then measured in the supernatants using ELISA. All of the four anti-PD-1 mAbs significantly increased IFN- γ production in PBMCs in comparison to those treated with control rat IgG (Figure III-3a). The treatments of both 2H7 and 5D2 upregulated the IFN- γ production in all sample cases. Furthermore, in PBMCs cultivated with PMA/ionomycin *in vitro*, 2C12 and 5D2, but not 2H7 and 3G2, significantly enhanced IFN- γ production (Figure III-3b), showing that the function of activated lymphocytes by PMA/ionomycin stimulation could be additionally enhanced by PD-1 blockade with 2C12 or 5D2 treatment. The most effective mAb was not determined because there were individual differences in INF- γ production, and no significant difference was observed within the degree of enhancement of IFN- γ production induced by the treatments with the four mAbs.

PD-1 expression is upregulated in CD4⁺ and CD8⁺ T cells in BCBL

A previous report claimed that *PD-1* mRNA expression in T cells isolated from BCBL was higher than that from BLV⁻ cattle [Ikebuchi et al., 2010]. To confirm the

PD-1 expression on the cell surface of T cells in BLV⁺ cattle, flow cytometric analysis was performed in PBMCs and mesenteric (BLV⁻ and BLV⁺ cattle) or tumor-bearing (BCBL) LN cells using anti-PD-1 mAb, 5D2. In blood, the mean percentages of PD-1⁺ CD4⁺ T cells were higher in BCBL than in BLV⁺ and BLV⁻ cattle (Figure III-4a). Meanwhile, in LN cells, the rates of PD-1 expression in both CD4⁺ and CD8⁺ T cells were significantly higher in BCBL than in BLV⁺ and BLV⁻ cattle (Figure III-4b; an example of the gating strategy is shown in Figure III-4c). IgM⁺ B cells in all sample barely showed PD-1 expression (data not shown), although CD19⁺ B cells in human patients of chronic lymphocytic leukemia (CLL) expressed strongly PD-1 [Grzywnowicz et al., 2012].

PD-1 blockade upregulates T cell function in PBMCs from BLV⁺ cattle

To assess whether PD-1 blockade activates T cell function in response to a BLV antigen, PBMCs from BLV⁺ cattle were cultured with BLV-gp51 peptide mixture in the presence or absence of anti-PD-1 mAb (5D2), and IFN- γ production and proliferation of lymphocytes were measured. The IFN- γ production in PBMCs was upregulated by gp51 peptide mixture relative to Flag peptide (Figure III-5a), indicating that anti-gp51 immune reaction was induced by gp51 peptide mixture. As expected, PD-1 blockade additionally enhanced IFN- γ production in the presence of gp51 peptide mixture, as compared to the treatment with rat IgG (Figure III-5b). The increasing rate of IFN- γ production in PD-1 blockade was correlated with frequencies of PD-1 expression in CD4⁺ T cells (Figure III-5c), but not CD8⁺ T cells (data not shown). On the contrary, the production of IL-10, which is one of the immunoinhibitory cytokines, was not altered by PD-1 blockade (Figure III-5d). Next, proliferation of lymphocytes was analyzed by CFSE staining. Detection of proliferating T cells was difficult because T cells were very few in cultivated PBMCs from BLV⁺ cattle causing abnormal B cell proliferation. Thus, the proliferation of lymphocytes other than B cells was investigated using the gating strategies described in Figures III-2a and III-4c. PD-1 blockade in PBMCs resulted in the increase in the frequencies of CFSE^{low} IgM⁻ lymphocytes relative to the treatment of control antibody (Figure III-5e).

PD-1 blockade inhibits BLV-gp51 expression and B cell activation

Next, whether PD-1 blockade altered B cell activation and BLV expression was evaluated. The incubation of PBMCs with gp51 peptide mixture in the presence of

anti-PD-1 mAb resulted in the reduction of the frequencies of gp51⁺ cells in IgM⁺ B cells (Figure III-6a). Because previous reports showed that B cell activation augments viral expression *ex vivo* [Gillet et al., 2007], it was hypothesized that one of the mechanisms responsible for the inhibition of gp51 expression resulting from PD-1 blockade was the downregulation of B cell activation. To test this hypothesis, the expression of activation markers, CD19-like (WC4) and CD80, of B cells were measured. PD-1 blockade resulted in the attenuation of WC4 and CD80 expression in B cells (Figure III-6b, c), and moreover, the reduction in the expression of *BAFF* mRNA (Figure III-6d), which was important cytokine for B cell survival [Lied et al., 2011]. Moreover, the frequencies of apoptotic B cells were also increased in PBMCs treated with anti-PD-1 mAb, as compared with control antibody (Figure III-6e). Finally, to assess whether PD-1 blockade have a direct effect on B cells, isolated B cells were cultivated with gp51 peptide mixture in the presence of anti-PD-1 mAb. As expected, the significant changes in gp51 expression in B cells by PD-1 blockade were not observed (Figure III-6f), suggesting that anti-PD-1 mAb did not affect directly on gp51 expression in B cells.

Table III-1. The number of positive hybridomas in each screening test

ELISA for PD-1-Ig	ELISA for bovine IgG	ELISA for PD-1-Ig (do not recognize bovine IgG)	Flow cytometry for PD-1 expressing Cos-7
47	78	14	4

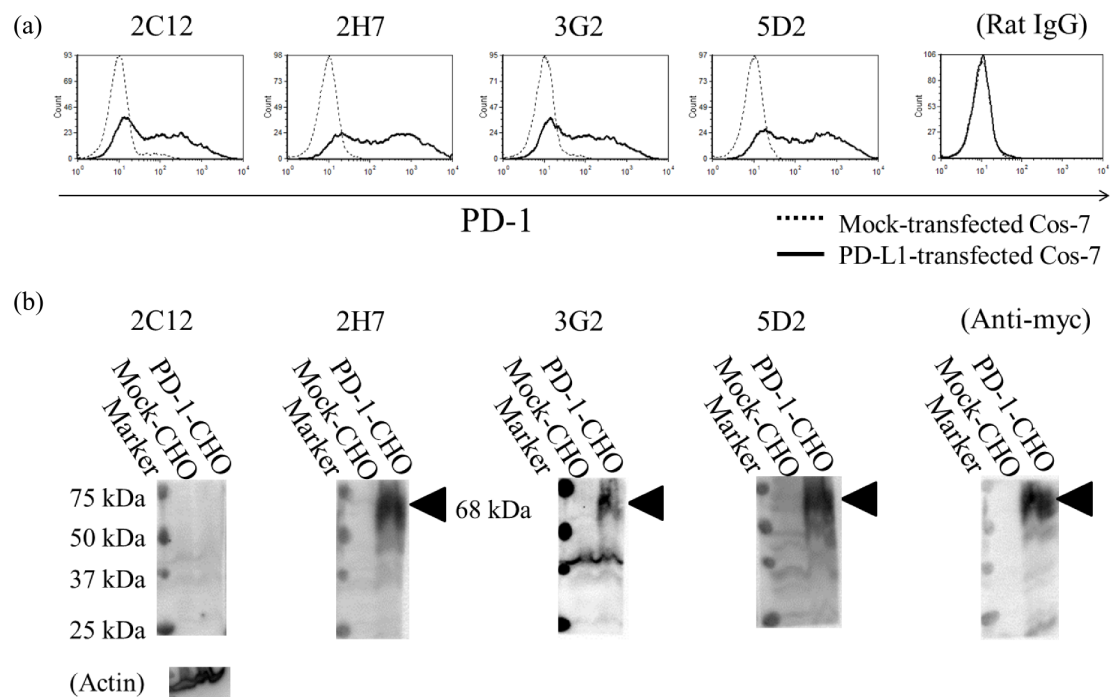


Figure III-1. Recognition of PD-1-expressing cells by anti-PD-1 mAbs.

(a) Flow cytometric analysis of surface expression of bovine PD-1. Cos-7 expressing PD-1 (black line) and Cos-7 transfected with the control vector (dashed line) were stained with four types of anti-PD-1 mAbs (2C12, 2H7, 3G2, and 5D2) and isotype control (Rat IgG). (b) Western blot analysis of bovine PD-1 expression in CHO-DG44 cells stably expressing bovine PD-1 with myc-tag. Three types of anti-PD-1 mAbs recognized PD-1 (triangle) at about 68 kDa. Anti-actin antibody and anti-myc antibody were used as a loading control and a positive control.

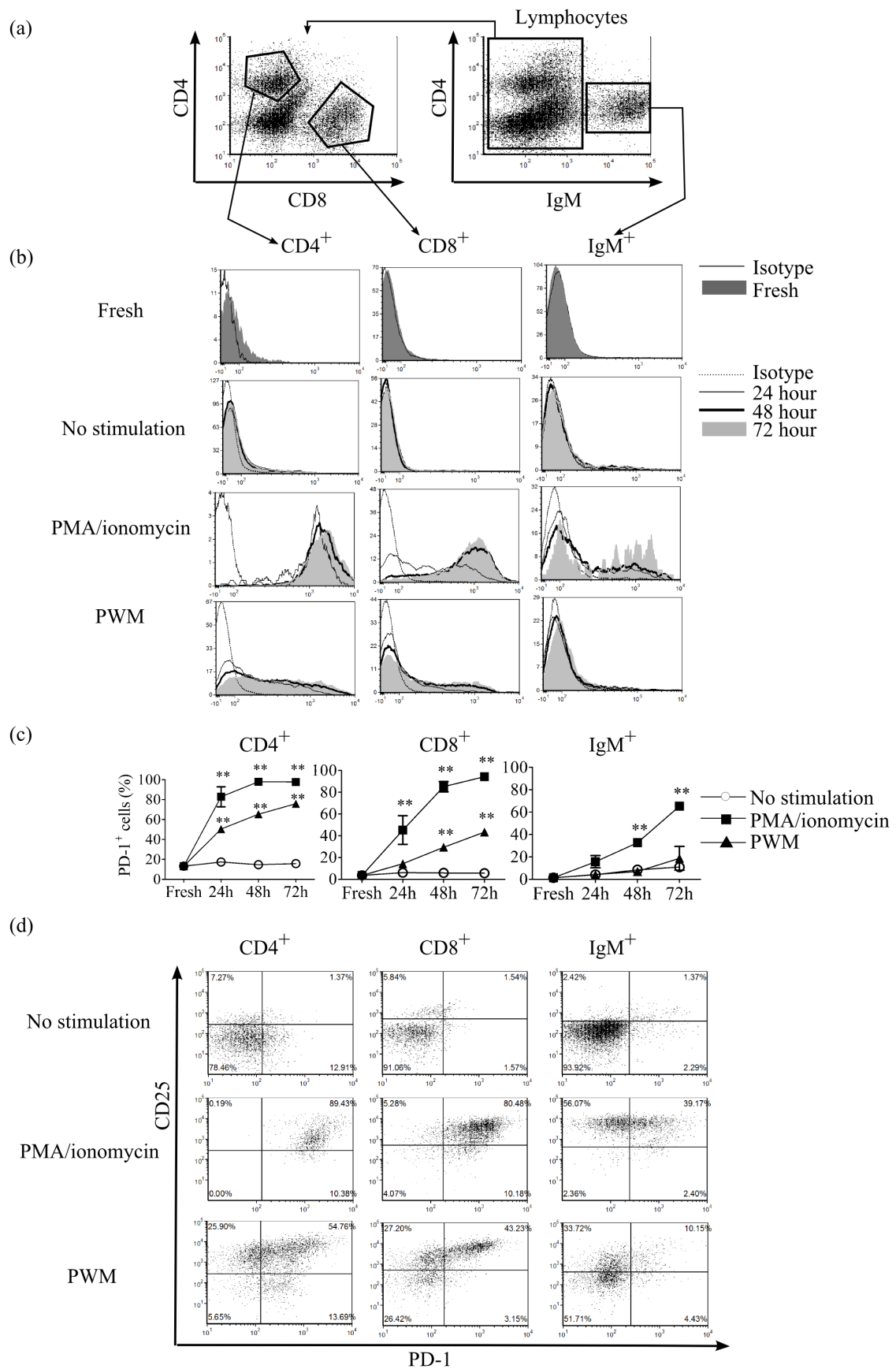


Figure III-2. Recognition of PD-1-expressing lymphocytes by anti-PD-1 mAb.

(a) Gating strategies for CD4⁺ T cells, CD8⁺ T cells and IgM⁺ B cells in bovine lymphocytes. (b) Representative histograms obtained by flow cytometry of PD-1 expression in CD4⁺ T cells, CD8⁺ T cells and IgM⁺ B cells isolated from three BLV⁻ cattle. Freshly isolated PBMCs were stained with anti-PD-1 (5D2), CD4, CD8, and IgM mAb. PBMCs were cultivated with PBS (No stimulation), PMA/ionomycin, and PWM for 24, 48, and 72 h, and stained in a similar way. (c) Proportions of PD-1 positive cells in CD4⁺ T cells, CD8⁺ T cells and IgM⁺ B cells. Statistical comparisons between percentages of PD-1 positive cells stimulated with PBS and PMA/ionomycin or PWM were made using the two-way ANOVA. Differences were considered statistically significant at $P < 0.05$ (** $P < 0.01$). (d) Representative dot plots of PD-1 and CD25 expression in CD4⁺ T cells, CD8⁺ T cells and IgM⁺ B cells 48h after cultivation. Values in the quadrant indicate the percentage of the cells in lymphocytes.

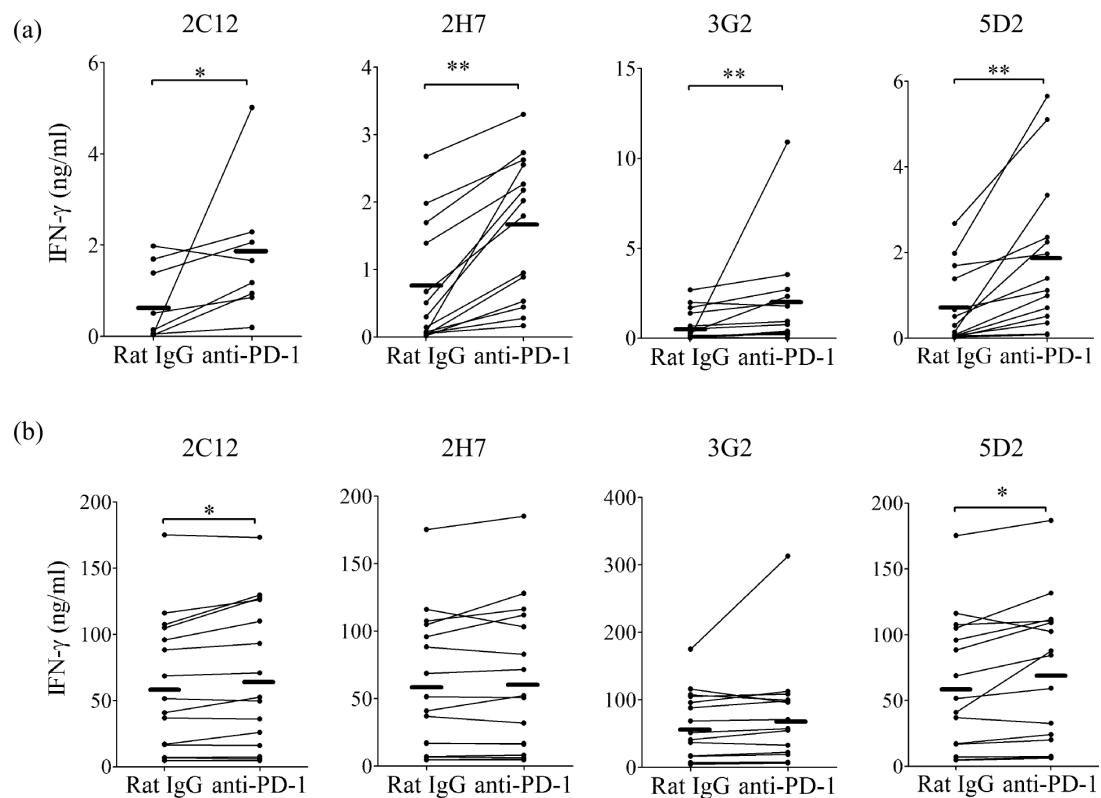


Figure III-3. Enhancement of cytokine production by anti-PD-1 mAbs in bovine lymphocytes PBMCs were cultivated with rat IgG control or four types of anti-PD-1 mAbs (20 μ g/ml) in the absence (a; $n = 8$ or 14) or presence (b; $n = 16$) of PMA/ionomycin. IFN- γ production was measured by ELISA. Each of line indicates the mean percentages in each group. Statistical comparisons between rat IgG control and anti-PD-1 mAb were made using the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

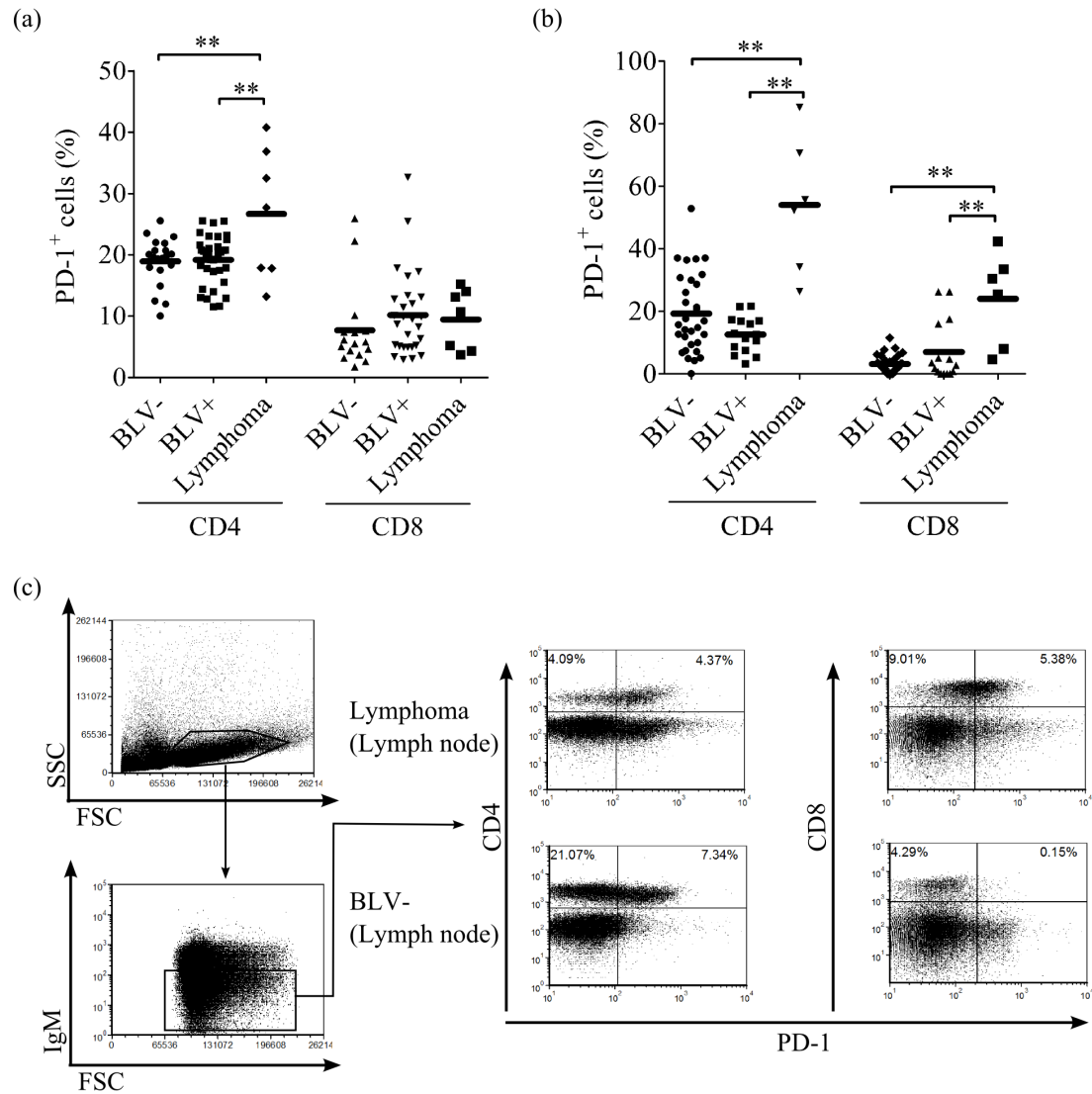


Figure III-4. Expression analysis of bovine PD-1 in BLV⁺ cattle.

(a, b) Percentages of PD-1-expressing CD4⁺ and CD8⁺ T cells in PBMCs (a) and LN (b) isolated from BLV⁻ cattle (CD4⁺ T cells; $n = 20$ and 31 , CD8⁺ T cells; $n = 16$ and 31), BLV⁺ cattle without lymphoma (CD4⁺ T cells; $n = 35$ and 15 , CD8⁺ T cells; $n = 28$ and 15) and BCBL (CD4⁺ T cells; $n = 7$ and 6 , CD8⁺ T cells; $n = 7$ and 6). (c) An example of gating strategies for PD-1 expression in CD4⁺ T cells and CD8⁺ T cells isolated from LN of BCBL and BLV⁻ cattle. Each of line indicates the mean percentages in each group. Values in the quadrant indicate the percentage of the cells in lymphocytes other than IgM⁺ B cells. Statistical comparisons were made using the one-way ANOVA with Tukey's test. Differences were considered statistically significant at $P < 0.05$ (** $P < 0.01$).

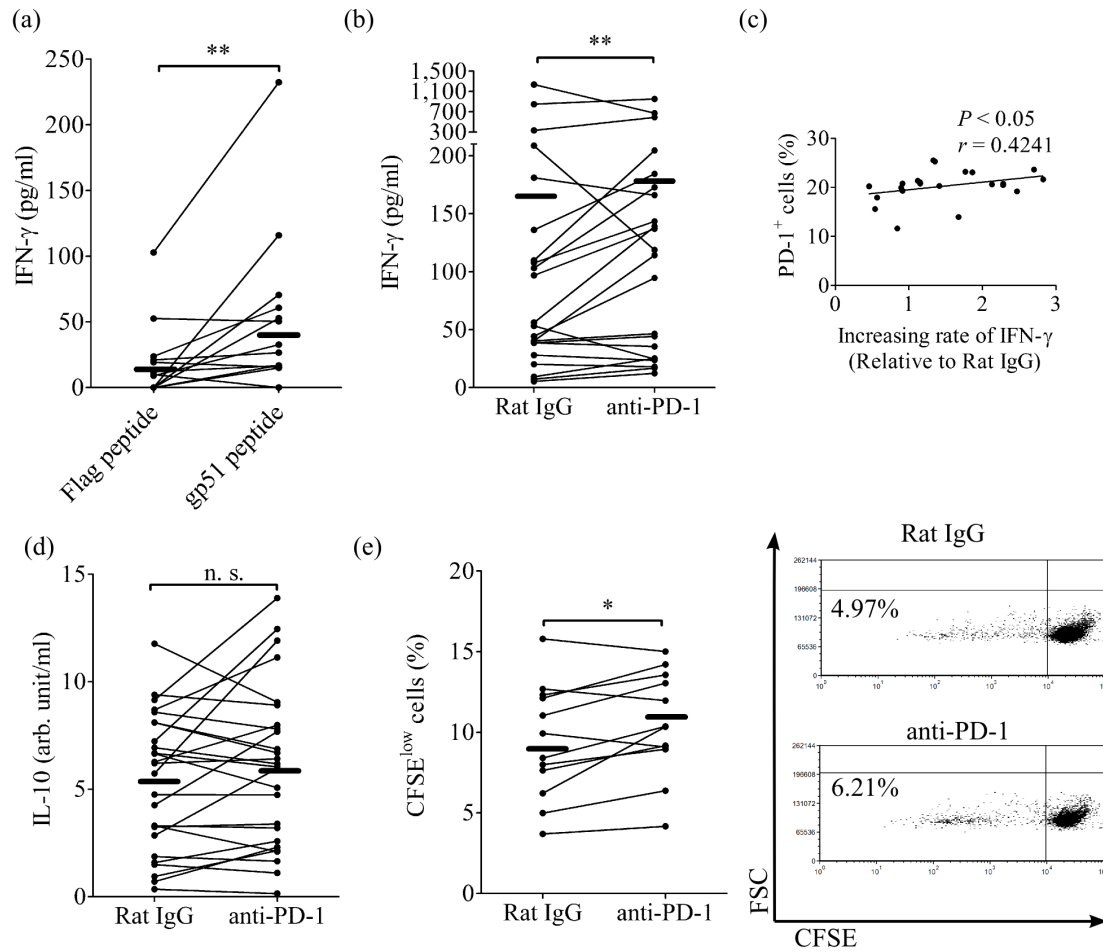


Figure III-5. Effect of PD-1 blockade on T cell function.

(a) PBMCs from BLV⁺ cattle were cultured with Flag peptide as negative control and gp51 peptide mixture. IFN- γ production was measured by ELISA ($n = 13$). (b, c, d) PBMCs were cultured with rat IgG control or anti-PD-1 mAb (5D2; 20 μ g/ml) in the presence of gp51 peptide mixture. Each IFN- γ and IL-10 production was measured by ELISA (b; $n = 22$, d; $n = 26$). Correlation statistics between increasing rate of IFN- γ production and percentages of PD-1⁺ cells in CD4⁺ T cells corresponding to Figure III-4a were analyzed using the Spearman correlation (c; $n = 22$). (e) The proliferative responses were evaluated by detection of CFSE^{low} cells in IgM⁻ lymphocytes by flow cytometry ($n = 12$). Representative dot plots of CFSE-staining in lymphocytes other than B cells are shown. Values in the quadrant indicate the percentage of the cells in CFSE^{low} cells in IgM⁻ lymphocytes. Each of line indicates the mean percentages in each group. Statistical comparisons between rat IgG control and anti-PD-1 mAb were made using the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

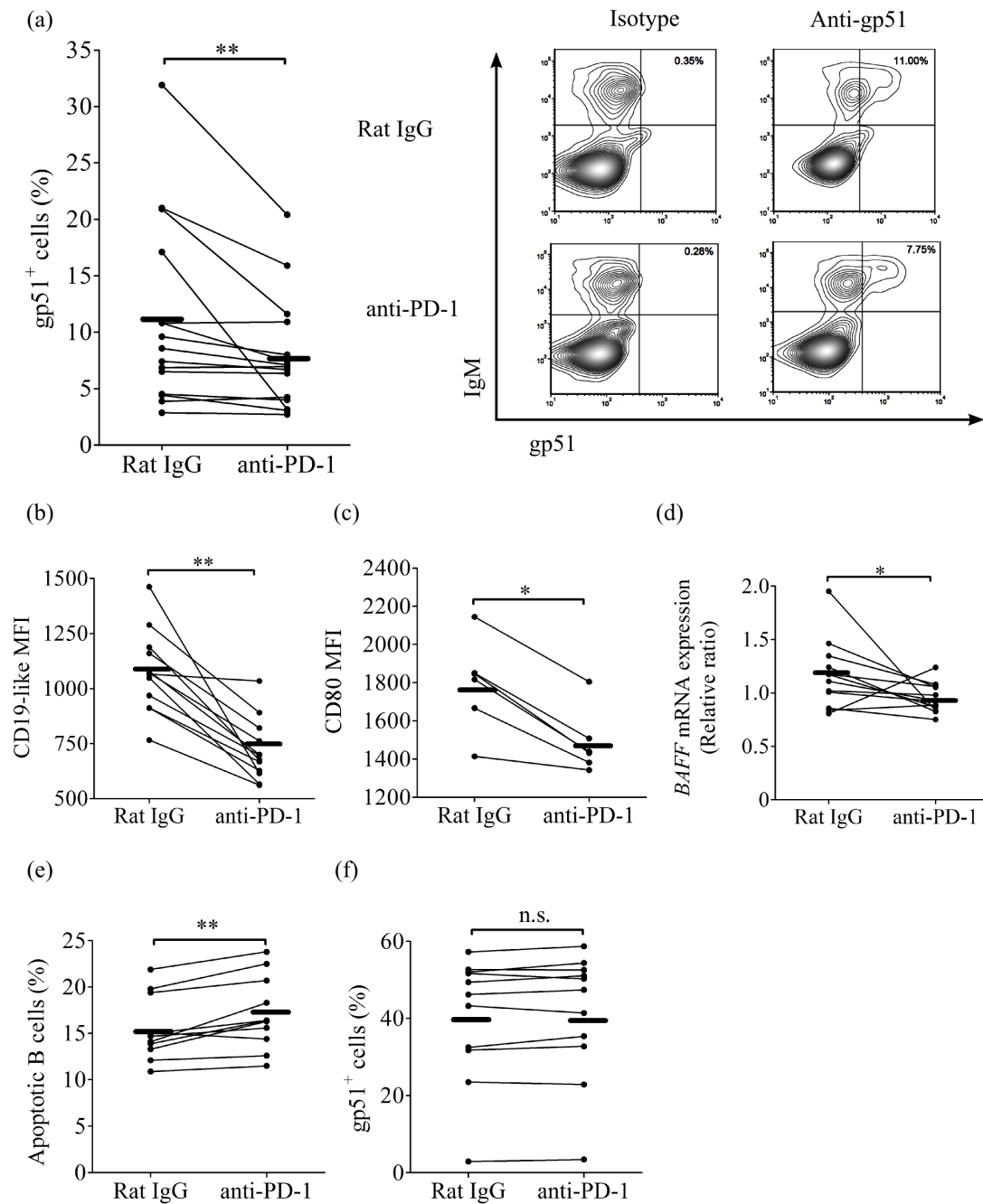


Figure III-6. Effect of PD-1 blockade on gp51 expression and B cell activation.

(a, b, c) Percentages of gp51-expressing cells (a; $n = 15$) and mean fluorescence index (MFI) of WC4 (CD19 like molecule) (b; $n = 12$) and CD80 (c; $n = 6$) in IgM⁺ B cells were evaluated by flow cytometry in PBMCs treated with rat IgG control or anti-PD-1 mAb (20 μ g/ml) in the presence of gp51 peptide mixture. Representative contour plots showing gp51 expression (right panels) in PBMCs treated with rat IgG control (upper panels) or anti-PD-1 mAb (lower panels) was showed in (a). No staining was observed in PBMCs stained with isotype control for anti-gp51 mAb (left panels). Values in the quadrant indicate the percentage of IgM⁺ gp51⁺

cells in lymphocytes. (d) Expression of *BAFF* mRNA was evaluated by real-time PCR ($n = 12$). The results were indicated as relative change to control (no antibody treatment) when the amount of *BAFF* mRNA expression was divided by *GAPDH* mRNA expression. (e) Percentages of apoptotic cells in IgM⁺ B cells were measured by flow cytometry. Apoptotic B cells were identified as annexin-V⁺ 7-AAD⁻ cells ($n = 11$). (f) Percentages of gp51-expressing cells were evaluated by flow cytometry in isolated B cells cultivated with rat IgG control or anti-PD-1 mAb ($n = 11$). Each of line indicates the mean percentages in each group. Statistical comparisons between rat IgG control and anti-PD-1 mAb were made using the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

DISCUSSION

PD-1 on lymphocytes is thought to be a major immunoinhibitory receptor involved in the maintenance of peripheral immune tolerance and immune evasion of tumors and infectious agents [Keir et al., 2008]. The expression of *PD-1* mRNA was previously analyzed in some animal species by real-time PCR because of lack of specific antibodies [Folkl et al., 2010; Matsuyama-Kato et al., 2012]. However, although the real-time PCR method can be used for quantitative analysis, the method targeting PD-1 requires attention because several splice variants and homologs of the human and mouse *PD-1* gene have been reported [Flies et al., 2011; Nielsen et al., 2005; Wan et al., 2006]. *PD-1* mRNA transcripts, in which the third exon of the *PD-1* gene encoding the transmembrane domain was spliced out, encoded a soluble form of PD-1 (sPD-1) [Nielsen et al., 2005], and sPD-1 blocked the immunoinhibitory effect of PD-1 expressed on the T cell membrane [Wan et al., 2006]. Thus, the primer set must be designed carefully to amplify only the membrane-bound form but not the soluble form or other splice variants of *PD-1* mRNA. Expression analysis of membrane-bound PD-1 antigen by flow cytometry is essential to investigate the exact immunoinhibitory effect of PD-1 in immune cells. In this study, anti-bovine PD-1 mAb was successfully prepared, and it was shown that the frequency of PD-1⁺ T cells was increased in BCBL. In addition, IFN- γ production and cellular proliferation were upregulated and BLV-gp51 expression and B cell activation were reduced in PBMCs from BLV⁺ cattle by the treatment with anti-PD-1 mAb.

Molecular weight of both human PD-1 and bovine PD-1 predicted by calculation from their amino acid sequences was approximately 30 kDa. However, the band of human PD-1 was found at about 55kDa in Western blotting [Agata et al., 1996]. In addition, in this study, bovine PD-1 was detected at about 68kDa. From these observations, human PD-1 and bovine PD-1 are thought to be heavily glycosylated, which is consistent with the potential *N*-glycosylation sites of human and bovine PD-1.

CD4⁺ and CD8⁺ T cells are the main targets of study when investigating immunoinhibition induced by PD-1 in human and mouse models. In cattle, CD4⁺ T cells were the main cells that express PD-1 in freshly isolated PBMCs, but the expression levels were not so high. In human and mice, PD-1 expression levels in total CD4⁺ and CD8⁺ T cells were also very low, even when detected by sensitive

instruments, such as flow cytometry [Agata et al., 1996; Shimauchi et al., 2007]. T cells were divided into naive and different memory subsets by the expression of CD45RO, CD45RA and CCR7, and the memory T cells expressed high levels of PD-1 [Rosignoli et al., 2009]. In this study, PD-1 expression in total T cells or B cells was investigated, but detailed expression analysis using many cell markers is required to clarify whether PD-1⁺ CD4⁺ T cells are the memory T cells or other phenotype.

Activated T and B cells expressed PD-1 antigen, perhaps, for the sake of preventing activation-induced cell death [Agata et al., 1996]. In this chapter, it was confirmed that PD-1 expression in CD4⁺ and CD8⁺ T cells and B cells was strongly enhanced by PMA/ionomycin treatment. Moreover, almost all of activated CD4⁺ T cells determined by the expression of CD25, which is the classical activation marker, strongly expressed PD-1 at 48 h after the stimulation. However, PD-1 upregulation was also confirmed in CD4⁺ CD25⁻ T cells and was not observed in all of CD8⁺ CD25⁺ T cells and IgM⁺ CD25⁺ B cells. These data suggested that the cell activation followed by CD25 expression was not the single cause of PD-1 upregulation. Further investigation is needed to clarify the kinetics of the induction of PD-1 expression in each cell population.

In chronic infection or tumors, production of inflammatory cytokines and proliferation in response to antigens by pathogen-specific CD4⁺ or CD8⁺ T cells were impaired by the PD-1/PD-L1 pathway. These T cells failed to eradicate the infected cells or cancer cells, and PD-1 was the most appropriate marker of these “exhausted” T cells [Matsuzaki et al., 2010; Said et al., 2010]. In this study, BLV infection was chosen as typical chronic infection and tumor in cattle for PD-1 expression and functional analysis. BLV and HTLV-1 are the member of deltaretroviruses, and higher percentages of PD-1 expression were observed in CD4⁺ T cells from blood of HTLV-1-infected patients with adult T cell leukemia [Shimauchi et al., 2007]. The frequencies of PD-1⁺ cells were also higher in CD4⁺ T cells in blood and both CD4⁺ and CD8⁺ T cells in tumor-bearing LN from BCBL than mesenteric LN from BLV⁺ and BVL⁻ cattle. These data suggested that anergic T cells, which may be specific for lymphoma cells were increased in total CD4⁺ or CD8⁺ T cells in BCBL. Moreover, the frequency of PD-1⁺ T cells is higher in tumor-bearing LN than peripheral blood in BCBL, and the same tendency was reported in patients with metastatic melanoma [Ahmadzadeh et al., 2009]. Immune tolerance by PD-1 expression in T cells follows T cell activation by continued epitope recognition in peripheral lymphoid tissue [Blattman

et al., 2009; Fife et al., 2008; Richter et al., 2009; Streeck et al., 2008; Tsushima et al., 2007]. Thus, one can speculate that most of tumor-infiltrating PD-1⁺ T cells could be specific for lymphoma cells, and be presented with tumor-associated antigen, but fail to be activated properly by the PD-1/PD-L1 pathway, resulting in the immune evasion of BLV-induced lymphoma cells.

To verify the relationship between PD-1 expression in T cells and antigen-specific immunosuppression, PD-1 expression analysis in antigen-specific T cells is required. Indeed, PD-1 upregulation was mainly observed in CD8⁺ T cells specific for pathogens that cause chronic infection and tumors, such as HIV and HTLV-1 infection and mesenteric melanoma [Ahmadzadeh et al., 2009; Kozako et al., 2009; Trautmann et al., 2006]. In this study, PD-1 upregulation in total CD4⁺ and CD8⁺ T cells from BLV⁺ cattle without lymphoma was not observed and PD-1 expression in BLV-specific T cells was not examined. The detection of BLV-specific T cells would enable more detailed analysis of PD-1, although the divergence of genetic background of cattle complicates the establishment of MHC-tetramer which is an essential tool for the detection. BLV⁺ B cells were not completely eradicated by immune system and proliferated in lymphoid tissue *in vivo* [Debacq et al., 2006], so that BLV-specific T cells from BLV⁺ cattle without lymphoma would recognize antigen continuously, express high PD-1, and lapse into anergic state by PD-L1 expression in BLV⁺ B cells.

In BLV⁺ cattle with advanced stage of the disease, proliferation of CD4⁺ T cells in response to BLV proteins, such as gag and env, was impaired [Orlik et al., 1996]. In this study, PD-1 blockade enhanced the IFN- γ production in PBMCs in response to gp51 peptide mixture. Moreover, the increasing rate of IFN- γ production was correlated with the percentages of PD-1⁺ cells in CD4⁺ T cells, suggesting that PD-1 blockade invigorated the function of PD-1⁺ CD4⁺ T cells, perhaps gp51-specific T cells. Meanwhile, IL-10 production was not altered in PBMCs by the treatment with anti-PD-1 mAb. Blockade of the PD-1/PD-L1 pathway seems not to upregulate all function of PD-1⁺ T cells.

In addition to T cell activation, PD-1 blockade resulted in the inhibition of gp51 expression, reduced the expression of activation marker of B cells, WC4 and CD80 and increased B cell apoptosis. Although the mechanism of BLV-gp51 expression both *in vitro* cultivation and *in vivo* is not dissolved, B cell activation by immune-mediated stimulation is known as the important factor for activation of viral protein synthesis [Gillet et al., 2007]. This study did not demonstrate that reactivated T cells by

anti-PD-1 mAb have a direct effect on the B cell activity. However, anti-PD-1 mAb did not directly affect B cells, because recognizable PD-1 expression was not observed in B cells and PD-1 treatment of isolated B cells did not alter gp51 expression. Changes in cytokine environment, such as reduction of BAFF or activation of cytotoxic T cells induced by PD-1 blockade could create disadvantageous environment for B cells, subsequently resulting in the reduction of gp51, WC4 and CD80 expression and increased B cell apoptosis. Further investigation about gp51 expression in B cells is described in Chapter IV.

PD-1 is expected to be a potential target for reinvigorating the function of exhausted T cells. Many researchers have investigated antibody treatment that blocks the PD-1/PD-L1 pathway [Hirano et al., 2005; Trautmann et al., 2006; Velu et al., 2009], and clinical trials in patients with cancer who were administered anti-PD-1 antibody are now ongoing [Berger et al., 2008; Brahmer et al., 2010]. Anti-bovine PD-1 mAbs augmented IFN- γ production in PBMCs from BLV⁻ cattle, indicating that the immune reactivation by PD-1 blockade was not a limited phenomenon in BLV infection. Although it is unknown whether PD-1⁺ T cells in BLV⁻ cattle are specific for some pathogens, there is the possibility that the treatment with anti-PD-1 mAb could be applied to new target of therapy for many types of infection in cattle via upregulation of immune responses. Moreover, this mAb could promote research regarding bovine immunology and clarify the mechanisms of the immunosuppression in various refractory diseases.

SUMMARY

PD-1 is a known immunoinhibitory receptor that contributes to immune evasion of various tumor cells and pathogens causing chronic infection, such as BLV infection. First, to establish a method for the expression and functional analysis of bovine PD-1, hybridomas producing mAb specific for bovine PD-1 were established. Treatment with these anti-PD-1 mAbs enhanced IFN- γ production in bovine PBMCs. Next, to examine whether PD-1 blockade by anti-PD-1 mAb could upregulate the immune reaction during chronic infection, the expression and functional analysis of PD-1 in PBMCs isolated from BLV⁺ cattle with or without lymphoma were performed using anti-PD-1 mAb. The frequencies of both PD-1⁺ CD4⁺ T cells in blood and LN and PD-1⁺ CD8⁺ T cells in LN were higher in BLV⁺ cattle with lymphoma than those without lymphoma or control uninfected cattle. PD-1 blockade enhanced IFN- γ production and cell proliferation and reduced BLV-gp51 expression and B cell activation in PBMCs from BLV⁺ cattle in response to BLV-gp51 peptide mixture. These data showed that anti-bovine PD-1 mAb could provide a new therapy to control BLV infection via upregulation of immune response.

CHAPTER IV

Viral Expression and Functional Analysis of IgM^{high} and IgM^{low} B Cells Isolated from Bovine Leukemia Virus-Infected Cattle

The original paper of this part will be submitted for publication, and thus can not be shown at the time the thesis has been submitted to Hokkaido University.

ABSTRACT

Bovine leukemia virus (BLV) causes lymphocytosis and BL in BLV⁺ cattle, and the BLV genome is inserted into the host genome as a provirus. BLV-infected bovine B cells barely express viral antigens *in vivo*, but the expression is upregulated in a part of BLV⁺ B cells by *ex vivo* cultivation. This observation suggests that BLV⁺ cells can be divided into BLV-silencing cells and -expressing cells, but the machinery for viral expression in B cells is still unknown. To identify the markers distinguishing BLV-silencing from -expressing cells, expression analyses of sIgM and BLV antigen were performed in B cells from BLV⁺ cattle. The sIgM expression was upregulated in BLV⁺ cattle as the clinical disease progressed, and IgM^{high} B cells were increased in number within blood lymphocytes from BLV⁺ cattle. BLV antigens, gp51 and BLV-p24, were mainly expressed in IgM^{high} B cells as compared to IgM^{low} B cells, although both of the subsets showed the similar provirus load. To clarify the cellular behavior of the two subsets, several parameters were measured. The ratio of PD-L1⁺ cells which inhibits T cell immune reaction, were higher in IgM^{low} as compared to IgM^{high} B cells. Higher expression of viral and endogenous oncogenes, *Tax/Rex*, *Maf*, *Jun* and *Fos* was observed in IgM^{low} than IgM^{high} B cells, determined by real-time PCR and microarray analyses. Moreover, IgM^{low/-} BL cells, not IgM^{high} BL cells were observed within enlarged LN in all fourteen BCBL observed in the field. These data suggest that IgM^{high} B cells are the main subset of BLV-expressing cells in blood from BLV⁺ cattle, whereas IgM^{low} B cells, which include a high proportion of BLV-silencing B cells, are superior in terms of immune evasion and could produce neoplastic B cells.

CONCLUSION

Bovine leukemia virus (BLV) induces B cell lymphoma named bovine leukosis in a subset of BLV-infected (BLV⁺) cattle. In BLV⁺ cattle before the development of bovine leukosis, abnormally proliferating B cells induce immunoinhibition, resulting in economic loss and disease progression. No effective vaccine has been developed against BLV although the number of BLV⁺ cattle is increased. In this study, it is hypothesized that BLV-induced immunoinhibition is induced by the programmed-death-1 (PD-1) and PD-ligand-1 (PD-L1) system which is involved in the exhaustion of immune cells. To develop a novel therapy for BLV infection, expressions and function of PD-1 and PD-L1 were analyzed in BLV⁺ cattle. In addition, to clarify the mechanism of BLV antigen expression and the development of leukosis, a marker discriminating BLV-expressing from -silencing cells was identified and cellular functions of both of the B cell subsets were investigated in BLV⁺ cattle.

CHAPTER I: PD-L1 expression in B cells was investigated in BLV⁺ cattle by flow cytometry. Previous reports showed that PD-L1 was involved in immune evasion of infectious agents and tumors. In BLV infection, high frequency of PD-L1⁺ B cells was observed in blood collected from BLV⁺ cattle at the late stage of the infection and cattle with bovine leukosis. The increase in the proportions of PD-L1⁺ B cells in lymphocytes was positively correlated with disease progression and negatively with *interferon-gamma* (IFN- γ) mRNA expression.

CHAPTER II: The effect of PD-L1 cross-linking on cellular death was investigated using PD-L1-expressing cell lines and bovine lymphocytes. Inhibition of the interaction between PD-1 and PD-L1 by specific antibodies or recombinant proteins is known to reactivate various functions of exhausted T cells. The treatments with anti-PD-1 or anti-PD-L1 antibody are now undergoing on the clinical application for refractory infection and cancer. However, some researchers reported that cross-linking of PD-L1 induced an inhibitory signal into PD-L1⁺ cells, although details on the mechanism are unknown. In Chapter II, PD-1-Ig, which is the recombinant soluble bovine PD-1 fused with bovine IgG Fc, and anti-bovine PD-L1 mAb were used to analyze the influence of PD-L1 cross-linking on cellular functions. The treatment of PD-1-Ig or anti-PD-L1 mAb led to increased frequency of dead cells in PD-L1^{high} cells among PD-L1-expressing cell lines. When bovine lymphocytes were incubated with

anti-PD-L1 mAb, cytokine production was augmented. Meanwhile, PD-1-Ig treatment upregulated the population of dead cells in PD-L1⁺ B cells, and reduced both cytokine production and cell proliferation in bovine lymphocytes.

CHAPTER III: Anti-bovine PD-1 mAb was established to investigate PD-1 expression and the effect of PD-1 blockade on T cell re-activation in BLV infection. PD-1 is expressed on the surface of T cells and interacts with PD-L1, resulting in T cell exhaustion. The high level of PD-1 expression is observed in pathogen-specific T cells in chronic infection and tumor. In this chapter, it was observed that high frequency of T cells expressed PD-1 in BLV⁺ cattle with bovine leukosis. *In vitro* PD-1 blockade by anti-PD-1 mAb increased IFN- γ production and cell proliferation, whereas decreased B cell activation and expression of BLV-gp51 in lymphocytes from BLV⁺ cattle.

CHAPTER IV: The differences in BLV antigen expressions, gene expression profiles and cellular behavior were investigated in IgM^{high} and IgM^{low} B cells isolated from BLV⁺ cattle. BLV particles and antigens are rarely detected in freshly isolated lymphocytes from BLV⁺ cattle, although, viral genome is inserted into host genome as provirus. Meanwhile, the subpopulation of B cells can express BLV antigens after *ex vivo* cultivation, although the molecular mechanism for this expression remains unknown. In this chapter, it was observed that IgM^{high} B cells were increased in number in blood from BLV⁺ cattle, and were prone to express BLV antigens as compared to IgM^{low} B cells. To identify the cellular behavior of IgM^{high} and IgM^{low} B cells, several parameters in these two subsets were investigated. IgM^{low} B cells showed higher expression levels of the *Tax/Rex* mRNA, PD-L1 and some proto oncogenes (e. g. *Maf*, *Jun* and *Fos*). These data indicated that IgM^{low} B cells are superior to evade from immune surveillance system and to become neoplastic clones.

In conclusion, this study suggests that IgM^{high} B cells are prone to express BLV antigens and IgM^{low} B cells contribute to the immune evasion and the production of neoplastic clones. This observation is valuable to clarify the mechanisms of BLV expansion *in vivo* and transformation by BLV. In addition, this study also clarified that the PD-1/PD-L1 pathway is involved in BLV-induced immunosuppression and disease progression. Both anti-bovine PD-1 and anti-bovine PD-L1 mAbs established in this study are suitable drug candidates to reactivate immune function in BLV⁺ cattle.

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SUMMARY IN JAPANESE

和文要旨

ウシ白血病ウイルス (BLV) は、感染牛の一部に B 細胞性リンパ腫である牛白血病を引き起こす。また、一部の BLV 感染牛では B 細胞が異常に増殖するリンパ球増多症を呈し、免疫抑制状態に陥ることが示唆されており、このことが BLV 感染牛における生産効率の低下や病態進行の原因であると考えられている。本疾病による経済的損害が大きいことより、防御法や治療法の確立が待たれているが、現在有効なワクチンまたは治療法は存在しない。本研究では、BLV 感染牛における免疫抑制状態の原因が免疫担当細胞の疲弊化に関与する免疫抑制受容体 programmed death-1 (PD-1) とそのリガンド PD-ligand 1 (PD-L1) であると予測し、新規治療法の開発に向けて種々の解析を行った。また、未解明である BLV 抗原発現機構を解明するために、BLV 抗原発現 B 細胞と未発現 B 細胞の機能解析を行った。

第 1 章：様々なウイルス感染症や腫瘍疾患において、PD-L1 の発現増加が病原体の免疫回避機構の一つであることが明らかにされている。BLV 感染牛における PD-L1 発現をフローサイトメトリー法にて解析を行った結果、病態が進行した BLV 感染牛および牛白血病発症牛の末梢血において、PD-L1⁺ B 細胞の増加が認められ、その増加は病態進行と正の相関を、*Interferon-gamma* (IFN- γ) mRNA の発現量と負の相関を示した。

第 2 章：PD-1 と PD-L1 の結合を抗体や組換え体により阻害することで、疲弊化 T 細胞の再活性化を促すことが知られており、本抗体を難治性感染症や腫瘍疾患の治療法へ応用する研究が盛んに行われている。しかし、PD-L1 は抗体により架橋されることで抑制性シグナルを発生する可能性が示唆されているが、その詳細は不明である。そこで、PD-1 にウシ IgG の Fc 部位を融合させた PD-1-Ig および抗ウシ PD-L1 モノクローナル抗体を作製し、これらの存在下で PD-L1 発現細胞を培養した場合の細胞死誘導、サイトカイン産生能及び細胞増殖への影響を検討した。PD-1-Ig あるいは抗 PD-L1 抗体存在下で、PD-L1 過剰発現細胞株を培養した場合には、PD-L1 発現細胞に死細胞の増加が認められた。リンパ球を培養した場合では、抗 PD-L1 抗体の添加によりサイトカイン産生の増加が認められたが、一方で PD-1-Ig の添加により PD-L1 陽性 B 細胞の細胞死が誘導され、さらにサイトカイン産生や細胞増殖能の低下が確認された。

第 3 章：PD-1 は T 細胞表面に発現しており、PD-L1 が結合することで T 細胞の疲弊化を引き起こす。慢性感染を引き起こす微生物や腫瘍の抗原に特異的な T 細胞において、PD-1 の発現が増加することが明らかにされている。そこで、抗ウシ PD-1 モノクローナル抗体を作製し、BLV 感染牛における PD-1 発現量を測定して、さらに抗ウシ PD-1 抗

体存在下での免疫応答の変化を検討した。その結果、牛白血病発症牛の T 細胞において高い PD-1 発現が認められた。また、抗 PD-1 抗体添加により、リンパ球の IFN- γ の産生量や増殖能が増加し、B 細胞の活性やウイルス抗原の発現量が低下した。

第 4 章：BLV は B 細胞に感染すると宿主ゲノムにプロウイルスとして組み込まれるが、感染牛のリンパ球や血清中に BLV 粒子及び抗原はほとんど検出されない。一方、感染牛由来リンパ球を数時間培養すると、一部の B 細胞が BLV 抗原を発現するが、その詳細な分子機構は不明である。そこで、BLV 感染 B 細胞を用いて詳細に解析した結果、病態の進行に伴い増加する IgM^{high} B 細胞は、IgM^{low} B 細胞よりも BLV 抗原を発現しやすい細胞群であることが明らかとなった。これら両細胞群は同様のプロウイルス感染率を示した。次に、IgM^{high} B 細胞と IgM^{low} B 細胞の機能解析及びマイクロアレイによる発現遺伝子の網羅的解析を行った。その結果、IgM^{low} B 細胞では、T 細胞性免疫を抑制する PD-L1、ウイルス由来癌遺伝子 *Tax/Rex* mRNA 及び数種類の前癌遺伝子の発現が高かった。また、14 頭の BLV 感染牛由来 B 細胞リンパ腫の主要な細胞群は、IgM^{low} または IgM^{high} B 細胞であり、IgM^{high} B 細胞はほとんど存在しなかった。

本研究により、IgM^{high} B 細胞は BLV 抗原をよく発現する細胞群、IgM^{low} B 細胞は抗原を発現しにくいと同時に腫瘍細胞を発生する可能性が高い細胞群であることが示された。今後、この結果を用いた BLV の体内伝播機構や牛白血病発症機構の解明が期待される。また、PD-1/PD-L1 が BLV 感染症に起因する免疫抑制及び病態進行に関与していることが示され、新たに作製した抗 PD-1 抗体及び抗 PD-L1 抗体が BLV 感染牛の免疫抑制を再活性化することが示唆された。今後、本抗体を基にした BLV 感染症に対する新規治療法やワクチンの開発が期待される。