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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CHAPTER IV.

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

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 $CD8^+$ effecter 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 μg/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/μl 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 permealized 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.

$PD\text{-}L1^+$ cells were increased in lymphocytes isolated from PL cattle and BCBL

To evaluate PD-L1 expression in BLV^+ animals, the percentages of $PD\text{-}L1^+$ 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 $PD\text{-}L1^+$ 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 $PD\text{-}L1^+$ cells was significantly increased in BCBL compared to BLV^- cattle. No difference in the mean percentage of $PD\text{-}L1^+$ 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.

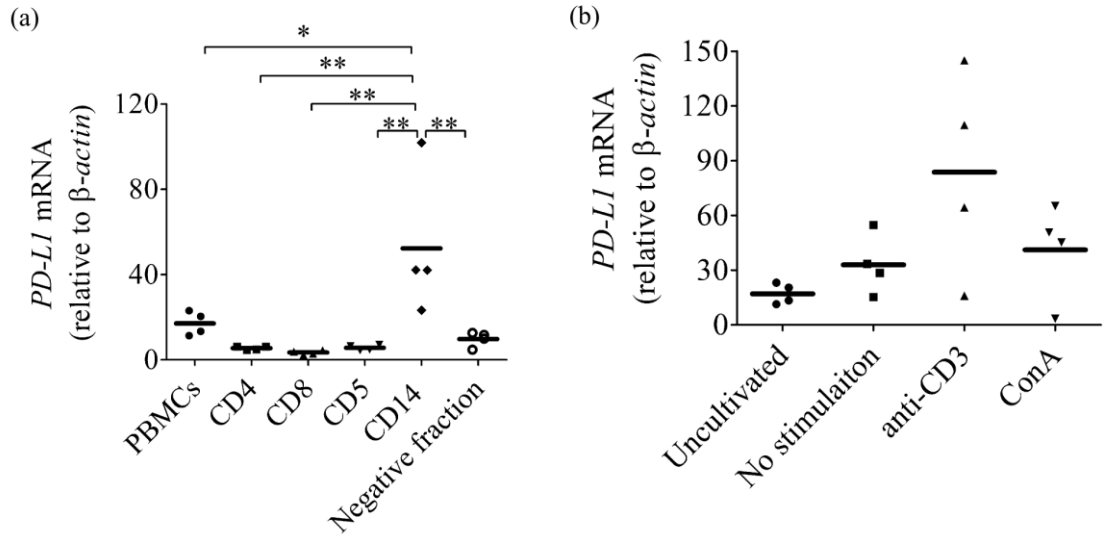

Figure I-1. Analysis of bovine *PD-L1* mRNA expression by real-time PCR.
(a) *PD-L1* mRNA expressions were determined in total PBMCs from healthy Holstein cattle, subpopulations of $CD4^+$, $CD8^+$, $CD5^+$, $CD14^+$ cells and negative fraction for these subpopulations (n = 4). (b) *PD-L1* mRNA expression 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 $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

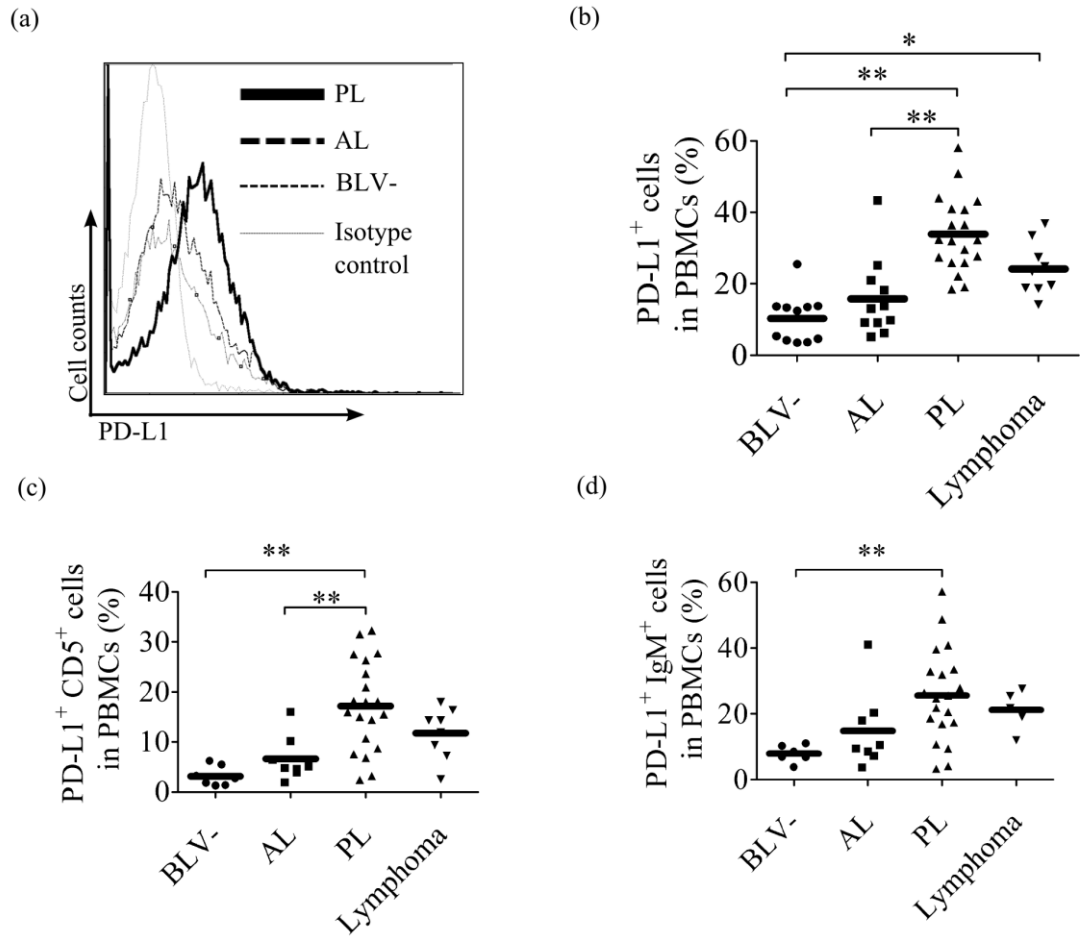

Figure I-2. Flow cytometric analysis of bovine PD-L1 expression in BLV^+ cattle at different disease stages.

(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 $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

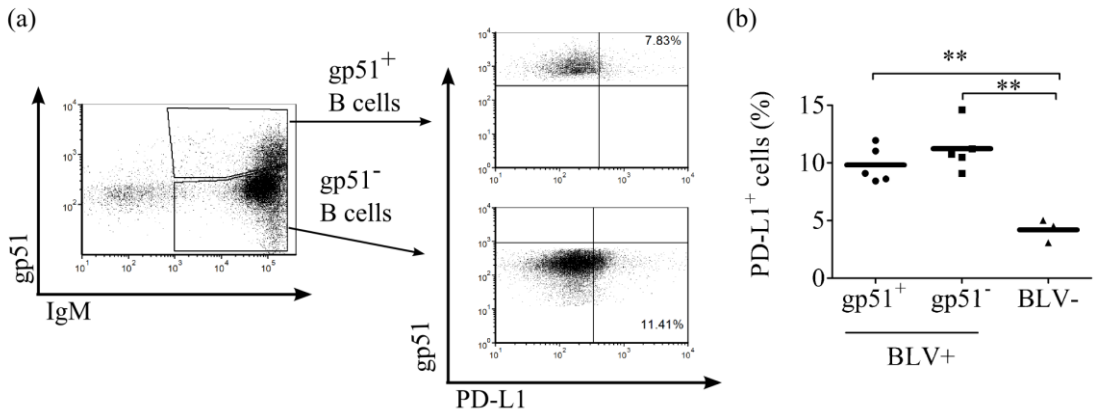

Figure I-3. PD-L1 expression in BLV antigen-expressing B cells.
(a) An example of expression of BLV-gp51 and PD-L1 in cultivated lymphocytes. Values in the quadrant indicate the percentage of PD-L1$^+$ cells in lymphocytes. (b) Percentages of PD-L1-expressing gp51$^+$ and gp51$^-$ (n = 5) IgM$^+$ B cells 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 $P < 0.05$ (** $P < 0.01$).

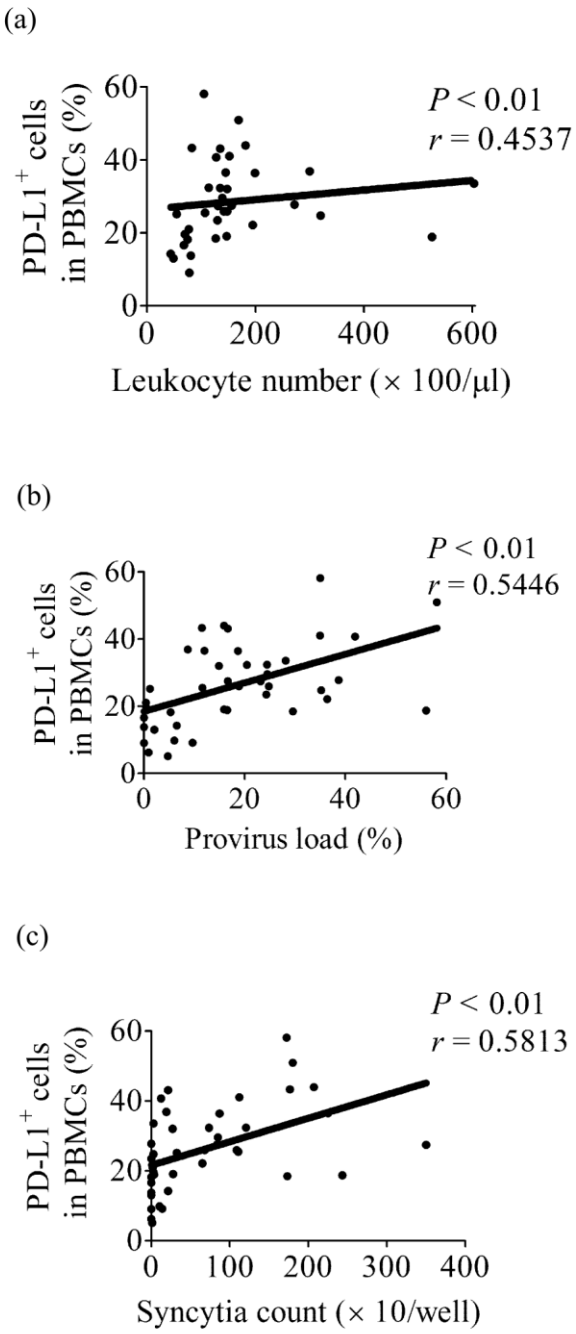

Figure I-4. Correlation between markers of disease progression and PD-L1 expression in BLV^+ cattle.

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 $PD\text{-}L1^+$ 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.

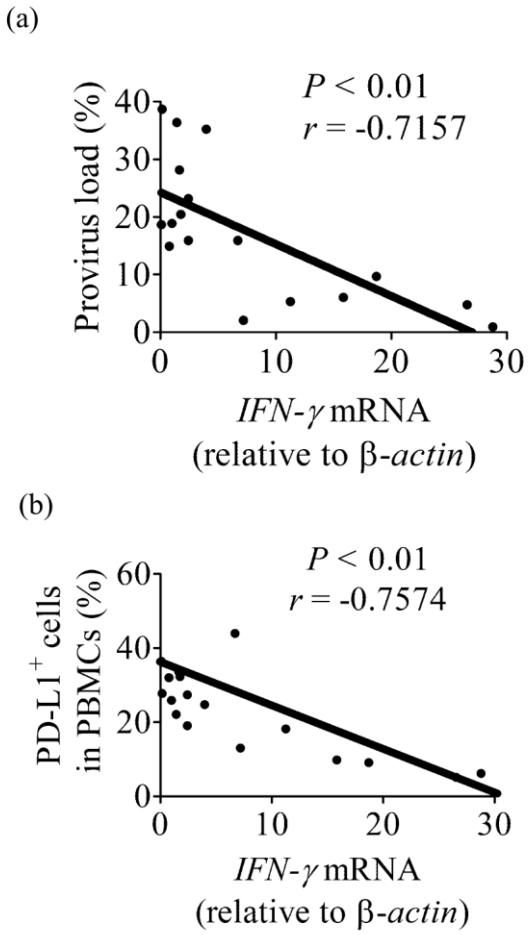

Figure I-5. Correlation between PD-L1 and IFN-γ expression in BLV^+ cattle.
Inverse correlation between *IFN-γ* mRNA expression and provirus load (a), and percentages of $PD\text{-}L1^+$ cells in PBMCs (b) (n = 17; AL: 6 PL: 8 BCBL: 3). The level of *IFN-γ* mRNA expression is shown as the ratios obtained by dividing the concentrations of the PCR products from *IFN-γ* 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 $PD\text{-}L1^+$ 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 $PD\text{-}L1^+$ 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-Immunogloblin 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 Fcγ 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 μg/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× 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 *Nhe*I and following reverse primers containing a restriction site for *Xho*I; 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. [^{3}H]-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.

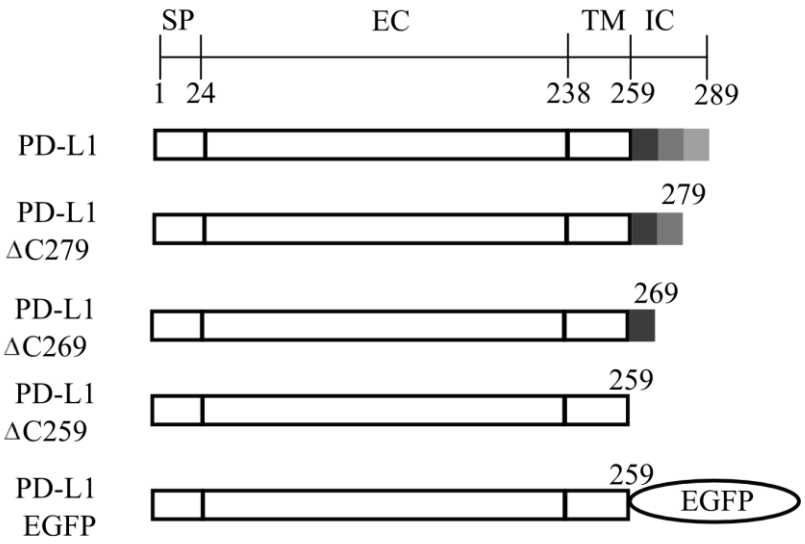

Figure II-2. Schematic representation of PD-L1, PD-L1-ΔC279, PD-L1-ΔC269, PD-L1-ΔC259 and PD-L1-EGFP.

PD-L1, PD-L1-ΔC279, PD-L1-ΔC269 and PD-L1-ΔC259 were inserted in pCIneo and PD-L1-EGFP was inserted pEGFP-N2. Numbers indicate amino acid number of bovine PD-L1. Gray region indicates intracellular domain of PD-L1. SP: signal peptide. EC: extracellular domain. TM: transmembrane domain. IC: intracellular domain.

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 $PD\text{-}L1^+$ 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 $PD\text{-}L1^+$ 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 $PD\text{-}L1^-$ B cells were also upregulated (Figure II-7d).

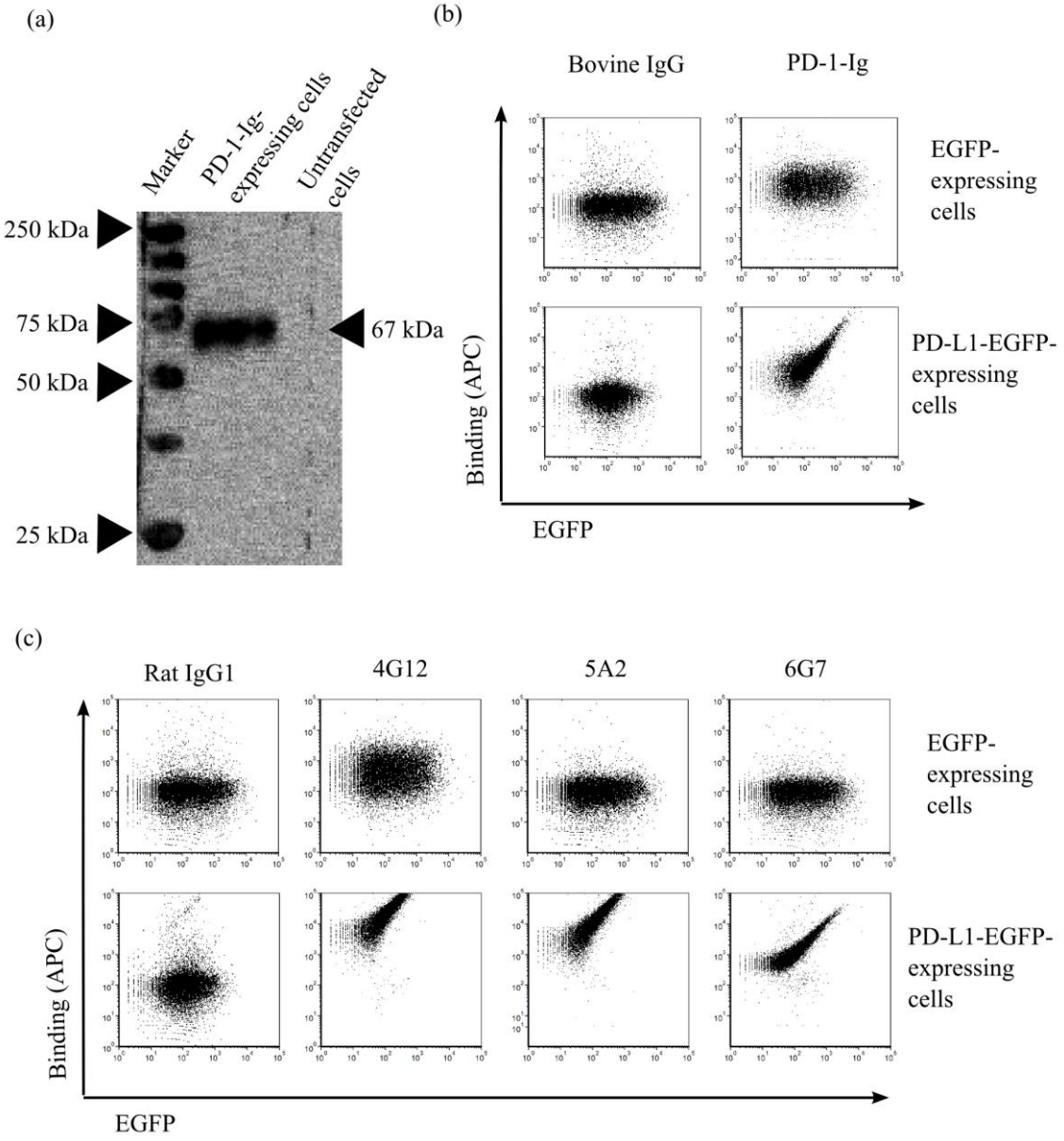

Figure II-3. Establishment of PD-1-Ig and anti-bovine PD-L1 mAbs.
(a) Western blot analysis of PD-1-Ig. Anti-bovine IgG Fc antibody recognized PD-1-Ig at about 67 kDa from supernatant of PD-1-Ig-expressing CHO-DG44 cells. (b, c) PD-1-Ig (b) and three types of anti-PD-L1 mAbs (c) recognized PD-L1-EGFP-expressing 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.

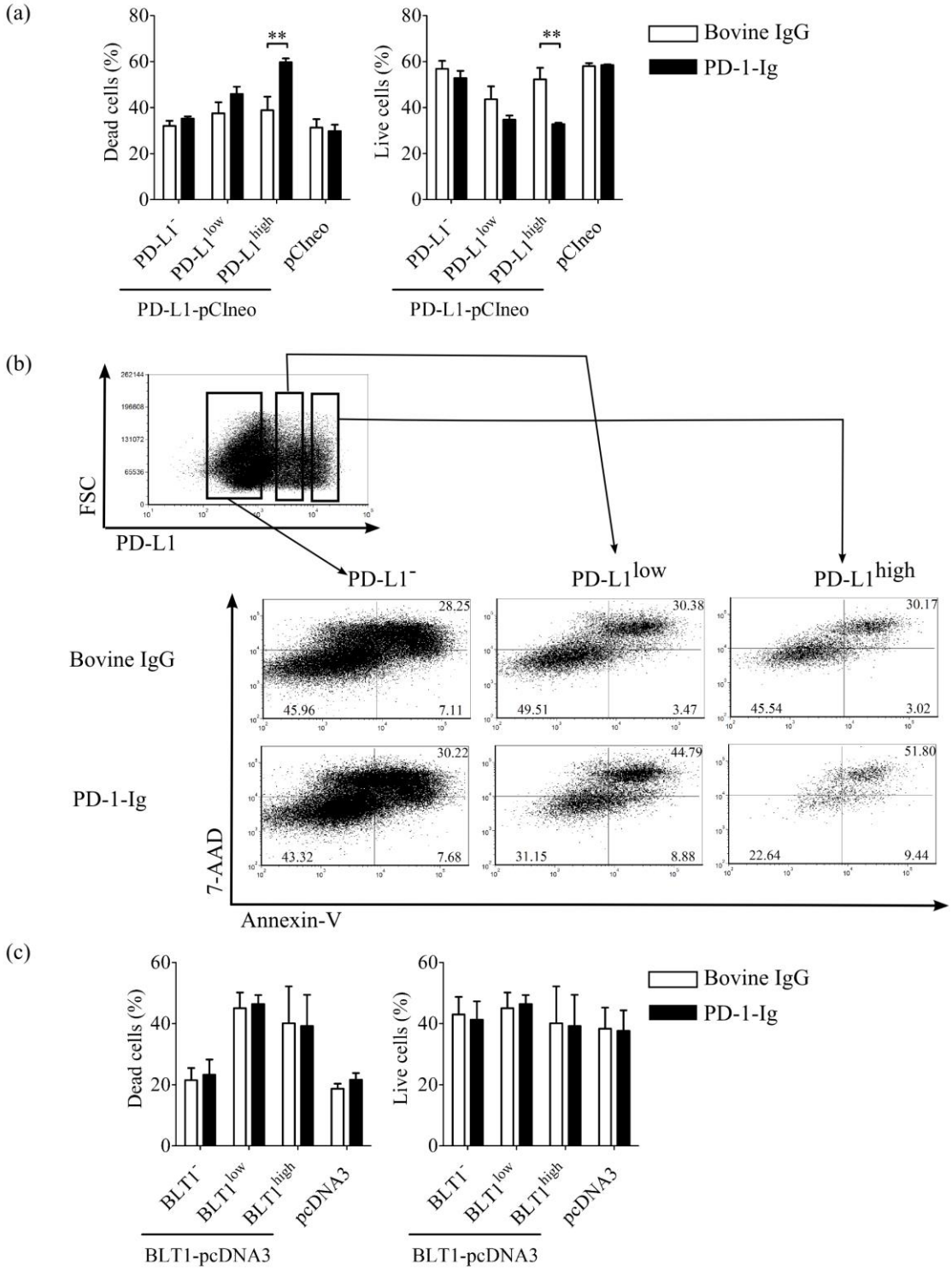

Figure II-4. Cell death in PD-L1-expressing cells treated with PD-1-Ig.
(a, c) Percentages of dead cells (Annexin-V$^+$ 7-AAD$^+$) and live cells (Annexin-V$^-$ 7-AAD$^-$) among Cos-7 cells transfected with pCIneo encoding PD-L1 (a) or pcDNA3 encoding BLT1 (c) and treated with bovine IgG or PD-1-Ig (50 μg/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 ± 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 $P < 0.05$ (** $P < 0.01$).

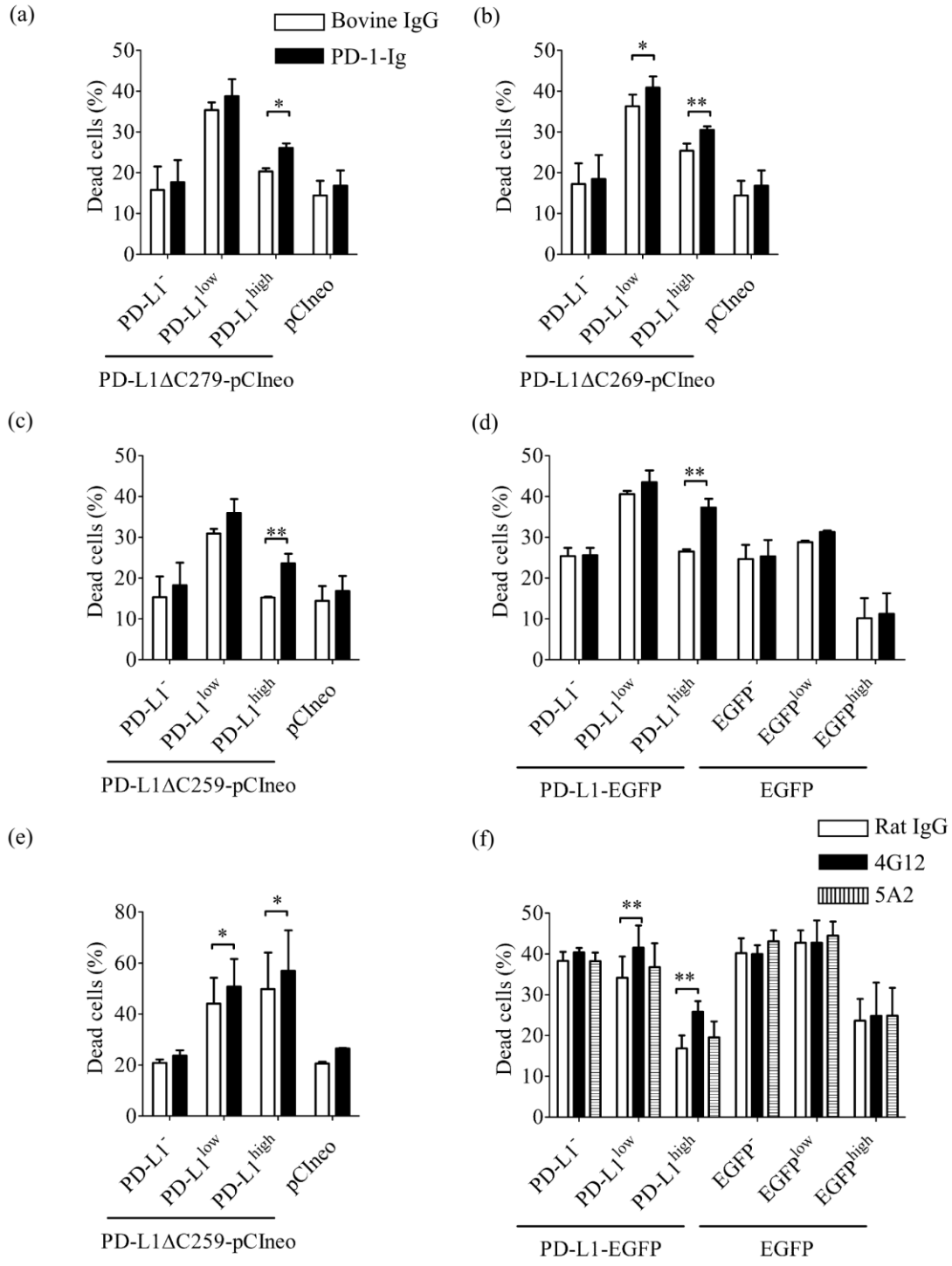

Figure II-5. Cell death in cells transfected with plasmids encoding C-terminal deletion mutants of PD-L1 and treated with PD-1-Ig.

(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 ± 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 $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

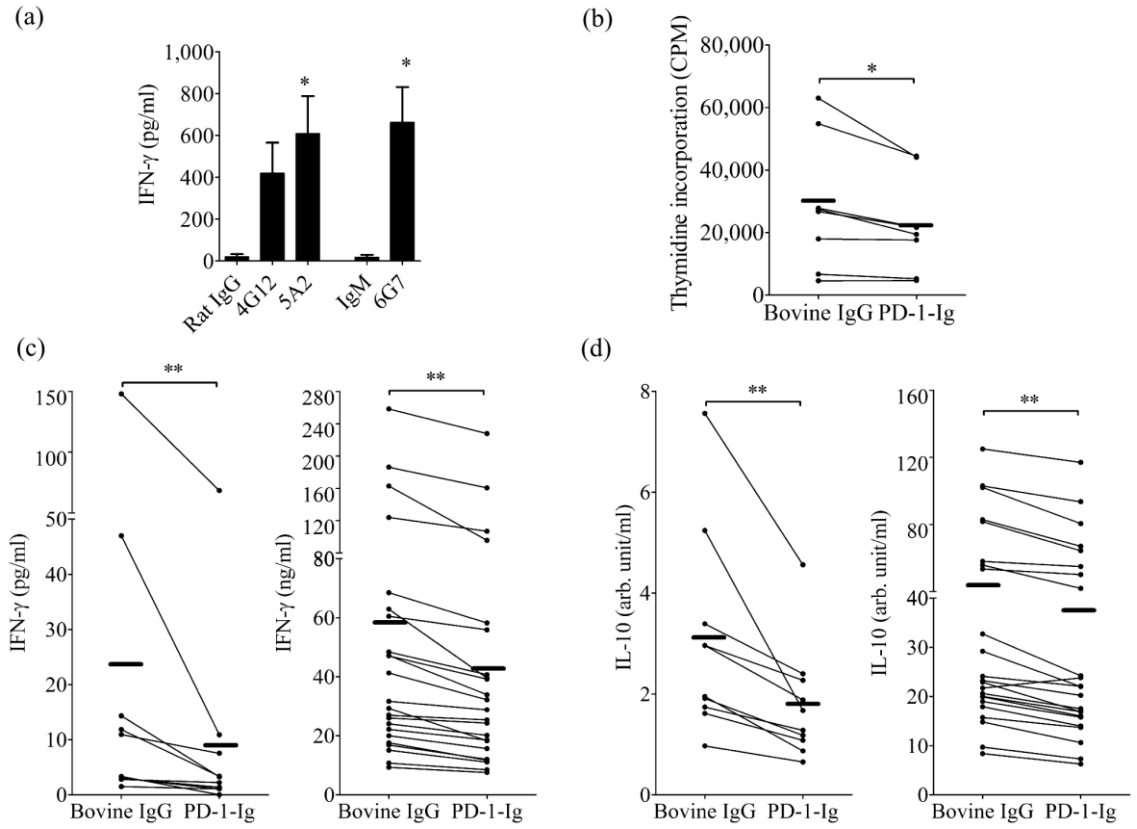

Figure II-6. Effect of PD-L1 blockade by anti-PD-L1 mAbs and PD-1-Ig treatment on immune reaction in bovine lymphocytes.

(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 μg/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 $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

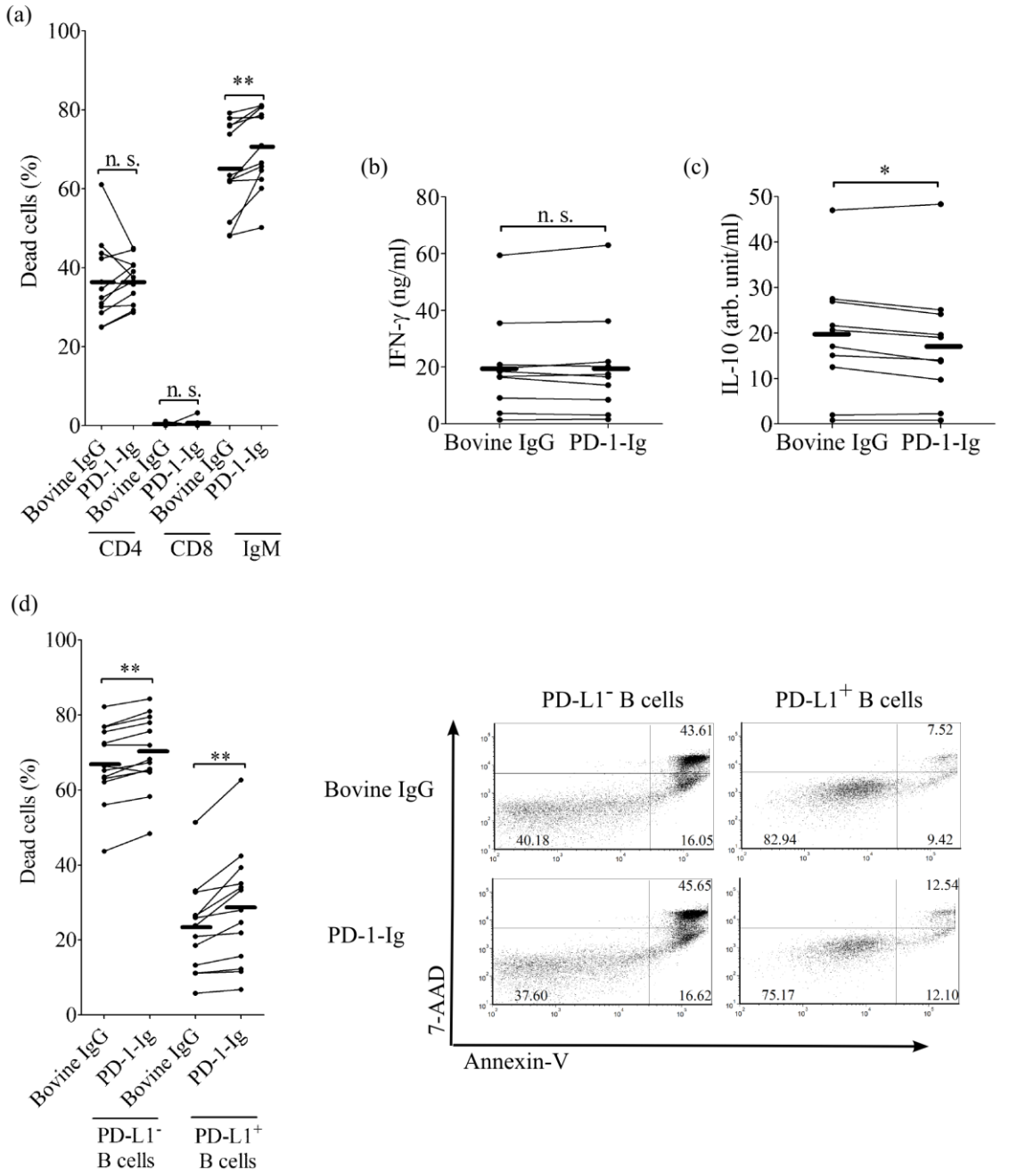

Figure II-7. Effect of PD-1-Ig treatment on B cell survival in bovine lymphocytes.
(a) Percentages of dead cells in $CD4^+$ T cells, $CD8^+$ T cells and IgM^+ B cells among PBMCs treated with bovine IgG or PD-1-Ig (n = 12). (b, c) IFN-γ (b) and IL-10 (c) productions in IgM^+ B cell-depleted PBMCs were 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 $PD\text{-}L1^-$ B cells were also increased in response to PD-1-Ig treatment. While increase in the number of apoptotic $PD\text{-}L1^+$ B cells might affect apoptosis in $PD\text{-}L1^-$ 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 $PD\text{-}L1^+$ 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 $PD\text{-}L1^{high}$ 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-expresing 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 μm; BD Biosciences) to obtain single cell suspension.

PD-1 expression analysis

To block nonspecific staining, 5-20 × 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% CO_2 in complete RPMI 1640 medium in the presence of pokeweed mitogen (PWM, 5 μg/ml, Sigma-Aldrich) or PMA and ionomycin (20 ng/ml and 1 μg/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 μg/ml of anti-PD-1 mAb or rat IgG in the presence or absence of PMA/ionomycin, or in the presence of 10 μg/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 μM 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 ($FITC^+$ $7\text{-}AAD^-$) 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 $PD\text{-}1^+$ $CD4^+$ 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

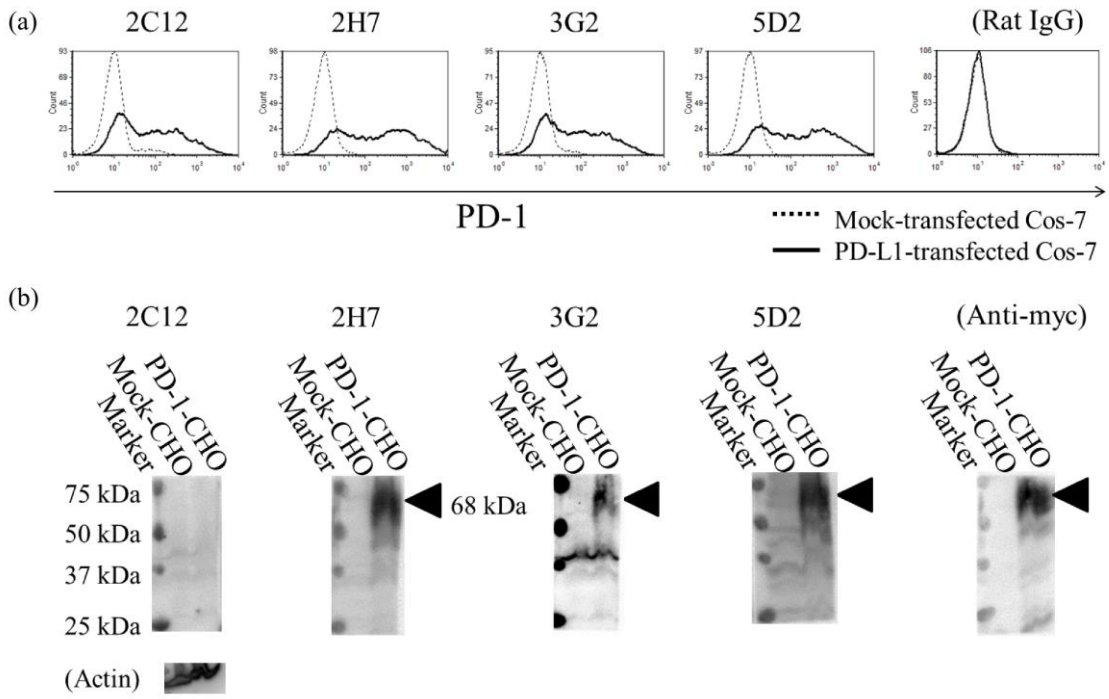

Figure III-1. Recognition of PD-1-expressing cells by anti-PD-1 mAbs.
(a) Flow cytometric analysis of surface expression of bovine PD-1. Cos-7 expressing PD-1 (black line) and Cos-7 transfected with the control vector (dashed line) were stained with four types of anti-PD-1 mAbs (2C12, 2H7, 3G2, and 5D2) 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.

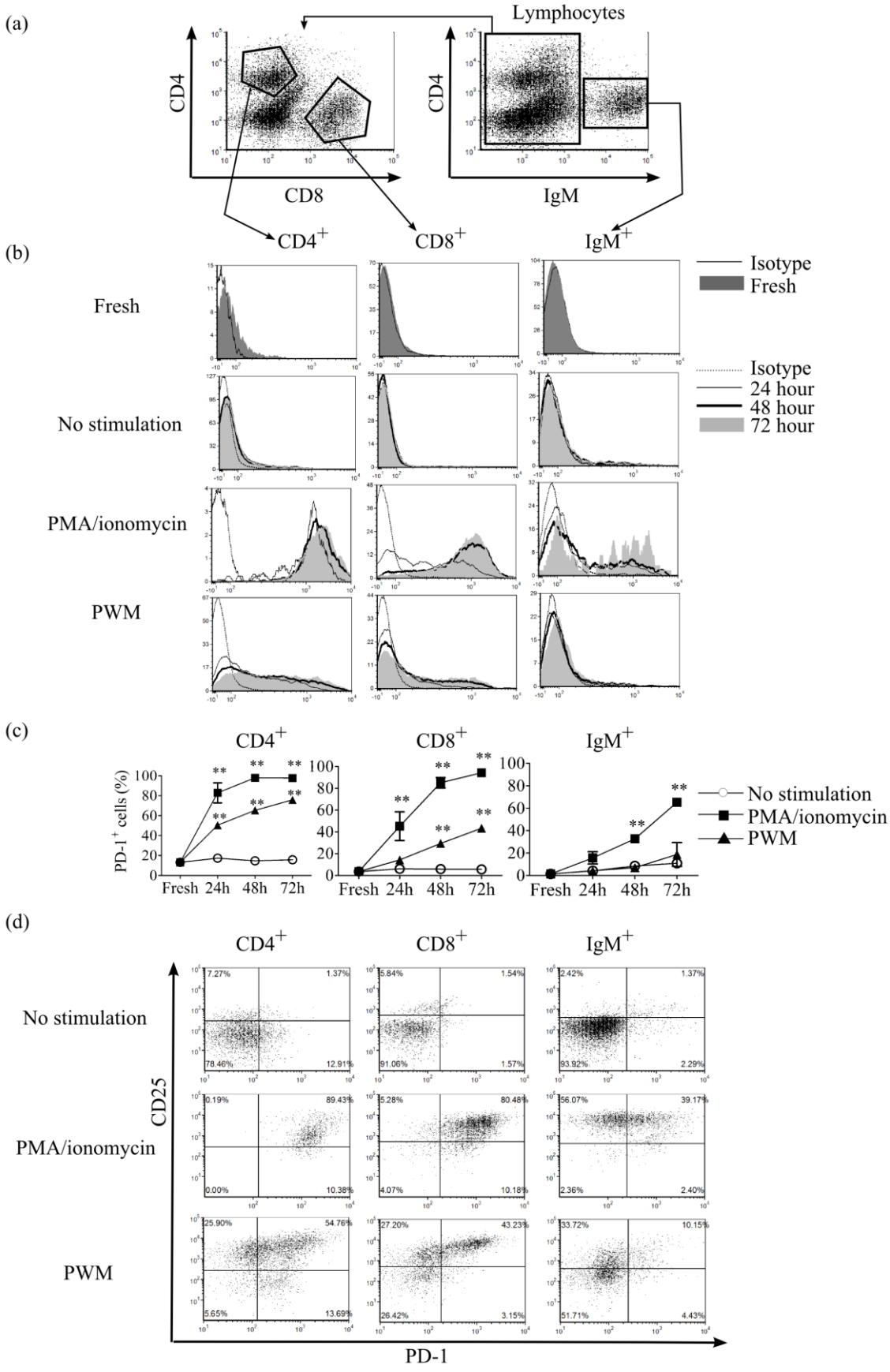
(a)
Lymphocytes
CD4
CD8
IgM
CD4+
CD8+
IgM+
(b)
Fresh
No stimulation
PMA/ionomycin
PWM
Isotype
Fresh
Isotype
24 hour
48 hour
72 hour
(c)
CD4+
CD8+
IgM+
PD-1+ cells (%)
Fresh 24h 48h 72h
No stimulation
PMA/ionomycin
PWM
(d)
CD4+
CD8+
IgM+
No stimulation
PMA/ionomycin
PWM
CD25
PD-1

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.

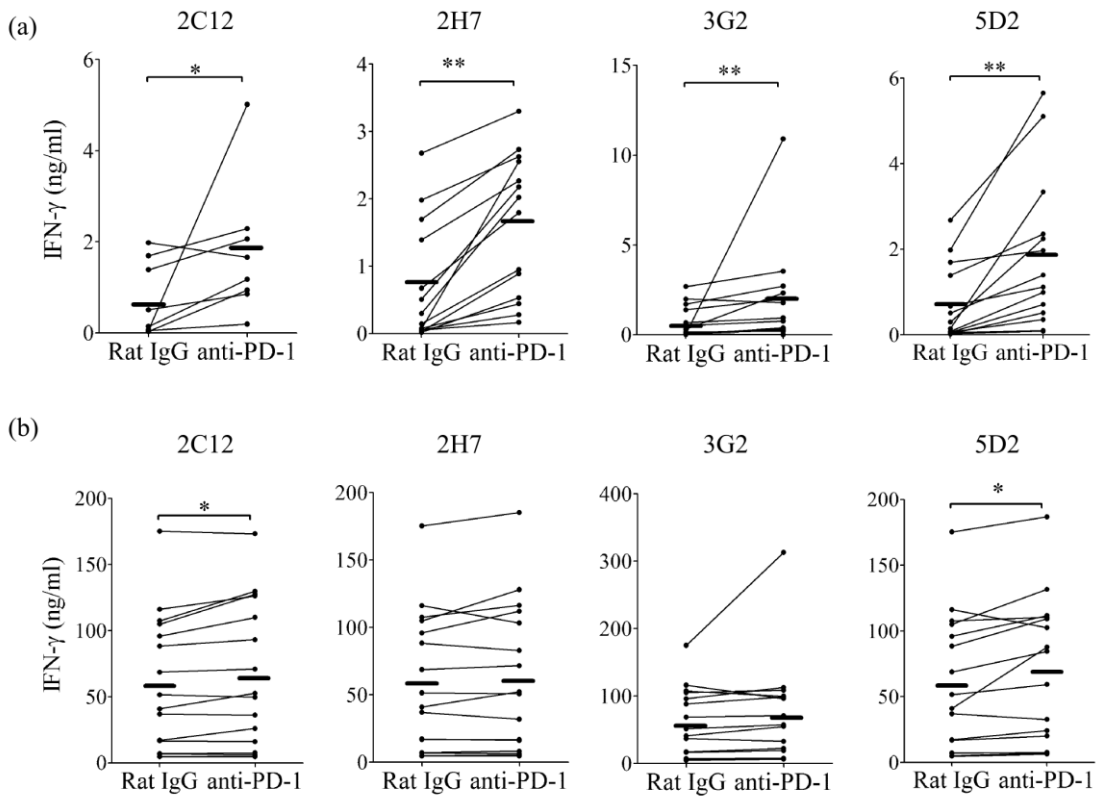

Figure III-3. Enhancement of cytokine production by anti-PD-1 mAbs in bovine lymphocytes
PBMCs were cultivated with rat IgG control or four types of anti-PD-1 mAbs (20 μg/ml) in the absence (a; n = 8 or 14) or presence (b; n = 16) of PMA/ionomycin. IFN-γ production was measured by ELISA. Each of line 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 $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

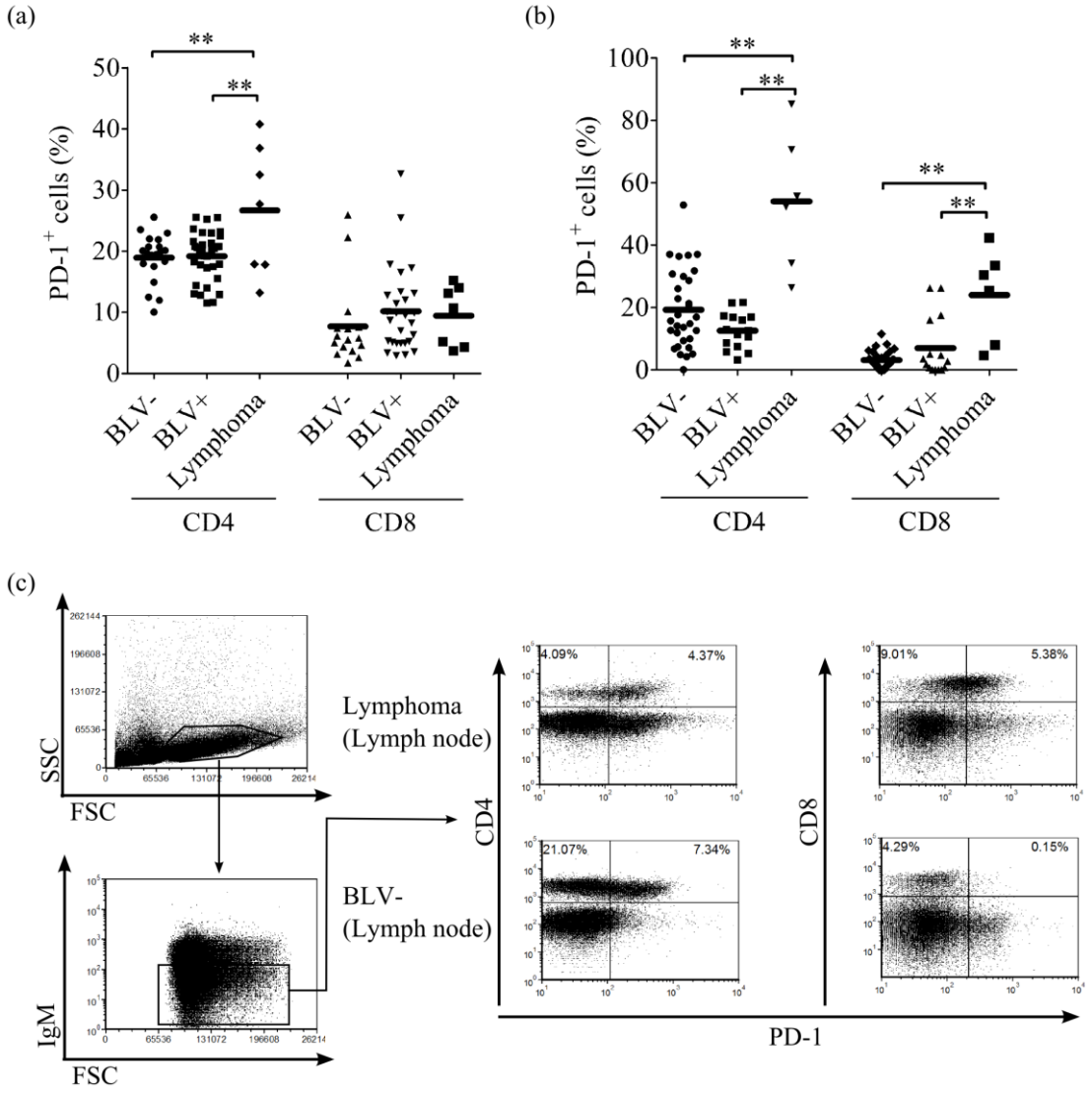

Figure III-4. Expression analysis of bovine PD-1 in BLV$^+$ cattle.
(a, b) Percentages of PD-1-expressing CD4$^+$ and CD8$^+$ T cells in PBMCs (a) and LN (b) isolated from BLV$^-$ cattle (CD4$^+$ T cells; n = 20 and 31, CD8$^+$ T cells; n = 16 and 31), BLV$^+$ cattle without lymphoma (CD4$^+$ T cells; 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 $P < 0.05$ (** $P < 0.01$).

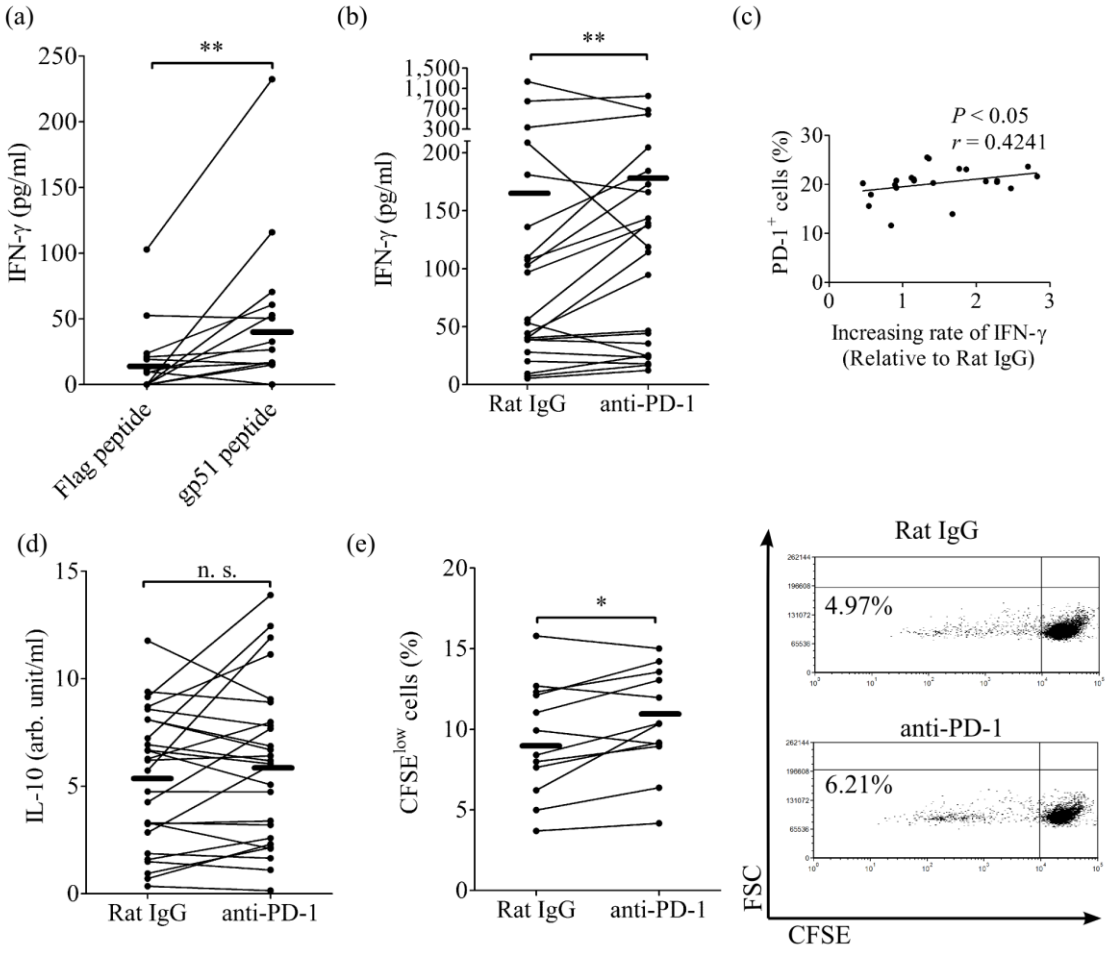

Figure III-5. Effect of PD-1 blockade on T cell function.
(a) PBMCs from BLV^+ cattle were cultured with Flag peptide as negative control and gp51 peptide mixture. IFN-γ production was measured by ELISA (n = 13). (b, c, d) PBMCs were cultured with rat IgG control or anti-PD-1 mAb (5D2; 20 μg/ml) in the 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 $PD\text{-}1^+$ 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 $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

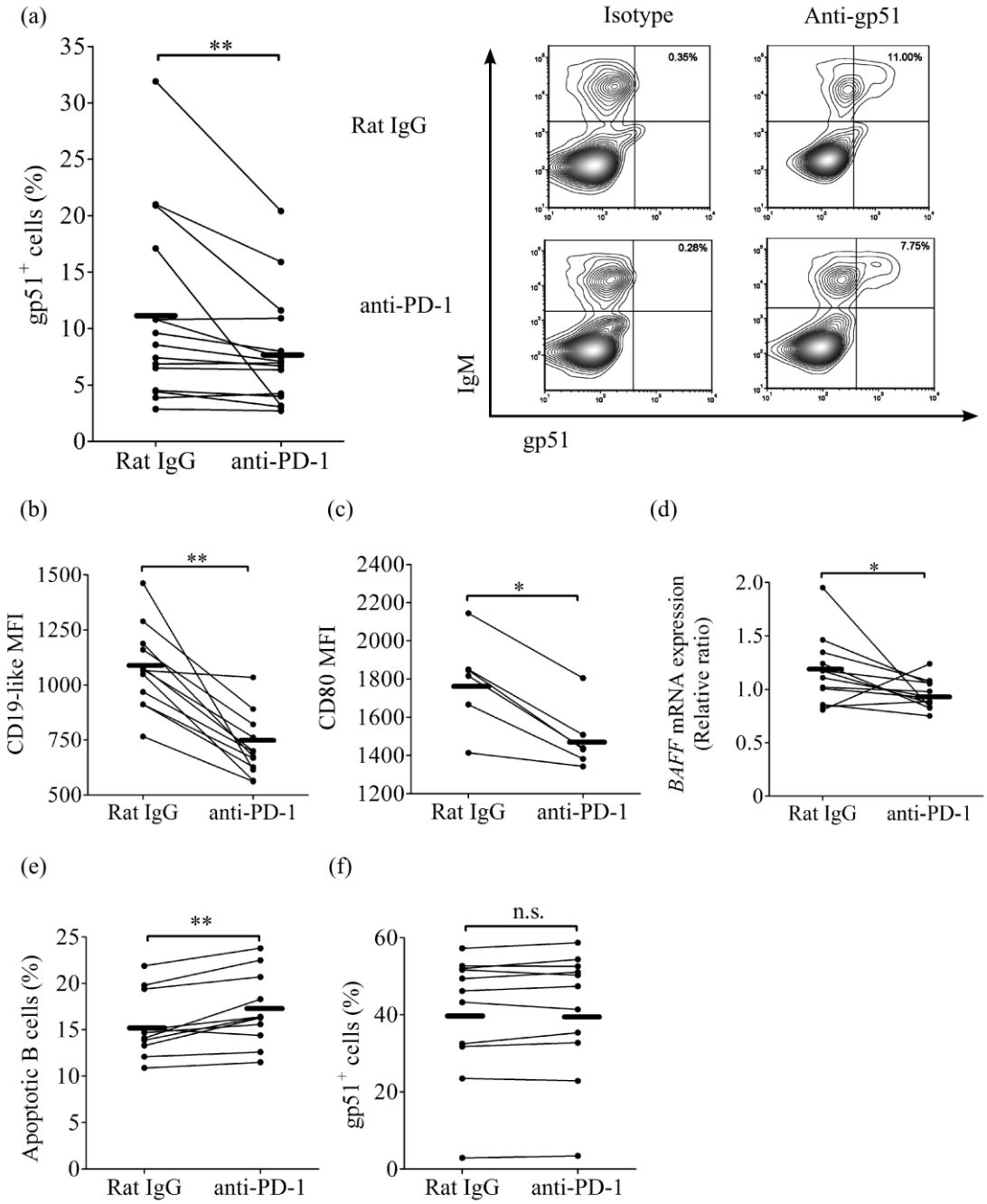

Figure III-6. Effect of PD-1 blockade on gp51 expression and B cell activation.
(a, b, c) Percentages of gp51-expressing cells (a; n = 15) and mean fluorescence index (MFI) of WC4 (CD19 like molecule) (b; n = 12) and CD80 (c; n = 6) in IgM^+ B cells were evaluated by flow cytometry in PBMCs treated with rat IgG control or anti-PD-1 mAb (20 μg/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 $PD\text{-}1^+$ 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 speculates 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 $PD\text{-}1^+$ 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 $PD\text{-}1^+$ $CD4^+$ T cells in blood and LN and $PD\text{-}1^+$ $CD8^+$ 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

INTRODUCTION

The major target cells for BLV seem to be B cells, although the possibility that other cell types are also infected can not be ruled out [Domenech et al., 2000; Fulton et al., 2006; Panei et al., 2013]. BLV^+ B cells and BL cells frequently express IgM, IgG, CD5, CD11b, MHCII and CD25 [Gillet et al., 2007; Meirom et al., 1997], but the apparent markers expressed exclusively in only BLV^+ B cells with provirus remain unknown. For these reasons, it is necessary to identify the specific marker for BLV^+ B cells for better understanding of the kinetics of excessive proliferation and transformation of the B cells.

It is not possible to identify all of BLV^+ B cells by using antibody specific to BLV antigen, because BLV expression was rarely observed *in vivo* and in freshly isolated lymphocytes [Asquith et al., 2005; Gillet et al., 2007; Hanon et al., 2000; Powers et al., 1992]. Short term of *ex vivo* cultivation or blood incubation at 37°C results in the viral antigen expression, but only in a part of BLV^+ B cells. Florins et al. [2012] reported that the viral antigen expression causes high death rates of B cells *in vivo* when BLV^+ lymphocytes were cultivated and transplanted into the BLV^+ donor cattle. These data suggests that the silencing and expressing of viral antigens seem to be a strategy to reduce immunogenicity, to evade immune surveillance, and to favor efficient propagation in the BLV^+ cattle.

The *ex vivo* expressions of viral antigens are upregulated by mitogen stimulations which activate B cell function [Kidd et al., 1996; Lagarias et al., 1989], but are reduced by inhibitor of protein kinase C [Jensen et al., 1992]. Thus, BLV antigen expression seems to be regulated by B cell activation. However, the mechanisms for the suppression of viral gene expression *in vivo* are not completely clarified in BLV-infected animal, and even in patients infected with HTLV-1, which is genetically related to BLV [Taniguchi et al., 2005].

Previous reports regarding the detection of BLV antigens by flow cytometry and microscope after *ex vivo* cultivation demonstrated that a part of, but not all, B cells expressed BLV antigens [Fulton et al., 2006; Gillet et al., 2007; Ikebuchi et al., 2013]. Moreover, a silent provirus was integrated into B cell tumors isolated from BLV^+ sheep and the cell lines derived from these tumors [Broeke et al., 1988; Merimi et al., 2007a, 2007b]. These reports suggested that “BLV-silencing” B cells are present among

lymphocytes in infected cattle. Thus, one can hypothesize that there are at least two subsets in B cells whose genome incorporates BLV-provirus; “BLV-expressing” B cells, which could transmit BLV to BLV^- cells, can be detected by immune surveillance system to activate immune cells and finally eradicated, and “BLV-silencing” B cells, which could proliferate without being attacked by immune cells [Gillet et al., 2007].

In this study, to explore the marker which distinguishes BLV-expressing from BLV-silencing B cells, the expression of surface markers and viral antigens in B cells were investigated in BLV^+ cattle. It was accordingly clarified that IgM^{high} B cells were prone to express BLV antigen and IgM^{low} B cells were not, although provirus loads in both subsets were similar. Thus, to investigate functional difference of IgM^{high} B cells and IgM^{low} B cells, gene expression profiling, several cellular functions and BLV gene expression in both subsets were characterized.

MATERIALS AND METHODS

Samples from cattle, BLV diagnosis and cell preparation

In this chapter, blood samples of 166 cattle altogether (BLV^+: 117, BLV^-: 49) bred in several farms were obtained. Blood collection, PBMCs isolation and diagnosis of BLV infection were performed as described in Chapter I, and diagnosis of BL was done as described in Chapter III. The number of lymphocytes in blood was counted by MEK-6450 Celltacα (Nihon Kohden). Enlarged LN and blood samples from BCBL were provided from several farms.

B cells were purified from PBMCs by autoMACS Pro using anti-bovine IgM (BIG73A) and anti-mouse IgG1 MicroBeads following the manufacturer's instructions. IgM^{high} and IgM^{low} B cells were sorted by Moflo Astrios (Beckman Coulter) from cultivated PBMCs stained with anti-bovine IgM pre-labeled with Zenon Alexa Fluor 488, anti-WC4 and Alexa Fluor 647-conjugated anti-mouse IgG. High purities of the IgM^+ B cells, IgM^{high} B cells and IgM^{low} B cells were confirmed by FACS Verse.

Expression of recombinant BLV-env-Ig fusion protein (BLV-env-Ig)

Soluble BLV-env-Ig was expressed in CHO-DG44 cells stably transfected with pCAGGS encoding a mouse CD150 signal sequence, extracellular region of BLV-env and a Fc fragment of rabbit IgG as described in Chapter II. Concentration of BLV-env-Ig in the supernatants was measured by Rabbit IgG ELISA Quantitation Set.

Flow cytometry

To measure the expression of surface IgM, WC4 (CD19-like) and B-B7 (CD21-like) in B cells isolated from BLV^+ cattle, PBMCs were incubated with anti-WC4 or anti-B-B7 diluted with PBS containing 10% goat serum at 4°C for 30 min. After washing, the cells were stained with Alexa Fluor 647-conjugated anti-mouse IgG, washed again, and stained with anti-bovine IgM (BIG73A) pre-labeled with Zenon Alexa Fluor 488.

To analyze BLV antigen expression, PBMCs were cultivated in complete RPMI 1640 medium overnight. The cells were collected, and stained with anti-IgM and anti-WC4 as described above. BLV-gp51 staining was performed as described in Chapter I. In the case of p24 expression analysis, the cells were stained with anti-IgM

and Alexa Fluor 647-conjugated anti-mouse IgG, and then fixed, permealized and stained with anti-p24 pre-labeled with Zenon Alexa Fluor 488. In the case of triple staining with anti-IgM, anti-gp51 and anti-p24, the intracellular staining was performed with anti-gp51 pre-labeled with Zenon Alexa Fluor 488 and anti-p24 pre-labeled with Zenon Alexa Fluor PE. Live cells were gated by staining with Fixable Viability Dye eFluor 780 (eBioscience).

To examine the expression of BLV-binding receptors, PBMCs were stained with anti-IgM pre-labeled with Zenon Alexa Fluor 488, anti-WC4 pre-labeled with Zenon Alexa Fluor 647 and the supernatants containing 5 μg/ml of BLV-env-Ig with PE conjugated anti-rabbit IgG (Beckman coulter). Labeling of BLV-env-Ig was performed at 37°C for 30min. Rabbit IgG isotype control was used as a negative control for the supernatants of BLV-env-Ig. Dead cells were removed by 7-AAD staining.

To analyze PD-L1 expression, freshly isolated PBMCs were stained with anti-bovine PD-L1 mAb (4G12) and anti-bovine IgM (BIG73A) followed by staining with APC-conjugated anti-rat IgM+IgG and PE-conjugated anti-mouse IgG.

The stained cells were analyzed by FACS Verse and FCS Express 4. The most of dead cells were isolated through a forward scatter (FSC) versus side scatter (SSC) plot. Appropriate isotype controls were used for each of the samples.

Anti-IgM and anti-WC4 stimulation and treatment of phosphatidylinositol 3-kinase (PI3-kinase) inhibitor

Isolated IgM^+ B cells were cultured in complete RPMI 1640 medium supplemented with goat polyclonal anti-bovine IgM (KPL; 20 μg/ml), anti-WC4 or PI3-kinase inhibitors: wortmannin (Sigma-Aldrich; 100 nM) and LY294002 (Sigma-Aldrich; 20 μM) for 48 h. The cells were collected, fixed, permealized, and stained with anti-gp51 pre-labeled with Zenon PE. Mouse IgG1 (Beckman Coulter) and dimethylsulfoxide (Nacalai tesque) were respectively used as a negative control for anti-WC4 and both of wortmannin and LY294002. PBS and goat IgG (Beckman coulter) were used as negative controls for polyclonal anti-IgM. The treatments with goat IgG and PBS was confirmed not to change gp51 expression in some of the samples (data not shown). Endotoxin levels in antibody solutions were decided by limulus test performed by Dr. S. Imamura, National Veterinary Assay Laboratory, Japan. The endotoxin concentrations

in the antibody solutions were confirmed to be less than 60 EU/ml, and the endotoxin included did not induce any artifact on lymphocytes.

Real-time PCR

IgM^{high} and IgM^{low} B cells were isolated from PBMCs cultivated overnight to express BLV antigens as described above. Total RNA extraction from IgM^{high} and IgM^{low} B cells by RNeasy Plus Mini, cDNA synthesis and quantitative real-time PCR was performed as described in Chapters I and III. Following primers were used to measure the expression level of Tax/Rex, R3 and G4: 5´-CGA TCA TCA GAT GGC AAG TG-3´ and 5´-CGA TGG TGA CAT CAT TGG AC-3´ for Tax/Rex, 5´-GAT CAT CAG ATG GGT CCT GAT GAA C-3´ and 5´-GCT GCT GGA TGT GGC TGG AAT GTC-3´ for R3, and 5´-TTC GGC GCC CAG CCA CAT C-3´ and 5´-GTC GTT ATC AGG TAA TGG ATC CCG A-3´ for G4. The amounts of gene expressions were divided by the mean expression of *RPLPO*, *B2M* and *ACTB* mRNA as internal control genes. Each amplification procedure was done in duplicate, and the results of IgM^{high} B cells were indicated as relative ratio to the expression levels in IgM^{low} B cells.

To measure provirus load, total cellular DNA was purified from IgM^{high} and IgM^{low} B cells sorted from cultivated PBMCs by Nucleospin Tissue XS (Macherey Nagel). Provirus was amplified by quantitative real-time PCR as described in Chapters I and III except that following primers were used: 5´-ACT TTC AGA CCC CCT TGA CTG ACA-3´ and 5´-AAA CCT CTG CCC TGG TGA TTA AGG-3´ designed from BLV provirus sequence (Genbank numbers; K02120 and AF033818). The *β-globin* gene was amplified to normalize the results of provirus to 100 cells using following primers: 5´-ACA CAA CCG TGT TCA CTA GC-3´ and 5´-CAA CTT CAT CCA CTT TCA CC-3´. Standard lines were generated from pGEM-T Easy vector (Promega) carrying the amplified products by the primers for provirus and β-globin. Copy numbers of plasmids were calculated based on DNA concentration measured by Nanodrop8000 (Thermo Scientific). Each amplification procedure was done in duplicate, and the results are presented as copy number of provirus in 100 cells obtained by normalized copy number of the provirus divided by that of the β-globin multiplied by 200.

Microarray analysis

Microarray analysis was performed using bovine oligo DNA microarray (Agilent technologies) in DNA Tip Laboratory, according to protocol of Agilent technologies.

PBMCs were isolated from three BLV^+ cattle, and IgM^{high} and IgM^{low} B cells were sorted from cultivated PBMCs to purify total RNA as described above. cDNA was synthesized and transcribed into cRNA from 100 ng of the total RNA purified from either IgM^{high} or IgM^{low} B cells by using Low RNA input fluorescent linear amplification kit (Agilent technologies). Each cRNA from total RNA of IgM^{high} or IgM^{low} B cells was labeled with cyanine 5 or cyanine 3. Labeled cRNA was purified by RNeasy Mini and pair of the cRNA from IgM^{high} and IgM^{low} B cells was hybridized using Gene expression hybridization kit (Agilent technologies). Then, the array was washed and scanned using DNA microarray scanner (Agilent technologies). Data were extracted by Feature extraction software (version 9.5.3, Agilent technologies), and normalized and analyzed using GeneSpring GX 11 (Agilent technologies). Differential expression of a gene within the experiment in all cases of three cattle was obtained if a 1.5-fold or greater difference was observed in the comparison. Gene ontology (GO) analysis of identified genes was performed using GeneSpring GX 11. *P*-value was corrected by false discovery rate method. To confirm the reliability of microarray data, expression levels of some genes (*MafB*, *Jun*, *Fos*, *FosB*, *Frat1*, *Fyn*, *Ccl3*, *Ccl4*, *Il8* and *Ccr1*) were measured using RNA samples from nine BLV^+ cattle by quantitative real-time PCR as described above.

Detection of live and dead cells

PBMCs were cultivated overnight in complete RPMI 1640 medium, stained and analyzed as described in Chapter III. The results are presented as the percentages of $FITC^+$ $7\text{-}AAD^+$ cells as dead cells and $FITC^-$ $7\text{-}AAD^-$ cells as live cells in total IgM^+ B cells.

Calcium mobilization

Isolated IgM^+ B cells stained with Alexa Fluor 647-conjugated anti-mouse IgG were suspended by Hanks' balanced salt solution (HBSS) containing 1% BSA (HBSS-BSA) and incubated at 30°C for 10 min, and then stained with 0.1 mM Fluo4-AM (Sigma-Aldrich) and 4% Pluronic F-127 (Life Technologies)-containing HBSS-BSA for 15 min in the dark. After washing with HBSS-BSA, the cells were suspended by 2.5 mM probenecid (Sigma-Aldrich)-containing HBSS-BSA, incubated at 30°C for 30 min in the dark, and kept on ice until analysis. The cells (300 μl solution) were warmed at 37°C for 10 min prior to the measurement of background fluorescence

for 30 sec followed by the addition of either 50 μg/ml of goat polyclonal anti-bovine IgM or 20 μg/ml of anti-WC4, and data were acquired for further 4 minutes. Then, 4 μl of 0.5 mg/ml ionomycin was added to the control for dye loading and cell activation potential. To confirm that polyclonal anti-IgM or anti-WC4-induced calcium mobilization was not due to technical artifacts, some samples were stimulated with 1 mM ethyleneglycoltetraacetic acid (Dojindo), 50 μg/ml of goat IgG and 20 μg/ml of mouse IgG1. The data acquired by FACS Fortessa (BD Biosciences) was analyzed by FCS Express 4. To calculate the percentage of cells responding to the stimulation, background fluorescence threshold was decided for each of the samples at the fluorescence intensity of 85% of unstimulated cells as described previously [Mockridge et al., 2007]. The results were indicated as the peak percentages of responding cells corresponding to fluorescence intensity above the threshold by the stimulation.

Statistical analysis

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

RESULTS

Surface IgM (sIgM) expression was augmented in B cells isolated from BLV$^+$ cattle

Firstly, the expressions of B cell-associated surface markers in B cells isolated from BLV$^+$ cattle at different disease stages were analyzed. The rate of IgM$^+$ B cells in PBMCs were increased in BLV$^+$ cattle in line with increasing numbers of lymphocytes in blood (Fig IV-1a, b). The percentage of IgM$^+$ B cells in BLV$^-$ cattle was up to around 50% of PBMCs. MFI of sIgM expression was also augmented in IgM$^+$ B cells from BLV$^+$ cattle compared to BLV$^-$ cattle (Figure IV-1c). Inversely, dual staining with anti-IgM and either anti-WC4 or anti-B-B7 revealed that both proportions of CD19-like$^+$ cells and CD21-like$^+$ cells and MFI of CD19-like and CD21-like expressions in IgM$^+$ B cells from BLV$^+$ cattle were reduced as compared with those from BLV$^-$ cattle (Figure IV-1d, e). Increase in IgM expression and decrease in CD19-like and CD21-like expressions in B cells were correlated with the proportions of IgM$^+$ B cells in PBMCs (Figure IV-1f). When IgM$^+$ B cells were divided into two subsets, IgM^{high} and IgM^{low} B cells, the percentages of IgM^{high} B cells in BLV$^+$ cattle were significantly higher than those of IgM^{low} B cells in BLV$^+$ cattle and IgM^{high} and IgM^{low} B cells in BLV$^-$ cattle (Figure IV-1g). The amplitude of IgM^{high} B cells was higher than that of IgM^{low} B cells in BLV$^+$ and BLV$^-$ cattle (Figure IV-1h). These data indicated that the number of IgM^{high} CD19-like^{mid} CD21-like^{mid} B cells is increased in blood lymphocytes during the disease progression of BLV infection.

IgM^{high} B cells were prone to express BLV antigen and IgM^{low} B cells were not

To investigate the relationship between surface marker and the expression of BLV antigens in B cells after *ex vivo* cultivation, PBMCs were cultivated for BLV expression and stained with anti-IgM, anti-WC4 and either anti-gp51 or anti-p24. The *ex vivo* cultivation facilitated to distinguish IgM^{high} from IgM^{low} B cells as compared to fresh lymphocytes (Figure IV-1a and 2a). In freshly-isolated lymphocytes, CD19-like expression in IgM^{low} B cells was higher than that in IgM^{high} B cells (Figure IV-1a and 2b), but the overnight cultivation led to a decrease of CD19-like expression in IgM^{low} B cells (Figure IV-2b). The CD19-like expression in IgM^{high} B cells was not altered in both freshly isolated and cultivated lymphocytes. Then, BLV-gp51 was mostly expressed in IgM^{high} B cells (Figure IV-2c). By contrast, IgM^{low} B cells rarely

expressed gp51. Similarly, higher levels of BLV-p24 expression were observed in IgM^{high} and IgM^{low} B cells (Figure IV-2d), and the percentages of B cells expressing both gp51 and p24 were also higher in IgM^{high} B cells than IgM^{low} B cells (Figure IV-2e). Few $gp51^{+}$ $p24^{-}$ B cells were present in both IgM^{high} and IgM^{low} B cells. These data suggested that IgM^{high} CD19-$like^{mid}$ cells included a high proportion of BLV-expressing cells, while IgM^{low} CD19-$like^{low}$ B cells included a high proportion of BLV-silencing cells. Thus, BLV-expressing and BLV-silencing B cells could be loosely discriminated by the difference in sIgM expression levels.

IgM and WC4 stimulation upregulated gp51 expression

To identify whether sIgM and CD19-like molecules play a role in the expression of BLV antigens, the change in BLV-gp51 expression was measured in B cells treated with either anti-IgM, anti-WC4 or PI3-kinase inhibitors which inhibit the signaling associated with CD19 [So et al., 2012]. Anti-IgM treatment demonstrated the upregulation of gp51 expression in B cells (Figure IV-3a), in good agreement with the previous report showing that IgM-crosslinking induced p24 expression in PBMCs and B cells [Kerkhofs et al., 1996]. There was no difference in gp51 expression between B cells treated with goat IgG and PBS in some samples (data not shown). The percentages of $gp51^{+}$ B cells were increased by anti-WC4 treatment as compared to mouse IgG1 treatment (Figure IV-3b), and decreased by the treatment of two types of PI3-kinase inhibitors (Figure IV-3c). Moreover, LY294002 treatment abolished the effect of anti-WC4 treatment (Figure IV-3c), indicating that CD19 signaling, as well as B cell receptor (BCR) signaling, could play important roles in BLV antigen expression.

Provirus loads and expression levels of BLV-binding receptors in IgM^{high} and IgM^{low} B cells were almost the same

The lower expression of BLV antigen in IgM^{low} B cells relative to IgM^{high} B cells could be attributed to lower proportion of BLV^{+} B cells in the subset. To investigate the percentages of BLV^{+} B cells in IgM^{high} and IgM^{low} B cells, provirus loads in both of the subsets were measured by real-time PCR from genome DNA. Both of the B cell subsets were isolated from PBMCs cultivated overnight to express BLV antigens. Unexpectedly, no differences were observed in provirus loads between IgM^{high} and IgM^{low} B cells (FigureIV-4a), although IgM^{high} B cells were prone to express BLV antigen (Figure IV-2b, d). Not all B cells would be infected with BLV in both of these

B cell subsets because the provirus loads were less than 80 copies per 100 cells in all samples, although the provirus loads did not accurately represent the percentages of BLV^+ cells due to the fact that they were calculated value from the data of real-time PCR.

Gult-1 and neuropilin-1 have been identified as cellular receptors for HTLV-1 entry [Ghez et al., 2006; Manel et al., 2003]. Although the receptors for BLV entry remain unidentified, the staining by using BLV-env-Ig facilitates to investigate the expression of "BLV-binding receptors" which are expected as BLV receptors [Lavanya et al., 2008]. Thus, IgM^{high} and IgM^{low} B cells equally expressed BLV-binding receptors in BLV^+ cattle (Figure IV-4b). Meanwhile, BLV-env-Ig bound to IgM^{low} B cells in BLV^- cattle at lower proportion than to IgM^{low} B cells in BLV^+ cattle and IgM^{high} B cells in both of the cattle. These data suggested that the relative susceptibility to BLV infection and the infection rate could be in the same range between IgM^{high} and IgM^{low} B cells of BLV^+ cattle.

Expression levels of *Tax/Rex* mRNA in IgM^{high} B cells were lower than those in IgM^{low} B cells

Non-structural proteins of BLV, Tax, Rex, R3 and G4 were involved in the activation of viral transcription, leukemogenesis and viral propagation [Broeke et al., 1988; Derse, 1987; Willems et al., 1993, 1994]. These viral proteins were thought to maintain the equilibrium of viral latency and viral replication *in vivo* [Baydoun et al., 2008; Matsuoka et al., 2007]. In this study, IgM^{high} B cells showed lower expression of *Tax/Rex* mRNA compared to IgM^{low} B cells (Figure IV-5a). The expression levels of *R3* and *G4* mRNA were equivalent in IgM^{high} and IgM^{low} B cells (Figure IV-5b, c). Relative value of the expression levels of *Tax/Rex* and *R3* mRNA in IgM^{high} B cells were negatively correlated with the number of lymphocytes in blood, indicating the degree of the disease progression (Figure IV-5d, e, f). These data suggested that IgM^{low} B cells including a higher proportion of $gp51^-$ or $p24^-$ BLV-silencing cells express the higher level of *Tax/Rex* mRNA compared to IgM^{high} B cells in line with disease progression.

Calcium influx in IgM^{high} B cells from BLV^+ cattle was higher than B cells from BLV^- cattle in response to BCR stimulation

Previous reports showed a correlation between the sIgM expression level in B cells

and their ability of calcium flux in response to BCR stimulation [Quach et al., 2011; Zikherman et al., 2012]. In this study, more responding cells stimulated by polyclonal anti-bovine IgM were present in IgM^{high} B cells from BLV^{+} cattle than IgM^{high} and IgM^{low} B cells from BLV^{-} cattle (Figure IV-6a, b). The percentages of IgM^{low} B cells in BLV^{+} cattle which responded to the BCR stimulation were also higher than those of IgM^{low} B cells isolated from BLV^{-} cattle. Unexpectedly, no significant difference in responsiveness to the BCR stimulation was observed between IgM^{high} and IgM^{low} B cells (Figure IV-6b). Meanwhile, responsiveness to WC4 stimulation was almost the same in all subsets of B cells.

IgM^{low} B cells from BLV^{+} cattle were prone to undergo spontaneous cell death after *ex vivo* cultivation

Previous studies showed that BLV infection in sheep protected their PBMCs from *ex vivo* apoptosis [Dequiedt et al., 1997], and $CD21^{low}$ B cells in human were sensitive to apoptosis [Charles et al., 2011; Isnardi et al., 2010]. To investigate the sensitivity to cell death of B cells from BLV^{+} cattle which were shown to express lower levels of CD21-like than BLV^{-} cattle (Figure IV-1c, d), PBMCs after *ex vivo* cultivation were stained with anti-IgM, annexin-V and 7-AAD. A higher frequency of dead cells (annexin-V^{+} 7-AAD^{+}) and lower frequency of live cells (Annexin-V^{-} 7-AAD^{-}) were observed in IgM^{low} B cells from BLV^{+} and BLV^{-} cattle compared to IgM^{high} B cells (Figure IV-6c, d). Moreover, IgM^{low} B cells from BLV^{+} cattle were more susceptible to cell death than those from BLV^{-} cattle.

IgM^{low} B cells contained a higher frequency of PD-$L1^{+}$ cells relative to IgM^{high} B cells

A previous study showed that the proportion of PD-$L1^{+}$ B cells was higher in BLV^{+} cattle with advanced disease stage as compared to BLV^{-} cattle (Chapter I) [Ikebuchi et al., 2011]. In this study, the PD-L1 expression was compared between IgM^{high} and IgM^{low} B cells isolated from BLV^{+} cattle by using anti-bovine PD-L1 mAb established in Chapter II. The percentages of PD-$L1^{+}$ cells in IgM^{low} B cells were higher than those in IgM^{high} B cells (Figure IV-6e). The fluorescence intensity of PD-L1 expression was not able to be compared between IgM^{high} and IgM^{low} B cells, because auto-fluorescence was different between these subsets (data not shown).

Higher expressions of proto-oncogenes and genes associated with chemotaxis and inflammatory in IgM^{low} B cells relative to IgM^{high} B cells

To investigate gene expression profile in IgM^{low} B cells which were prone to be silent in BLV expression, microarray analysis was performed using RNA isolated from both IgM^{high} and IgM^{low} B cells sorted from cultivated PBMCs of three BLV^{+} cattle. Expressions of 164 genes were upregulated and those of 28 genes were downregulated in IgM^{low} B cells compared to IgM^{high} B cells isolated from all three cattle (Table IV-1, 2). Expressions of several proto-oncogenes such as, *MafB* [Eychene et al., 2008], *Jun* [Jochum et al., 2001], *Fos/FosB* [Jochum et al., 2001; Milde-Langosch, 2005], *Frat1* [Jonkers et al., 1997], *Bcl9L* [Anastas et al., 2013] and *Fyn* [Saito et al., 2010] were augmented in IgM^{low} B cells. Then, GO analysis of the differentially expressed genes revealed that gene expressions associated with several different functions such as, chemokine activity, chemotaxis and inflammatory response were significantly augmented in IgM^{low} B cells (Table IV-3), indicating that IgM^{low} B cells could be at inflammatory state. The expression levels of *MafB*, *Jun*, *Fos*, *FosB*, *Frat1*, *Fyn*, *Ccl3*, *Ccl4*, *Il8* and *Ccr1* genes were also confirmed by quantitative real-time PCR to be upregulated in IgM^{low} B cells from nine other BLV^{+} cattle (Table IV-4).

IgM^{high} B cells were not observed in BL cells from LN

Actual surface markers of BL cells in BLV^{+} cattle have not been determined. Thus, the expression level of sIgM was measured in BL cells isolated from enlarged LNs in BCBL which was diagnosed based on CD5, CD19-like and CD21-like expression as described in Chapter III (data not shown). BL cells from 8 out of 14 cattle expressed low degrees of sIgM (Figure IV-7, Table IV-5), whereas IgM^{high} BL cells were not observed in LN of BCBL in this study. IgM^{-} B cells were also observed in BL cells isolated from 5 cattle. Heterogeneous level of IgM expression was detected in BL cells from one cattle. These data indicated that lymph node cells in BCBL mostly consist of IgM^{low} or IgM^{-} B cells, although IgM^{high} B cells were present in blood.

Table IV-1. Genes upregulated 1.5 times or more in IgM^{low} B cells relative to IgM^{high} B cells in all cases of three cattle.

Gene name	Genbank accession number	Gene symbol	Mean fold difference
chemokine (C-C motif) ligand 2	NM_174006	CCL2	35.90
T-cell receptor delta chain	D90419	TRD@	22.21
thrombospondin 1	NM_174196	THBS1	33.06
chemokine (C-X-C motif) ligand 2	NM_174299	CXCL2	30.36
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_175700	GRO1	28.65
interleukin 8	NM_173925	IL8	27.62
chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	NM_174300	CXCL6	28.50
chemokine (C-X-C motif) ligand 3	NM_001046513	CXCL3	28.60
chemokine (C-C motif) receptor 1	NM_001077839	CCR1	24.77
serpin peptidase inhibitor, clade B (ovalbumin), member 2	NM_001192079	SERPINB2	19.36
matrix metallopeptidase 1 (interstitial collagenase)	NM_174112	MMP1	16.02
v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	XM_610891	MAFB	27.57
activity-regulated cytoskeleton-associated protein	NM_001206407	ARC	12.20
interleukin 1, alpha	NM_174092	IL1A	17.97
S100 calcium binding protein A8	NM_001113725	S100A8	17.17
serum amyloid A 3	NM_181016	SAA3	13.56
interleukin 1 receptor antagonist	NM_174357	IL1RN	13.71
lymphocyte-specific protein tyrosine kinase	NM_001034334	LCK	9.48
matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	NM_174744	MMP9	12.18
potassium channel tetramerisation domain containing 12	EV787249	KCTD12	8.64
interleukin 1, beta	NM_174093	IL1B	10.68
heat shock 70kDa protein 1A	NM_174550	HSPA1A	8.59
annexin A1	NM_175784	ANXA1	9.27
CD14 molecule	BC148880	CD14	11.46
S100 calcium binding protein A12	NM_174651	S100A12	8.67
colony stimulating factor 1 receptor	NM_001075403	CSF1R	8.56
CD3e molecule, epsilon (CD3-TCR complex)	NM_174011	CD3E	7.46
inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	NM_001097568	ID1	7.83
mammary serum amyloid A3.2	NM_001242573	M-SAA3.2	7.60
coagulation factor XIII, A1 polypeptide	NM_001167894	F13A1	9.87

GTP cyclohydrolase 1	AF081949	GCH1	6.00
gTPase, IMAP family member 5-like	FE016509	LOC530077	6.22
transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyl transferase)	NM_001101848	TGM3	7.10
regulator of G-protein signaling 10	NM_001046540	RGS10	9.76
plasminogen activator, urokinase receptor	NM_174423	PLAUR	6.78
S100 calcium binding protein A10	NM_174650	S100A10	5.79
leucine rich repeat containing 25	NM_174688	LRRC25	6.97
thrombomodulin	NM_001166522	THBD	5.92
T cell receptor, alpha	BC149630	TCRA	6.25
annexin A5	NM_001040477	ANXA5	5.52
immediate early response 2	NM_001075871	IER2	4.74
dual specificity phosphatase 6	NM_001046195	DUSP6	5.75
docking protein 2, 56kDa	NM_001102002	DOK2	5.67
CD8a molecule	NM_174015	CD8A	4.65
histone cluster 1, H2ai-like	XM_602557	LOC524236	4.41
histone cluster 1, H2ag	NM_001098720	HIST1H2AG	4.52
GATS protein-like 2	XM_602920	GATSL2	4.24
neurofilament, heavy polypeptide	XM_865632	NEFH	4.63
phosphoribosyl pyrophosphate synthetase 2	XM_002700438	PRPS2	4.00
histone H3.2	NM_001166569	LOC504599	4.05
pleckstrin homology-like domain, family A, member 1	NM_001105631	PHLDA1	3.99
histone cluster 1, H2ba	XM_002697533	HIST1H2BA	3.92
chromosome 3 open reading frame, human C1orf162	XM_002705653	C3H1orf162	4.11
CCAAT/enhancer binding protein (C/EBP), beta	NM_176788	CEBPB	3.47
notch 1	XM_002691620	NOTCH1	3.65
sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	BC102274	SULT1A1	3.78
histone H2A type 1-A-like	XR_084054	LOC521825	3.36
keratin 17	NM_001105322	KRT17	3.34
histone cluster 1, H2bh-like	XM_002695159	LOC526915	3.36
serum amyloid A2	NM_001075260	SAA2	3.61
jun proto-oncogene	NM_001077827	JUN	3.30
histone cluster 1, H2ah	XM_868705	HIST1H2AH	3.30
GATA binding protein 3	NM_001076804	GATA3	3.36
histone cluster 2, H2be	NM_001099384	HIST2H2BE	3.17
hexamethylene bis-acetamide inducible 1	NM_001076181	HEXIM1	3.13
CD99 molecule	NM_001244214	CD99	3.34
chemokine (C-C motif) ligand 4	NM_001075147	CCL4	3.06
histone cluster 2, H2be-like	XM_002687403	LOC617908	3.09
adenylate cyclase 6	NM_001143877	ADCY6	2.99
histone H2B-like	NM_001114854	H2B	3.05

histone cluster 1, H2bl-like	XM_602005	LOC523702	3.03
Rho guanine nucleotide exchange factor (GEF) 10-like	NM_001046297	ARHGEF10L	2.92
G protein-coupled receptor 171	NM_001077002	GPR171	3.82
transthyretin	NM_173967	TTR	2.82
argininosuccinate synthase 1	NM_173892	ASS1	3.11
cyclin-dependent kinase inhibitor 1C (p57, Kip2)	NM_001077903	CDKN1C	2.99
LEM domain containing 3	NM_001192699	LEMD3	2.82
complement factor B	NM_001040526	CFB	3.31
S100 calcium binding protein A11	NM_001098856	S100A11	2.94
plexin D1	XM_001789172	PLXND1	3.19
phenylethanolamine N-methyltransferase	NM_177505	PNMT	2.67
histone cluster 2, H2be-like	XM_002687190	LOC521525	2.73
histone H2B type 1-like	XM_002699033	LOC512612	2.72
FBJ murine osteosarcoma viral oncogene homolog	NM_182786	FOS	2.71
FBJ murine osteosarcoma viral oncogene homolog B	NM_001102248	FOSB	2.73
tracheal antimicrobial peptide	NM_174776	TAP	2.73
leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3-like	XR_138959	LOC617702	2.98
histone H2B type 1-like	XM_002691912	LOC617043	2.65
histone cluster 3, H2bb	XM_598354	HIST3H2BB	2.60
adrenergic, beta-2-, receptor, surface	NM_174231	ADRB2	2.58
histone H2B type 1-like	XM_002696342	LOC614206	2.63
histone H2B type 1-like	XM_002693492	LOC520044	2.64
SRY (sex determining region Y)-box 4	NM_001078128	SOX4	2.57
MARCKS-like 1	NM_001076172	MARCKSL1	2.54
WDFY family member 4	NM_001205945	WDFY4	2.62
immediate early response 5-like	NM_001206835	IER5L	2.56
CD68 molecule	NM_001045902	CD68	2.82
pyroglutamylated RFamide peptide	NM_198222	QRFP	2.48
frequently rearranged in advanced T-cell lymphomas	XM_002698415	FRAT1	2.45
elastin microfibril interfacer 2	NM_001143869	EMILIN2	2.82
histone H2B type 1-like	XM_001249778	LOC528342	2.47
tumor necrosis factor receptor superfamily, member 1A	NM_174674	TNFRSF1A	2.67
RAS p21 protein activator 3	NM_174676	RASA3	2.55
interleukin 13 receptor, alpha 1	NM_001206677	IL13RA1	2.57
chromosome 21 open reading frame, human C14orf180	NM_001205388	C21H14orf180	2.57
neurocan	NM_001193082	NCAN	2.37
SH2 domain containing 2A	NM_001105401	SH2D2A	2.39
CUE domain containing 1	NM_001099064	CUEDC1	2.36
protein phosphatase 1, regulatory	NM_001046178	PPP1R15A	2.36

subunit 15A			
uncharacterized LOC615263	NM_001103298	LOC615263	2.39
chromosome 7 open reading frame, human C19orf29	XM_607390	C7H19orf29	2.41
polymerase I and transcript release factor	NM_001111103	PTRF	2.30
ATG16 autophagy related 16-like 2 (S. cerevisiae)	NM_001192280	ATG16L2	2.29
matrix metallopeptidase 15 (membrane-inserted)	NM_001191434	MMP15	2.28
CD3g molecule, gamma (CD3-TCR complex)	NM_001040472	CD3G	2.30
lymphocyte cytosolic protein 2 (SH2 domain containing leukocyte protein of 76kDa)	NM_001076844	LCP2	2.40
keratin 19	NM_001015600	KRT19	2.26
tyrosine kinase with immunoglobulin-like and EGF-like domains 1	NM_173965	TIE1	2.28
stomatin	NM_001105473	STOM	2.29
N-myc downstream regulated 1	NM_001035009	NDRG1	2.35
solute carrier organic anion transporter family, member 3A1	NM_001001134	SLCO3A1	2.25
family with sequence similarity 57, member B	NM_001105465	FAM57B	2.19
diacylglycerol kinase, delta 130kDa	NM_001035282	DGKD	2.24
regulator of G-protein signaling 2, 24kDa	NM_001075596	RGS2	2.19
chromosome 15 open reading frame, human C11orf53	XM_870687	C15H11orf53	2.15
transmembrane BAX inhibitor motif containing 1	NM_205798	TMBIM1	2.19
UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 4	NM_001038078	B4GALT4	2.10
SRY (sex determining region Y)-box 17	NM_001206251	SOX17	2.25
major vault protein	NM_001035317	MVP	2.17
H2A histone family, member X	NM_001079780	H2AFX	2.09
protein kinase C, eta	NM_001076863	PRKCH	2.10
B-cell CLL/lymphoma 9-like	NM_001205656	BCL9L	2.05
leukocyte receptor cluster (LRC) member 8	NM_001102100	LENG8	2.08
tumor necrosis factor receptor superfamily, member 12A	NM_001206328	TNFRSF12A	2.10
nuclear protein, transcriptional regulator, 1	NM_001114515	NUPR1	2.09
olfactory receptor, family 7, subfamily D, member 2	XM_585830	OR7D2	2.06
chromosome 10 open reading frame, human C15orf59	NM_001105432	C10H15orf59	2.04
olfactory receptor, family 4, subfamily D, member 11-like	XM_001789446	LOC788587	2.01

glutaminyl-peptide cyclotransferase-like	NM_001075940	QPCTL	1.96
early growth response 1	NM_001045875	EGR1	1.95
heat shock 70kDa protein 6 (HSP70B')	XM_002685850	HSPA6	1.95
serum/glucocorticoid regulated kinase 1	NM_001102033	SGK1	1.95
serine palmitoyltransferase, small subunit B	NM_001110774	SPTSSB	1.93
regulator of cell cycle	NM_001102276	RGCC	1.92
transmembrane and immunoglobulin domain containing 2	CO883749	TMIGD2	1.97
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	NM_001075148	HSPA5	1.92
FYN oncogene related to SRC, FGR, YES	NM_001077972	FYN	1.89
chemokine (C-C motif) ligand 3	CK769448	CCL3	1.84
histone H2A type 1-D-like	XM_003583895	LOC100848734	1.82
protein tyrosine phosphatase, non-receptor type 7	NM_001040576	PTPN7	1.81
calcium channel, voltage-dependent, beta 3 subunit	NM_174509	CACNB3	1.81
aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	DV787875	AKR1C3	1.77
capping protein (actin filament), gelsolin-like	NM_178574	CAPG	1.78
chromosome 8 open reading frame, human C9orf3	NM_001206980	C8H9orf3	1.73
potassium voltage-gated channel, Shaw-related subfamily, member 1	NM_001113281	KCNC1	1.73
RAS-like, family 11, member A	EV691903	RASL11A	1.72
CD97 molecule	AJ627191	CD97	1.70
alanyl-tRNA synthetase 2, mitochondrial (putative)	NM_001191211	AARS2	1.69
Kruppel-like factor 6	NM_001035271	KLF6	1.66
ras homolog gene family, member C	NM_001046138	RHOC	1.61
prostaglandin E receptor 2 (subtype EP2), 53kDa	NM_174588	PTGER2	1.61
PARP1 binding protein	XM_867341	PARPBP	1.60
RAS-like, family 11, member B	NM_001015635	RASL11B	1.59
regulator of calcineurin 1	NM_001034679	RCAN1	1.57

Table IV-2. Genes downregulated 1.5 times or more in IgM^{low} B cells relative to IgM^{high} B cells in all cases of three cattle.

Gene name	Genbank accession number	Gene symbol	Mean fold difference
glycoprotein 2 (zymogen granule membrane)	NM_001075950	GP2	-2.63
SH3 domain binding glutamic acid-rich protein like 2	NM_001083791	SH3BGRL2	-1.97
cat eye syndrome chromosome region, candidate 1	DV882019	CECR1	-1.95
thromboxane A2 receptor	NM_001167919	TBXA2R	-1.91
acyloxyacyl hydrolase (neutrophil)	NM_001078096	AOAH	-1.86
interleukin 32	BC134507	IL32	-1.82
uncharacterized LOC618541	BC133492	LOC618541	-1.81
tripartite motif containing 44	NM_001105014	TRIM44	-1.81
uncharacterized protein MGC137030	NM_001077081	MGC137030	-1.82
dynamin 1	NM_001076820	DNM1	-1.78
4-aminobutyrate aminotransferase	NM_001081581	ABAT	-1.78
EF-hand calcium binding domain 9	BC102622	EFCAB9	-1.76
phospholipase D family, member 4	XM_002696851	PLD4	-1.75
protein kinase C and casein kinase substrate in neurons 1	NM_001101101	PACSIN1	-1.73
neurotrophin receptor associated death domain	NM_001076398	NRADD	-1.70
complement component (3d/Epstein Barr virus) receptor 2	NM_001198991	CR2	-1.70
cysteinyl leukotriene receptor 1	NM_001099726	CYSLTR1	-1.69
transmembrane protein 110	NM_001076117	TMEM110	-1.68

mannosidase, endo-alpha-like	NM_001102105	MANEAL	-1.67
euchromatic histone-lysine N-methyltransferase 2	NM_001206263	EHMT2	-1.66
CD1a molecule-like	NM_001102024	LOC515418	-1.64
H2.0-like homeobox	NM_001101097	HLX	-1.63
napsin A aspartic peptidase	XM_001790617	NAPSA	-1.62
growth arrest-specific 7	NM_001102280	GAS7	-1.61
slit homolog 2 (Drosophila)	NM_001191516	SLIT2	-1.59
dihydropyrimidinase-like 2	NM_001076000	DPYSL2	-1.54
nephronophthisis 4	XM_002694114	NPHP4	-1.54
phytanoyl-CoA dioxygenase domain containing 1	NM_001076243	PHYHD1	-1.54

Table IV-3. Functional grouping of differentially expressed genes in IgM^{low} B cells.

Functional group / gene (GO accession number and corrected *P*-value)	Gene symbol	Mean fold difference
chemokine activity (0008009; 1.077E-07)		
chemokine (C-C motif) ligand 2	CCL2	35.90
chemokine (C-X-C motif) ligand 2	CXCL2	30.36
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	GRO1	28.65
chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CXCL6	28.50
interleukin 8	IL8	27.62
chemokine (C-C motif) ligand 4	CCL4	3.06
chemokine (C-C motif) ligand 3	CCL3	1.84
chemotaxis (0006935; 0.03309)		
chemokine (C-C motif) receptor 1	CCR1	24.77
S100 calcium binding protein A8	S100A8	17.17
GATA binding protein 3	GATA3	3.36
inflammatory response (0006954; 4.750E-08)		
interleukin 1, alpha	IL1A	17.97
interleukin 1 receptor antagonist	IL1RN	13.71
CD14 molecule	CD14	11.46
interleukin 1, beta	IL1B	10.68
S100 calcium binding protein A12	S100A12	8.67
serum amyloid A2	SAA2	3.61
tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	2.67

Among 37 GO terms enriched by GO analysis, selected three terms and represented genes in the three were indicated.

Table IV-4. Differentially expressed genes confirmed by quantitative real-time PCR in IgM^{low} B cells from nine BLV^{+} cattle.

Gene name	Gene symbol	Mean fold difference
v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	MAFB	196.62
jun proto-oncogene	JUN	66.95
FBJ murine osteosarcoma viral oncogene homolog	FOS	51.84
FBJ murine osteosarcoma viral oncogene homolog B	FOSB	23.00
frequently rearranged in advanced T-cell lymphomas	FRAT1	132.25
FYN oncogene related to SRC, FGR, YES	FYN	17.66
chemokine (C-C motif) ligand 3	CCL3	2.62
chemokine (C-C motif) ligand 4	CCL4	5.94
interleukin 8	IL8	155.43
chemokine (C-C motif) receptor 1	CCR1	39.38

Expression levels of all indicating genes in IgM^{low} B cells were significantly higher than those in IgM^{high} B cells ($P < 0.01$). Statistical comparisons between IgM^{high} and IgM^{low} B cells were made using the Wilcoxon matched-pairs test.

Table IV-5. IgM expression in BL cells isolated from fourteen BCBL.

IgM expression in enlarged LN	IgM^{high}	$IgM^{high/low}$	IgM^{low}				IgM^{-}		
Number	0	1	8				5		
IgM expression in blood		N. D.	high/low	low	-	N. D.	high/low	low	-
Number		1	4	2	0	2	2	1	2

N. D. indicates not determined because of sample limitation.

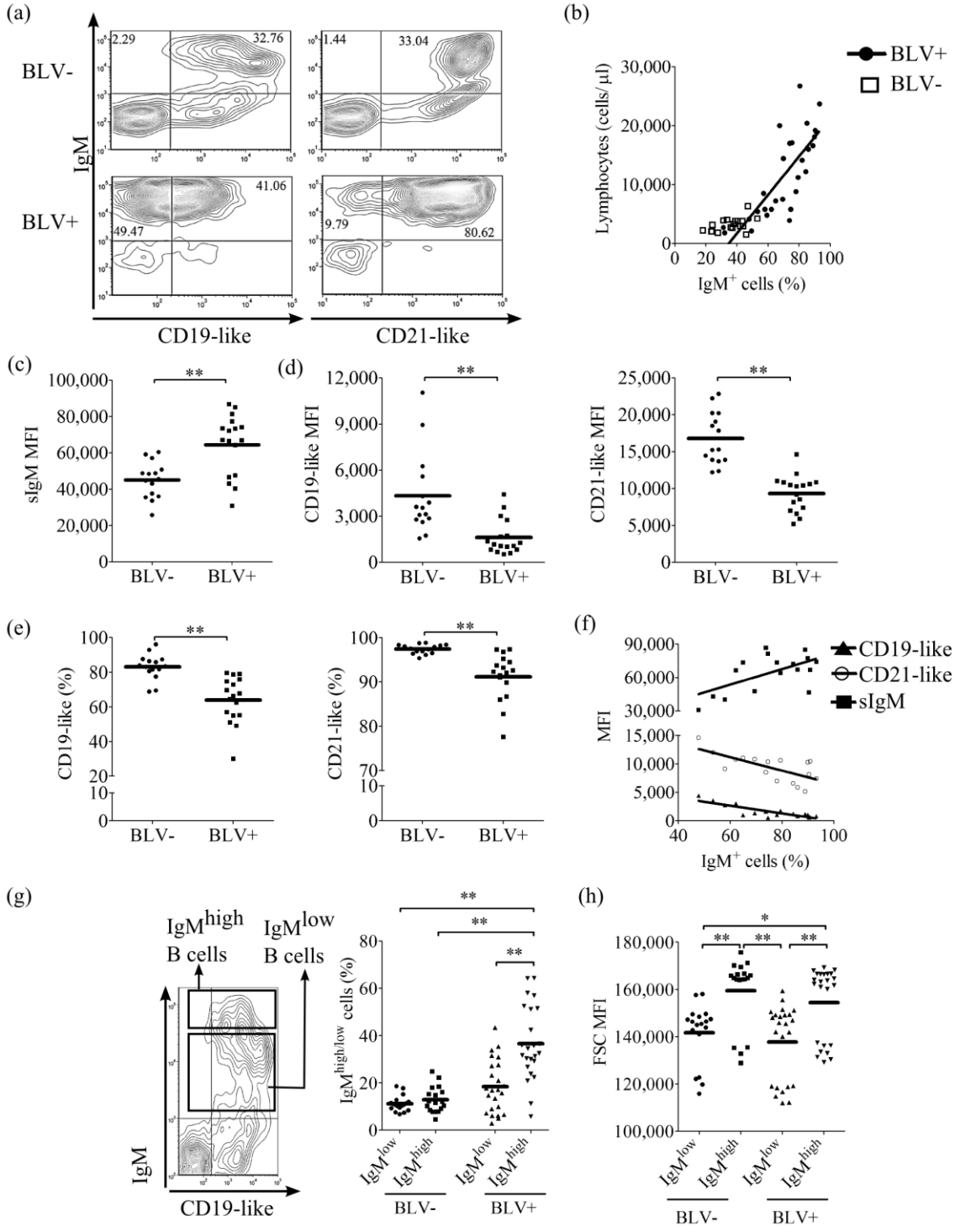

Figure IV-1. Phenotypic profile of B cells in BLV^+ cattle.
(a) Representative contour plots of IgM versus CD19-like, and IgM versus CD21-like molecules in BLV^+ and BLV^- cattle. Values in the quadrant and histograms indicate the percentage of the cells. (b) Correlation between the percentages of IgM^+ cells in PBMCs and

number of lymphocytes in blood isolated from BLV^+ and BLV^- cattle (n = 29 and 18; $P < 0.01$, r = 0.8038). (c, d) MFI of sIgM (c), CD19 and CD21 (d) expression in in IgM^+ B cells from BLV^+ and BLV^- cattle (n = 17 and 15). (e) Percentages of $CD19^+$ cells and $CD21^+$ cells in IgM^+ B cells from BLV^+ and BLV^- cattle (n = 17 and 15). (f) Correlations between the percentages of IgM^+ cells in PBMCs and MFI of CD19, CD21 and sIgM expression in BLV^+ cattle (n = 17; $P < 0.01$, r = -0.7353 for CD19; $P < 0.01$, r = -0.6397 for CD21; $P < 0.05$, r = 0.4485 for sIgM). (g, h) Gating strategy, percentages (g) and FSC MFI (h) of IgM^{high} and IgM^{low} B cells in PBMCs isolated from BLV^+ and BLV^- cattle (n = 24 and 18). Lines in correlation diagrams indicate the approximation straight lines of data of BLV^+ cattle and were created by fitted linear regression (b, f). Correlation statistics were analyzed using the Spearman correlation. Each of line in data of BLV^+ and BLV^- cattle indicates the mean values in each group (c, d, e, g). Statistical comparisons between the data of BLV^+ and BLV^- cattle were made using the Mann Whitney test or the one-way ANOVA with Tukey's test. Differences were considered statistically significant at $P < 0.05$ (** $P < 0.01$).

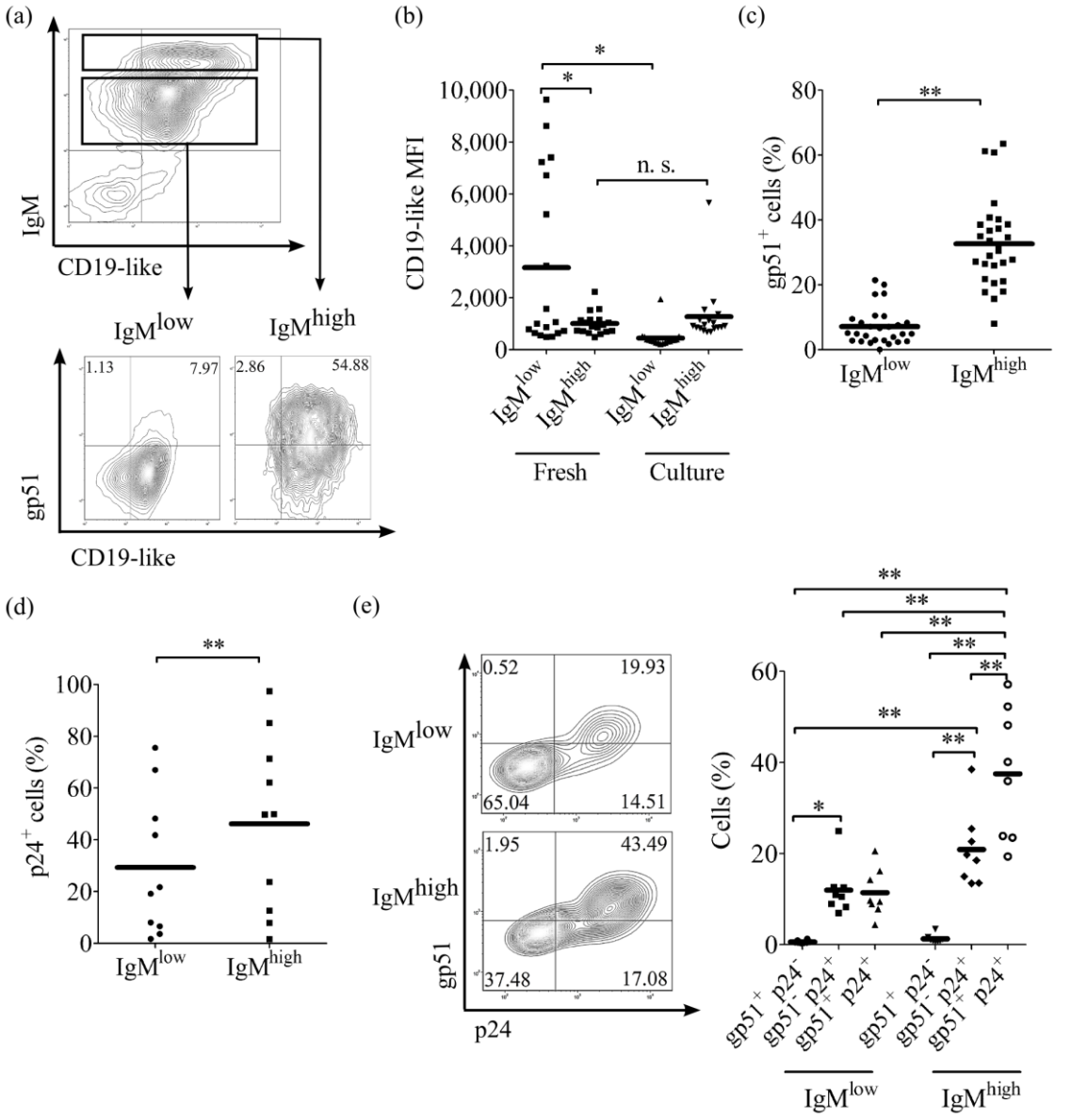

Figure IV-2. Expression of BLV antigens, gp51 and p24 in IgM^{high} and IgM^{low} B cells after *ex vivo* cultivation.

(a) Representative contour plots and histograms of IgM and gp51 expression in cultivated lymphocytes from BLV^+ cattle. (b) MFI of CD19 expression in IgM^{high} and IgM^{low} B cells isolated from fresh and cultivated PBMCs from BLV^+ cattle (n = 18). (c, d) Percentages of $gp51^+$ cells (c; n = 28) and $p24^+$ cells (d; n = 10) in IgM^{high} and IgM^{low} B cells. (e) Representative contour plots of gp51expression versus p24 expression and percentages of $gp51^+$ $p24^-$, $gp51^-$ $p24^+$ and $gp51^+$ $p24^+$ cells in IgM^{high} and IgM^{low} B cells (n = 8). Values in the quadrant and histograms indicate the percentage of the cells. Each of line indicates the mean values in each group. Statistical comparisons were made using the Wilcoxon matched-pairs test and the one-way ANOVA with Tukey's test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

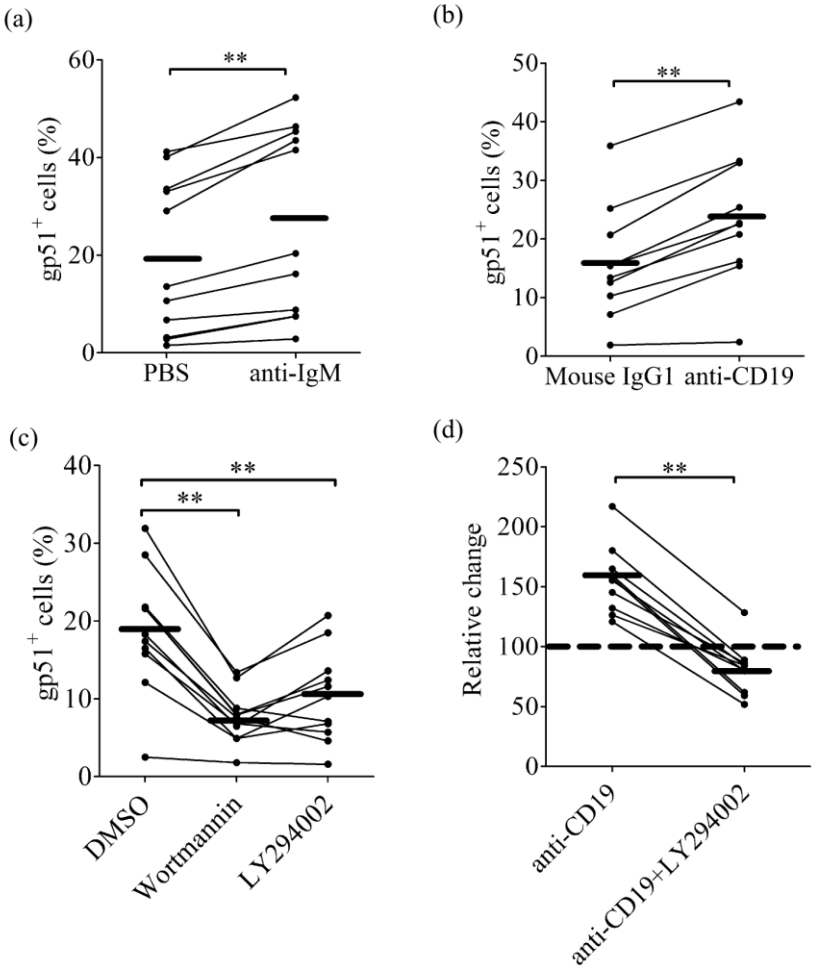

Figure IV-3. BLV-gp51 expression in B cells after anti-IgM and anti-WC4 stimulation.
(a, b, c) Percentages of $gp51^+$ cells in IgM^+ B cells purified by autoMACS and treated with anti-IgM (a; $n = 11$), anti-WC4 (b; $n = 10$) and PI3-kinase inhibitors (c; $n = 11$; wortmannin and LY294002). (d) Relative change to control (mouse IgG1; indicated as a dashed line) in proportions of $gp51^+$ cells among IgM^+ B cells treated with both anti-WC4 and LY294002 ($n = 10$). Each of line indicates the mean values in each group. Statistical comparisons between cells treated with the stimulants were made using the one-way ANOVA with Tukey's test and the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (** $P < 0.01$).

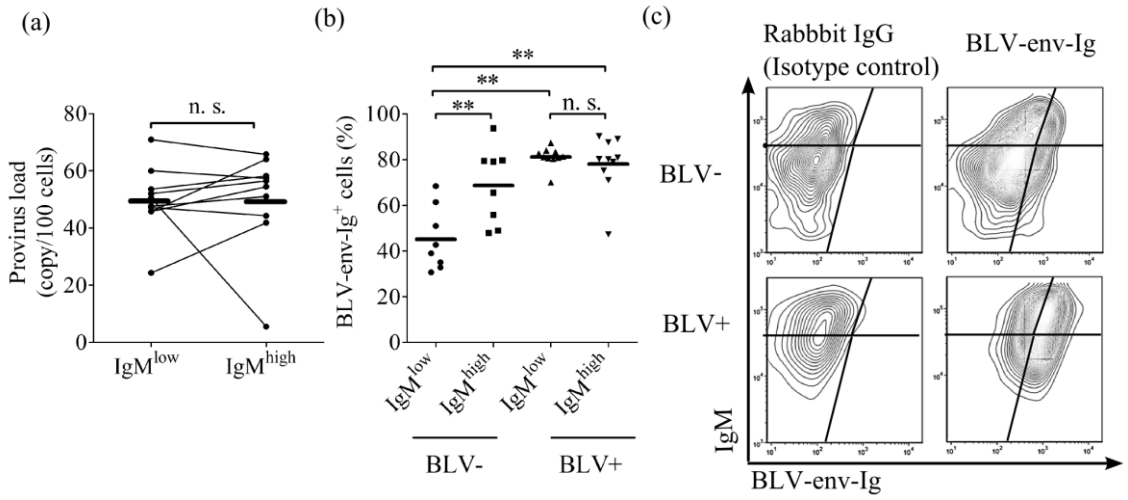

Figure IV-4. Provirus load and expression of BLV-binding receptors in IgM^{high} and IgM^{low} B cells.

(a) Calculated copy number of provirus in 100 cells among IgM^{high} and IgM^{low} B cells. Provirus load was measured by real-time PCR with DNA purified from IgM^{high} and IgM^{low} B cells isolated by cell sorter from cultivated PBMCs (n = 10). Statistical comparisons between IgM^{high} and IgM^{low} B cells were made using the Wilcoxon matched-pairs test. (b) Percentages of BLV-binding receptors-expressing cells in IgM^{high} and IgM^{low} B cells from BLV^+ and BLV^- cattle (n = 10 and 8). BLV-binding receptors were detected by BLV-env-Ig. Statistical comparisons between BLV^+ and BLV^- cattle were made using the one-way ANOVA with Tukey's post test. (c) Representative contour plots of BLV-env-Ig staining in PBMCs isolated from BLV^+ cattle. Each of line indicates the mean values in each group. Differences were considered statistically significant at $P < 0.05$ (** $P < 0.01$).

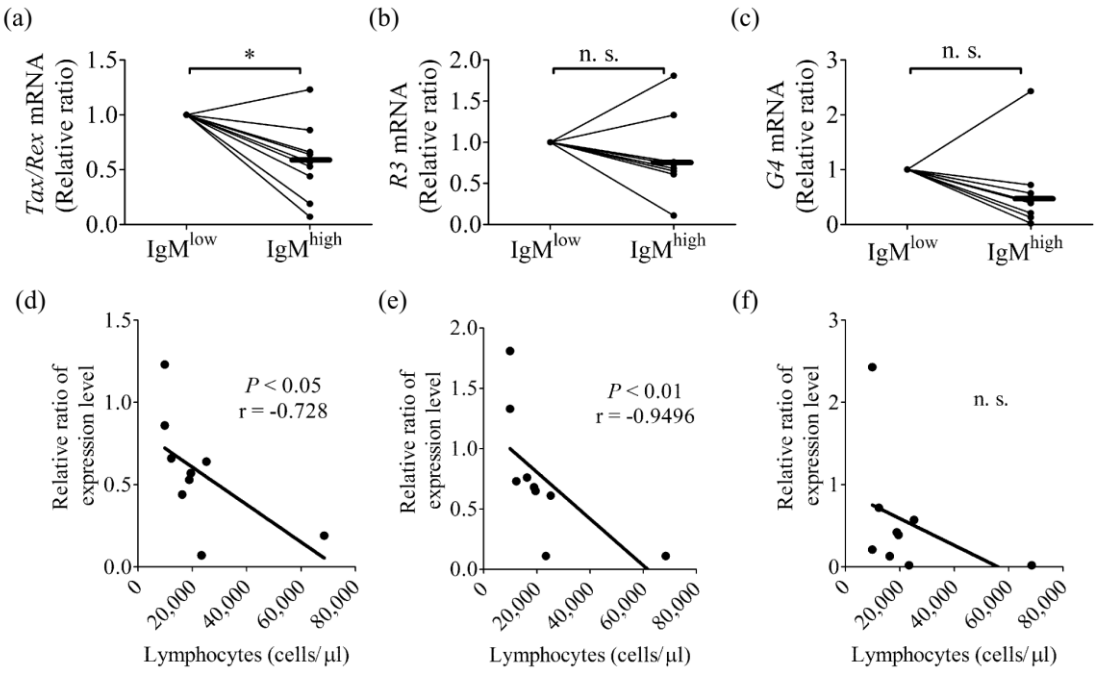

Figure IV-5. Expression analyses of BLV-associated genes in IgM^{high} and IgM^{low} B cells.
(a, b, c) Relative ratio of *Tax/Rex* (a), *R3* (b) and *G4* (c) mRNA expression levels in IgM^{high} B cells compared with IgM^{low} B cells (n = 9). Each of line indicates the mean values in each group. Statistical comparisons between IgM^{high} and IgM^{low} B cells were made using the Wilcoxon matched-pairs test. (d, e, f) Correlation between lymphocytes number as progenitor of disease progression and relative ratios of *Tax/Rex* (d), *R3* (e) and *G4* (f) mRNA expression (n = 9). Correlation statistics were analyzed using the Spearman correlation. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$).

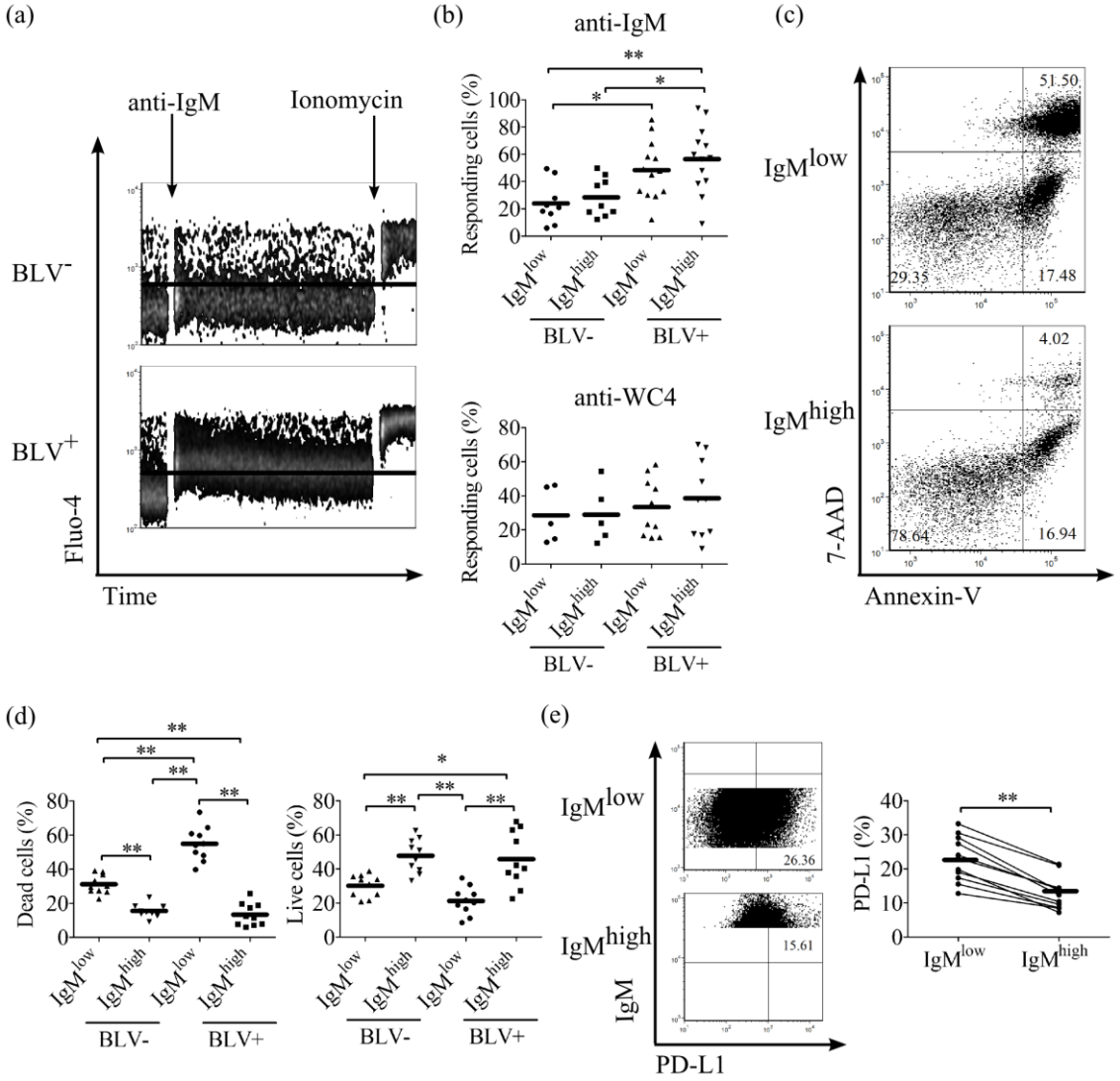

Figure IV-6. Functional analyses of IgM^{high} and IgM^{low} B cells.
(a) Representative density plots of calcium mobilization in IgM^{high} B cells from BLV^{+} and BLV^{-} cattle in stimulation of anti-IgM. Ionomycin was added 4 min after the addition of anti-IgM or anti-WC4. Responses were measured by detecting the peak percentage of the cells with fluorescence intensity above the threshold determined from unstimulated sample (lines). (b) Percentages of responding cells in IgM^{high} and IgM^{low} B cells. IgM^{+} B cells isolated from BLV^{+} and BLV^{-} cattle were stimulated with anti-IgM (BLV^{-} and BLV^{+} cattle; n = 9 and 13) and anti-WC4 (n = 5 and 10). (c) Representative dot plots of Annexin-V and 7AAD staining in IgM^{high} and IgM^{low} B cells among cultivated PBMCs from BLV^{+} cattle. PBMCs isolated from BLV^{+} and BLV^{-} cattle were cultivated overnight and stained with Annexin-V and 7AAD. (d) Percentages of dead (Annexin-V^{+} 7-AAD^{+}) and live cells (Annexin-V^{-} 7-AAD^{-}) in IgM^{high} and IgM^{low} B cells. (e) Representative dot plots of PD-L1 expression and percentages of PD-L1^{+} cells in IgM^{high} and IgM^{low} B cells from BLV^{+} cattle. Values in the quadrant indicate the percentage of the cells. Each of line indicates the mean values in each group. Statistical comparisons were made using the one-way ANOVA with Tukey's test and the Wilcoxon matched-pairs test. Differences were considered statistically significant at $P < 0.05$ (* $P < 0.05$; ** $P < 0.01$).

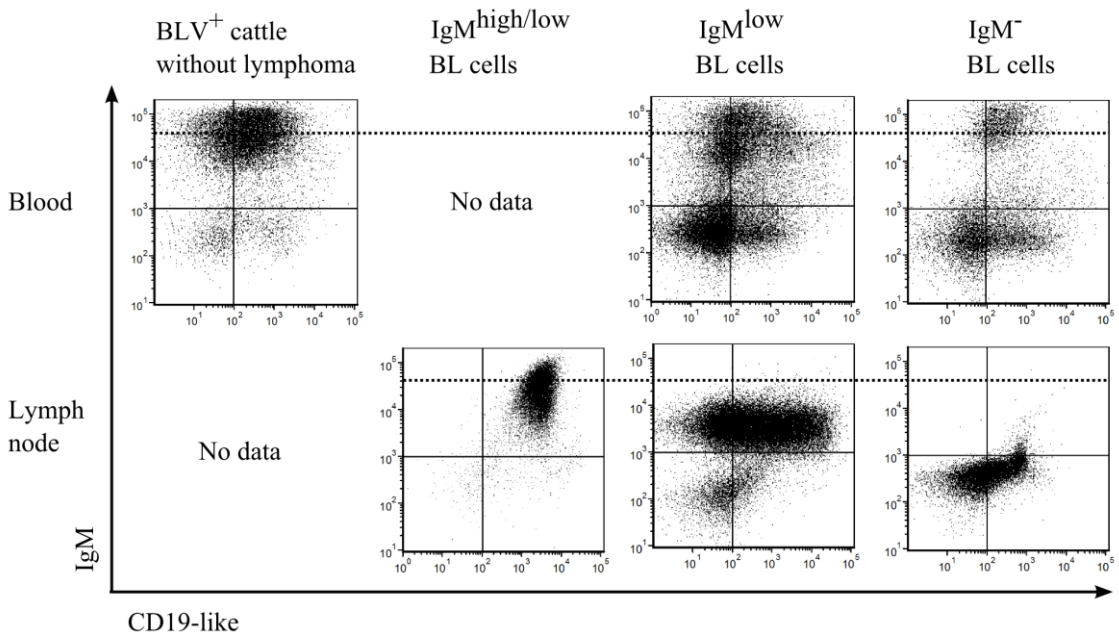

Figure IV-7. sIgM expression in BL cells.
Representative dot plots of $IgM^{high/low}$, IgM^{low} and IgM^{-} BL cells in LN isolated from BCBL (lower plots). Upper plots show lymphocytes in blood collected from BLV^{+} cattle with or without lymphoma. Dashed lines indicate boundary between fluorescence intensity of IgM^{high} and IgM^{low} B cells.

DISCUSSION

It is well known that B cell activation by mitogens upregulated BLV expression *ex vivo* [Kidd et al., 1996; Lagarias et al., 1989]. In this study, it was demonstrated that IgM^{high} B cells from BLV^{+} cattle were prone to elevate the levels of intracellular calcium in response to BCR stimulation as compared to IgM^{+} B cells from BLV^{-} cattle. This high responsiveness to antigen stimulation could contribute to cellular activation and BLV antigen expression in IgM^{high} B cells, which include a high proportion of BLV-expressing B cells. Thus, IgM^{high} B cells seem to play a central role as a source to expand BLV infection to BLV^{-} B cells *in vivo*.

Almost of all IgM^{low} B cells seemed to be BLV-silencing cells which were able to evade from immune surveillance [Gillet et al., 2007]. In fact, p24 antigen was expressed in 29.3% of IgM^{low} B cells (average value in Figure IV-2d), but only 7.1% of IgM^{low} B cells express gp51. The gp51 expression is essential for cell-to-cell infection, due to the instable nature of BLV particles outside the cell membrane [Derse et al., 2001; Gatot et al., 1998; Igakura et al., 2003; Johnston et al., 1996]. Thus, $p24^{+}$ IgM^{low} B cells seem to be an inefficient population to expand BLV infection. In the future work, identification of the additional markers other than sIgM is essential to study the mechanism for BLV-silencing, because it is still difficult to completely distinguish BLV-expressing from -silencing cells through the detection of sIgM expression.

The increased numbers of IgM^{high} B cells in blood from BLV^{+} cattle are likely not to be caused by susceptibility to BLV infection, because the expression levels of BLV-binding receptors are equivalent in IgM^{high} and IgM^{low} B cells from BLV^{+} cattle. Another possibility to increase the proportion of IgM^{high} B cells could be that production of immature B cells in primary lymphoid organs is augmented by BLV infection. Many studies showed that high sIgM expression was observed in the immature B cells emigrating from ileal Peyer's patch in sheep [Griebel et al., 1992; Yasuda et al., 2006] and immature transitional 1 B cells in mice models [Cambier et al., 2007; Teague et al., 2007]. Furthermore, Fulton et al. [2006] clarified that the emergence of IgM^{high} B cells was detected only in blood, not in efferent lymph after BLV infection in sheep. From these reports, one may imply that IgM^{high} B cells increasing in blood from BLV^{+} cattle could be non-recirculating immature B cells

derived from either bone marrow or Peyer's patch, which is the primary lymphoid organs for B cell development and B cell diversity in cattle and sheep [Yasuda et al., 2006]. Meanwhile, in the case of HTLV-1, immature thymocytes are targets for the infection and could be selected over time for the emergence of malignant clones in the thymus of HTLV-1-infected patients [Feuer et al., 1996; Hasegawa et al., 2006; Matsuoka et al., 2007]. Thus, progenitor cells of B cells could be infected with BLV in the primary lymphoid organs. To test this hypothesis, further studies about the mechanism of bovine B cell development are required.

CD 19 expression on B cells is essential to induce germinal center (GC) reaction in LN after the recognition of a cognate antigen with follicular DCs [Depoil et al., 2008]. The GC reaction is thought to be an important step for the development of BL induced by EBV [Kuppers, 2003]. Furthermore, CD19 promotes oncogene expression and tumor growth in the case of BL in human, and a longer survival was observed in the patients with low CD19 expression levels [Chung et al., 2012]. In this study, the high expression of CD19-like molecule was observed in IgM^{low} B cells among lymphocytes freshly-isolated from BLV^{+} cattle. Thus, IgM^{low} CD19-$like^{high}$ B cells could possess a tendency to expand and develop into the lymphoma cells via GC reaction.

The results obtained in this study showed that both IgM^{high} and IgM^{low} B cell subsets possessed the inherent capability to survive *in vivo* (Table IV-6). From the data concerning the proportions of live and dead cells in cultivated PBMCs, IgM^{high} B cells seem to be superior to survive. However, the capability to expand *in vivo* was also shown in IgM^{low} B cells. Higher PD-L1 expression and lower gp51 expression in this subset are involved in immune escape from virus-specific T cells. Higher expression of CD19-like molecule seems to operate in favor of antigen recognition and cellular activation in the antigen presentation from DCs [Depoil et al., 2008]. Moreover, the expression levels of the *Tax/Rex* gene and some proto-oncogenes (*MafB*, *Jun*, *Fos*, *FosB*, *Frat1*, and *Fyn*) were increased in IgM^{low} B cells, and IgM^{high} BL cells were not observed in LN from all of the fourteen BCBL, raising the possibility that either IgM^{low} B cells or clones derived from these cells are prone to become neoplastic B cells as compared to IgM^{high} B cells. This possibility is also supported by microarray data; chemokine genes and genes associated with chemotaxis, such as CCL3, CCL4, IL-8 and CCR1 were upregulated in IgM^{low} B cells. In CLL patients, the recirculation and the spread of CLL cells in the body were preferentially induced by the CCL3 and CCR1 system [Trentin et al., 2004]. Moreover, CCL3, CCL4 and IL-8

derived from lymphoma cells induce the homing of accessory cells which create supportive microenvironment for the maintenance and the expansion of the neoplastic cells [Burger, 2010]. In a similar fashion, IgM^{low} B cells in BLV^{+} cattle could induce trafficking and migration of other B cells and accessory cells into lymphoid organs, resulting in the development of enlarged LN in BCBL. Future work should be directed to investigate immune escape, migration and transformation in IgM^{low} B cells *in vivo*.

IgM^{-} BL cells were observed in LN from five BCBL in this study, and it was also reported that some cases of B cells transformed by EBV express no surface immunoglobulin [Kuppers, 2003]. This loss of BCR expression is thought to be caused by destructive immunoglobulin gene mutations in GC reaction. B cells which acquire such mutation normally tend to undergo apoptosis, but IgM^{-} BL cells could overcome the apoptotic signal through some transforming events. Thus, IgM^{-} BL cells in BLV^{+} cattle could be developed after antigen recognition and the somatic hypermutation in LN.

Contrary to BCR expression of BLV^{+} B cells, the expression level of T cell receptor, CD3, in HTLV-1-infected T cells is decreased gradually in line with disease progression. Such progressive losses of CD3 expression were observed in both HTLV-1-infected cell lines and $CD4^{+}$ T cells from HTLV-1-infected patients [Akl et al., 2007; Kobayashi et al., 2013]. A study using $CD4^{+}$ T cell lines experimentally infected with HTLV-1 also revealed that $CD3^{high}$ and $CD3^{low}$ T cells were equivalent in their capacity to express HTLV-1 core protein [Willard-Gallo et al., 2001]. Moreover, despite structural and functional homology between HTLV-1 and BLV, the treatment of calcium ionophore to HTLV-1-infected T cells reduced viral expression [Copeland et al., 1994], but upregulated in the case of BLV^{+} B cells [Kerkhofs et al., 1996]. These reports and the data obtained in this study suggest that HTLV-1-infected T cells and BLV^{+} B cells appear to use different signaling pathways and strategies to express viral antigens and to expand the infection to uninfected cells.

This study showed that IgM^{high} B cells were increased in number in blood of BLV^{+} cattle, prone to express BLV antigen after *ex vivo* cultivation, and could play a role as the source of the BLV infection. On the other hand, IgM^{low} B cells include a high proportion of BLV-silencing cells, express high levels of *Tax/Rex* mRNA and some proto-oncogenes and could subsequently produce some clones of neoplastic B cells. This is the first report investigating IgM^{high} and IgM^{low} B cells in BLV^{+} cattle. The information on these two B cell subsets is useful to investigate the mechanisms of BLV

silencing, disease progression and transformation in BLV infection.

Table IV-6. Contradistinction of expected function between IgM^{high} B cells versus IgM^{low} B cells.

Prospective function *in vivo* (Suggesting data)	IgM^{high} B cells	IgM^{low} B cells
Reactivity to antigen presentation by DCs (CD19-like expression)	+	+ +
BLV propagation/immune evasion (gp51 expression)	+ +	+
Infectious rate (provirus load)	+ +	+ +
Apoptosis resistant (cell death rate after *ex vivo* cultivation)	+ +	+
Immune evasion from BLV/tumor-specific T cells (PD-L1 expression)	+	+ +
Transformation? (expressions of *Tax/Rex* and some proto-oncogenes)	+	++

Underlines indicate the subset seems to be superior to survive *in vivo*. (++; strong, +; weak)

SUMMARY

BLV, which is closely related to HTLV-1, induces abnormal B cell proliferation and BL in BLV^+ cattle, and its provirus is integrated into the host genome. Bovine B cells infected with BLV rarely express viral proteins *in vivo*, but a short term *ex vivo* cultivation augments the BLV expression in a part of B cells, not all BLV^+ B cells. This observation showed that two subsets, BLV-silencing cells and -expressing cells, are present among B cells in BLV^+ cattle, although the mechanism of viral expression in B cells is not still determined. In this study, to identify markers discriminating BLV-expressing from -silencing B cells, sIgM expression and viral antigen expression in B cells from BLV^+ cattle were examined. The expression level of sIgM was augmented in BLV^+ cattle in line with disease progression of BLV infection, and the percentages of IgM^{high} B cells were increased in blood lymphocytes from BLV^+ cattle. After *ex vivo* cultivation, IgM^{high} B cells mainly expressed BLV-gp51 and BLV-p24 while IgM^{low} B cells did not, although the provirus load was equivalent in IgM^{high} and IgM^{low} B cells. To identify the cellular behavior, several parameters of these two subsets were investigated. Percentages of cells expressing PD-L1, which inhibits T cell immunity, were higher in IgM^{low} than IgM^{high} B cells. The expression levels of the *Tax/Rex* mRNA, viral oncogene, were higher in IgM^{low} B cells. Moreover, microarray analyses revealed higher expression levels of some proto-oncogenes, such as *Maf*, *Jun* and *Fos* in IgM^{low} B cells. BL cells from enlarged LN in fourteen BCBL consisted of IgM^{low} or IgM^- B cells, but not IgM^{high} B cells. This is the first report to clarify that IgM^{high} B cells in blood are mainly consist of BLV-expressing B cells in BLV^+ cattle, whereas IgM^{low} B cells include a high proportion of BLV-silencing B cells, are superior to evade from immune surveillance system and could become neoplastic clones.

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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REFERENCES

Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H, Honjo T: **Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes.** *International Immunology* 1996, **8**:765–772.

Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, Rosenberg SA: **Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired.** *Blood* 2009, **114**:1537–1544.

Akl H, Badran B, Dobirta G, Manfouo-Foutsop G, Moschitta M, Merimi M, Burny A, Martiat P, Willard-Gallo KE: **Progressive loss of CD3 expression after HTLV-I infection results from chromatin remodeling affecting all the CD3 genes and persists despite early viral genes silencing.** *Virology Journal* 2007, **4**:85.

Anastas JN, Moon RT: **WNT signalling pathways as therapeutic targets in cancer.** *Nature Reviews Cancer* 2013, **13**:11–26.

Andorsky DJ, Yamada RE, Said J, Pinkus GS, Betting DJ, Timmerman JM: **Programmed death ligand 1 is expressed by non-hodgkin lymphomas and inhibits the activity of tumor-associated T cells.** *Clinical Cancer Research* 2011, **17**:4232–4244.

Arase H, Mocarski ES, Campbell AE, Hill AB, Lanier LL: **Direct recognition of cytomegalovirus by activating and inhibitory NK cell receptors.** *Science* 2002, **296**:1323–1326.

Armour KL, Clark MR, Hadley AG, Williamson LM: **Recombinant human IgG molecules lacking Fcgamma receptor I binding and monocyte triggering activities.** *European Journal of Immunology* 1999, **29**:2613–2624.

Asquith B, Mosley AJ, Heaps A, Tanaka Y, Taylor GP, McLean AR, Bangham CRM: **Quantification of the virus-host interaction in human T lymphotropic virus I infection.** *Retrovirology* 2005, **2**:75.

Azuma T, Yao S, Zhu G, Flies AS, Flies SJ, Chen L: **B7-H1 is a ubiquitous antiapoptotic receptor on cancer cells.** *Blood* 2008, **111**:3635–3643.

Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R: **Restoring function in exhausted CD8 T cells during chronic viral infection.** *Nature* 2006, **439**:682–687.

Baydoun HH, Bellon M, Nicot C: **HTLV-1 Yin and Yang: Rex and p30 master regulators of viral mRNA trafficking.** *AIDS Review* 2008, **10**:195–204.

Berger R, Rotem-Yehudar R, Slama G, Landes S, Kneller A, Leiba M, Koren-Michowitz M, Shimoni A, Nagler A: **Phase I safety and pharmacokinetic study of CT-011, a humanized antibody interacting with PD-1, in patients with advanced hematologic malignancies.** *Clinical Cancer Research* 2008, **14**:3044–3051.

Blank C, Mackensen A: **Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion.** *Cancer Immunology Immunotherapy* 2007, **56**:739–745.

Blattman JN, Wherry EJ, Ha SJ, Most RG, Ahmed R: **Impact of epitope escape on PD-1 expression and CD8 T-cell exhaustion during chronic infection.** *Journal of Virology* 2009, **83**:4386-4394.

Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, Stankevich E, Pons A, Salay TM, McMiller TL, Gilson MM, Wang C, Selby M, Taube JM, Anders R, Chen L, Korman AJ, Pardoll DM, Lowy I, Topalian SL: **Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates.** *Journal of Clinical Oncology* 2010, **28**:3167–3175.

Broeke AVD, Cleuter Y, Chen G, Portetelle D, Mammerickx M, Zagury D, Fouchard M, Coulombel L, Kettmann R, Burny A: **Even transcriptionally competent proviruses are silent in bovine leukemia virus-induced sheep tumor cells.** *Journal of Virology* 1988, **85**:9263–9267.

Burger JA: **Chemokines and chemokine receptors in chronic lymphocytic leukemia (CLL): from understanding the basics towards therapeutic targeting.** *Seminars in Cancer Biology* 2010, **20**:424–430.

Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ: **Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses.** *Immunity* 2007, **27**:111–122.

Butte MJ, Pena-Cruz V, Kim M-J, Freeman GJ, Sharpe AH: **Interaction of human PD-L1 and B7-1.** *Molecular Immunology* 2008, **45**:3567–3572.

Cambier JC, Gauld SB, Merrell KT, Vilen BJ: **B-cell anergy: from transgenic models to naturally occurring anergic B cells?** *Nature Reviews Immunology* 2007, **7**:633–643.

Charles ED, Brunetti C, Marukian S, Ritola KD, Talal AH, Marks K, Jacobson IM, Rice CM, Dustin LB: **Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21^{low} B-cell subset.** *Blood* 2011, **117**:5425–5437.

Chen L: **Co-inhibitory molecules of the B7–CD28 family in the control of T-cell immunity.** *Nature Reviews Immunology* 2004, **4**:336–347.

Chen L, Zhang Z, Chen W, Zhang Z, Li Y, Shi M, Zhang J, Chen L, Wang S, Wang F-S: **B7-H1 up-regulation on myeloid dendritic cells significantly suppresses T Cell immune function in patients with chronic hepatitis B.** *Journal of Immunology* 2007, **178**:6634–6641.

Chung EY, Psathas JN, Yu D, Li Y, Weiss MJ, Thomas-Tikhonenko A: **CD19 is a major B cell receptor–independent activator of MYC-driven B-lymphomagenesis.** *Journal of Clinical Investigation* 2012, **122**:2257–2266.

Copeland KF, Haaksma AG, Goudsmit J, Heeney JL: **Calcium-mediated inhibition of phorbol ester and Tax trans-activation of the human T cell leukemia virus type 1.** *Journal of General Virology* 1994, **75**:1623–1631.

Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJR, Klenerman P, Ahmed R, Freeman GJ, Walker BD: **PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression.** *Nature* 2006, **443**:350–354.

Debacq C, Gillet N, Asquith B, Sanchez-Alcaraz MT, Florins A, Boxus M, Schwartz-Cornil I, Bonneau M, Jean G, Kerkhofs P, Hay J, Thewis A, Kettmann R, Willems L: **Peripheral blood B-cell death compensates for excessive proliferation in lymphoid tissues and maintains homeostasis in bovine leukemia virus-infected sheep.** *Journal of Virology* 2006, **80**:9710–9719.

Depoil D, Fleire S, Treanor BL, Weber M, Harwood NE, Marchbank KL, Tybulewicz VLJ, Batista FD: **CD19 is essential for B cell activation by promoting B cell receptor-antigen microcluster formation in response to membrane-bound ligand.** *Nature Immunology* 2008, **9**:63–72.

Dequiedt F, Hanon E, Kerkhofs P, Pastoret P, Burny NE, Kettmann R, Willems L: **Both wild-type and strongly attenuated bovine leukemia viruses protect peripheral blood mononuclear cells from apoptosis.** *Journal of Virology* 1997, **71**:630–639.

Derse D: **Bovine leukemia virus transcription is controlled by a virus-encoded trans-acting factor and by cis-acting response elements.** *Journal of Virology* 1987, **61**:2462–2471.

Derse D, Hill SA, Lloyd PA, Chung H, Morse BA: **Examining human T-lymphotropic virus type 1 infection and replication by cell-free infection with recombinant virus vectors.** *Journal of Virology* 2001, **75**:8461–8468.

Domenech A, Goyache J, Llames L, Jesus Paya M, Suarez G, Gomez-Lucia E: **In vitro infection of cells of the monocytic/macrophage lineage with bovine leukaemia virus.** *Journal of General Virology* 2000, **81**:109–118.

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

Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L: **Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion.** *Nature Medicine* 2002, **8**:793–800.

Dong H, Strome SE, Matteson EL, Moder KG, Flies DB, Zhu G, Tamura H, Driscoll CLW, Chen L: **Costimulating aberrant T cell responses by B7-H1 autoantibodies in rheumatoid arthritis.** *Journal of Clinical Investigation* 2003, **111**:363–370.

Dunn GP, Old LJ, Schreiber RD, Louis S, Burnet FM, Thomas L: **The immunobiology of cancer immunosurveillance and immunoediting.** *Immunity* 2004, **21**:137–148.

Elrefaei M, Baker CAR, Jones NG, Bangsberg DR, Cao H: **Presence of suppressor HIV-specific $CD8^+$ T cells is associated with increased PD-1 expression on effector $CD8^+$ T cells.** *Journal of Immunology* 2008, **180**:7757–7763.

Eychene A, Rocques N, Pouponnot C: **A new MAFia in cancer.** *Nature Reviews Cancer* 2008, **8**:683–693.

Feuer G, Fraser JK, Zack JA, Lee F, Feuer R, Chen IS: **Human T-cell leukemia virus infection of human hematopoietic progenitor cells: maintenance of virus infection during differentiation in vitro and in vivo.** *Journal of Virology* 1996, **70**:4038–4044.

Fife BT, Bluestone JA: **Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways.** *Immunological Reviews* 2008, **224**:166–182.

Finnefrock AC, Tang A, Li F, Freed C, Feng M, Cox KS, Sykes KJ, Guare JP, Miller MD, Olsen DB, Hazuda J, Shiver JW, Casimiro DR, Freed DC, Hazuda DJ, Fu T, Daniel C, Feng M, Cox KS, Sykes KJ, James P, Miller MD, Olsen DB, Hazuda DJ, Shiver JW, Casimiro DR, Fu T, Freed DC, Guare JP: **PD-1 blockade in rhesus macaques: impact on chronic infection and prophylactic vaccination.** *Journal of Immunology* 2009, **182**:980–987.

Flies DB, Wang S, Xu H, Chen L: **Cutting Edge: A monoclonal antibody specific for the programmed death-1 homolog prevents graft-versus-host disease in mouse models.** *Journal of Immunology* 2011, **187**:1537–1541.

Florins A, Brogniez AD, Elemans M, Bouzar A-B, François C, Reichert M, Asquith B, Willems L: **Viral expression directs the fate of B cells in bovine leukemia virus-infected sheep.** *Journal of Virology* 2012, **86**:621–624.

Folkl A, Wen X, Kuczynski E, Clark ME, Bienzle D: **Feline programmed death and its ligand: characterization and changes with feline immunodeficiency virus infection.** *Veterinary Immunology and Immunopathology* 2010, **134**:107–114.

Francisco LM, Sage PT, Sharpe AH: **The PD-1 pathway in tolerance and autoimmunity.** *Immunological Reviews* 2010, **236**:219–242.

Fulton BE, Portella M, Radke K: **Dissemination of bovine leukemia virus-infected cells from a newly infected sheep lymph node.** *Journal of Virology* 2006, **80**:7873–7884.

Gatot J-S: **Conservative Mutations in the immunosuppressive region of the bovine leukemia virus transmembrane protein affect fusion but not infectivity in vivo.** *Journal of Biological Chemistry* 1998, **273**:12870–12880.

Geng H, Zhang G-M, Xiao H, Yuan Y, Li D, Zhang H, Qiu H, He Y-F, Feng Z-H: **HSP70 vaccine in combination with gene therapy with plasmid DNA encoding sPD-1 overcomes immune resistance and suppresses the progression of pulmonary metastatic melanoma.** *International Journal of Cancer* 2006, **118**:2657–2664.

Ghez D, Lepelletier Y, Lambert S, Fourneau J-M, Blot V, Janvier S, Arnulf B, Endert PM, Heveker N, Pique C, Hermine O: **Neuropilin-1 is involved in human T-cell lymphotropic virus type 1 entry.** *Journal of Virology* 2006, **80**:6844–6854.

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

Gillet N, Florins A, Boxus M, Burteau C, Nigro A, Vandermeers F, Balon H, Bouzar A-B, Defoiche J, Burny A, Reichert M, Kettmann R, Willems L: **Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human.** *Retrovirology* 2007, **4**:18.

Griebel PJ, Kennedy L, Graham T, Davis WC: **Characterization of B-cell phenotypic changes during ileal and jejunal Peyer's patch development in sheep.** *Immunology* 1992, **77**:564–570.

Grzywnowicz M, Zaleska J, Mertens D, Tomczak W, Wlasiuk P, Kosior K, Piechnik A, Bojarska-Junak A, Dmoszynska A, Giannopoulos K: **Programmed death-1 and its ligand are novel immunotolerant molecules expressed on leukemic B cells in chronic lymphocytic leukemia.** *PloS One* 2012, **7**:e35178.

Gupta P, Ferrer JF: **Expression of bovine leukemia virus genome is blocked by a nonimmunoglobulin protein in plasma from infected cattle.** *Science* 1982, **215**:405-407.

Gupta P, Kashmiri SV, Ferrer JF: **Transcriptional control of the bovine leukemia virus genome: role and characterization of a non-immunoglobulin plasma protein from bovine leukemia virus-infected cattle.** *Journal of Virology* 1984, **50**:267-270.

Hanon E, Asquith RE, Taylor GP, Tanaka Y, Weber JN, Bangham CR: **High frequency of viral protein expression in human T cell lymphotropic virus type 1-infected peripheral blood mononuclear cells.** *AIDS Research and Human Retroviruses* 2000, **16**:1711–1715.

Hase M, Yokomizo T, Shimizu T, Nakamura M: **Characterization of an orphan G protein-coupled receptor, GPR20, that constitutively activates Gi proteins.** *Journal of Biological Chemistry* 2008, **283**:12747–12755.

Hasegawa H, Sawa H, Lewis MJ, Orba Y, Sheehy N, Yamamoto Y, Ichinohe T, Tsunetsugu-Yokota Y, Katano H, Takahashi H, Matsuda J, Sata T, Kurata T, Nagashima K, Hall WW: **Thymus-derived leukemia-lymphoma in mice transgenic for the Tax gene of human T-lymphotropic virus type I.** *Nature Medicine* 2006, **12**:466–472.

He Y-F, Zhang G-M, Wang X-H, Zhang H, Yuan Y, Li D, Feng Z-H: **Blocking programmed death-1 ligand-PD-1 interactions by local gene therapy results in enhancement of antitumor effect of secondary lymphoid tissue chemokine.** *Journal of Immunology* 2004, **173**:4919–4928.

Hirano F, Kaneko K, Tamura H, Dong H, Wang S, Ichikawa M, Rietz C, Flies DB, Lau JS, Zhu G: **Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity.** *Cancer Research* 2005, **65**:1089–1096.

Holets LM, Carletti MZ, Kshirsagar SK, Christenson LK, Petroff MG: **Differentiation-induced post-transcriptional control of B7-H1 in human trophoblast cells.** *Placenta* 2009, **30**:48–55.

Hori J, Wang M, Miyashita M, Tanemoto K, Takahashi H, Takemori T, Okumura K, Yagita H, Azuma M: **B7-H1-induced apoptosis as a mechanism of immune privilege of corneal allografts.** *Journal of Immunology* 2006, **177**:5928–5935.

Igakura T, Stinchcombe JC, Goon PKC, Taylor GP, Weber JN, Griffiths GM, Tanaka Y, Osame M, Bangham CRM: **HTLV-I spreads between lymphocytes by virus-induced polarization of the cytoskeleton.** *Science* 2003, **299**:1713–1716.

Ikebuchi R, Konnai S, Sunden Y, Onuma M, Ohashi K: **Molecular cloning and expression analysis of bovine programmed death-1.** *Microbiology and Immunology* 2010, **54**:291–298.

Ikebuchi R, Konnai S, Shirai T, Sunden Y, Murata S, Onuma M, Ohashi K: **Increase of cells expressing PD-L1 in bovine leukemia virus infection and enhancement of anti-viral immune responses in vitro via PD-L1 blockade.** *Veterinary Research* 2011, **42**:103.

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

Isnardi I, Ng Y-S, Menard L, Meyers G, Saadoun D, Srdanovic I, Samuels J, Berman J, Buckner JH, Cunningham-Rundles C, Meffre E: **Complement receptor 2/CD21$^-$ human naive B cells contain mostly autoreactive unresponsive clones.** *Blood* 2010, **115**:5026–5036.

Janossy G, Gomez DLCE, Waxadal MJ, Platts-Mills T: **The effects of purified mitogenic proteins (Pa-1 and Pa-2) from pokeweed on human T and B lymphocytes in vitro.** *Clinical and Experimental Immunology* 1976, **26**:108-117.

Jazayeri JA, Carroll GJ: **Fc-based cytokines : prospects for engineering superior therapeutics.** *Biodrugs* 2008, **22**:11–26.

Jensen WA, Wicks-Beard BJ, Cockerell GL: **Inhibition of protein kinase C results in decreased expression of bovine leukemia virus.** *Journal of Virology* 1992, **66**:4427–4433.

Jeon D-H, Oh K, Oh BC, Nam DH, Kim CH, Park H-B, Cho J, Lee JR, Lee D-S, Lee G: **Porcine PD-L1: cloning, characterization, and implications during xenotransplantation.** *Xenotransplantation* 2007, **14**:236–242.

Jochum W, Passegue E, Wagner EF: **AP-1 in mouse development and tumorigenesis.** *Oncogene* 2001, **20**:2401–2412.

Johnston ER, Powers MA, Kidd LC, Radke K: **Peripheral blood mononuclear cells from sheep infected with a variant of bovine leukemia virus synthesize envelope glycoproteins but fail to induce syncytia in culture.** *Journal of Virology* 1996, **70**:6296–6303.

Jonkers J, Korswagen HC, Acton D, Breuer M, Berns A: **Activation of a novel proto-oncogene, Frat1, contributes to progression of mouse T-cell lymphomas.** *EMBO Journal* 1997, **16**:441–450.

Kabeya H, Ohashi K, Onuma M: **Host immune responses in the course of bovine leukemia virus infection.** *Journal of Veterinary Medical Science* 2001, **63**:703–708.

Kalla M, Hammerschmidt W: **Human B cells on their route to latent infection--early but transient expression of lytic genes of Epstein-Barr virus.** *European Journal of Cell Biology* 2012, **91**:65–69.

Keir ME, Butte MJ, Freeman GJ, Sharpe AH: **PD-1 and its ligands in tolerance and immunity.** *Annual Review of Immunology* 2008, **26**:677–704.

Kerkhofs P, Adam E, Droogmans L, Portetelle D, Mammerickx M, Burny A, Kettmann R, Willems L: **Cellular pathways involved in the ex vivo expression of bovine leukemia virus.** *Journal of Virology* 1996, **70**:2170–2177.

Kidd LC, Radke K: **Lymphocyte activators elicit bovine leukemia virus expression differently as asymptomatic infection progresses.** *Virology* 1996, **217**:167–177.

Kim Y, Park G, Lee H, Song H, Choi I-H, Lee JW, Hur YD: **Cross-linking of B7-H1 on EBV-transformed B cells induces apoptosis through reactive oxygen species production, JNK signaling activation, and fasL expression.** *Journal of Immunology* 2008, **181**:6158–6169.

Kinter AL, Godbout EJ, McNally JP, Sereti I, Roby GA, Shea MAO, Fauci AS: **The common -chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression**

of programmed death-1 and its ligands. *Journal of Immunology* 2008, **181**:6738–6746.

Kobayashi S, Tian Y, Ohno N, Yuji K, Ishigaki T, Isobe M, Tsuda M, Oyaizu N, Watanabe E, Watanabe N, Tani K, Tojo A, Uchimaru K: **The CD3 versus CD7 plot in multicolor flow cytometry reflects progression of disease stage in patients infected with HTLV-I.** *PloS One* 2013, **8**:e53728.

Konnai S, Usui T, Ohashi K, Onuma M: **The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR.** *Veterinary Microbiology* 2003, **94**:283–294.

Konnai S, Usui T, Ikeda M, Kohara J, Hirata T-I, Okada K, Ohashi K, Onuma M: **Imbalance of tumor necrosis factor receptors during progression in bovine leukemia virus infection.** *Virology* 2005, **339**:239–248.

Konnai S, Usui T, Ikeda M, Kohara J, Hirata T, Okada K, Ohashi K, Onuma M: **Tumor necrosis factor-alpha up-regulation in spontaneously proliferating cells derived from bovine leukemia virus-infected cattle.** *Archives of Virology* 2006, **151**:347–360.

Kozako T, Yoshimitsu M, Fujiwara H, Masamoto I, Horai S, White Y, Akimoto M, Suzuki S, Matsushita K, Uozumi K, Tei C, Arima N: **PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients.** *Leukemia* 2009, **23**:375–382.

Kuipers H, Muskens F, Willart M, Hijdra D, Assema FBJV, Coyle AJ, Hoogsteden HC, Lambrecht BN: **Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated $CD4^+$ T cell activation.** *European Journal of Immunology* 2006, **36**:2472–2482.

Kuppers R: **B cells under influence: transformation of B cells by Epstein-Barr virus.** *Nature Reviews Immunology* 2003, **3**:801–812.

Lagarias DM, Radke K: **Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections.** *Journal of Virology* 1989, **63**:2099–2107.

Lavanya M, Kinet S, Mongellaz C, Battini J, Taylor N, Sitbon M: **Cell surface expression of the bovine leukemia virus-binding receptor on B and T lymphocytes is induced by receptor engagement 1.** *Journal of Immunology* 2008, **181**:891–898.

Lee S-J, Jang B-C, Lee S-W, Yang Y-I, Suh S-I, Park Y-M, Oh S, Shin J-G, Yao S, Chen L, Choi I-H: **Interferon regulatory factor-1 is prerequisite to the constitutive expression and IFN-gamma-induced upregulation of B7-H1 (CD274).** *FEBS Letters* 2006, **580**:755–762.

Liang SC, Latchman YE, Buhlmann JE, Tomczak MF, Horwitz BH, Freeman GJ, Sharpe AH: **Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses.** *European Journal of Immunology* 2003, **33**:2706–2716.

Lied GA, Berstad A: **Functional and clinical aspects of the B-cell-activating factor (BAFF): a narrative review.** *Scandinavian Journal of Immunology* 2011, **73**:1–7.

Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, Saudemont A, Quesnel B: **Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-γ and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway.** *Blood* 2007, **110**:296–304.

Manel N, Kim FJ, Kinet S, Taylor N, Sitbon M, Battini J-L: **The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV.** *Cell* 2003, **115**:449–459.

Manigold T, Racanelli V: **T-cell regulation by CD4 regulatory T cells during hepatitis B and C virus infections: facts and controversies.** *Lancet Infectious diseases* 2007, **7**:804–813.

Matsuoka M, Jeang K-T: **Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation.** *Nature Reviews Cancer* 2007, **7**:270–280.

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

Matsuzaki J, Gnjatic S, Mhawech-Fauceglia P, Beck A, Miller A, Tsuji T, Eppolito C, Qian F, Lele S, Shrikant P, Old LJ, Odunsi K: **Tumor-infiltrating NY-ESO-1-specific $CD8^+$ T cells are negatively regulated by LAG-3 and PD-1 in human ovarian cancer.** *Proceedings of the National Academy of Sciences of the United States of America* 2010, **107**:7875–7880.

Meirom R, Moss S, Brenner J: **Bovine leukemia virus-gp51 antigen expression is associated with CD5 and IgM markers on infected lymphocytes.** *Veterinary Immunology and Immunopathology* 1997, **59**:113–119.

Merimi M, Klener P, Szynal M, Cleuter Y, Bagnis C, Kerkhofs P, Burny A, Martiat P, Broeke AVD: **Complete suppression of viral gene expression is associated with the onset and progression of lymphoid malignancy : observations in Bovine Leukemia Virus-infected sheep.** *Retrovirology* 2007a, **4**:1–9.

Merimi M, Klener P, Szynal M, Cleuter Y, Kerkhofs P, Burny A, Martiat P, Broeke AVD: **Suppression of viral gene expression in bovine leukemia virus-associated B-cell malignancy: interplay of epigenetic modifications leading to chromatin with a repressive histone code.** *Journal of Virology* 2007b, **81**:5929–5939.

Milde-Langosch K: **The Fos family of transcription factors and their role in tumourigenesis.** *European Journal of Cancer* 2005, **41**:2449–2461.

Mirsky ML, Olmstead CA, Da Y, Lewin HA: **The prevalence of proviral bovine leukemia virus in peripheral blood mononuclear cells at two subclinical stages of infection.** *Journal of Virology* 1996, **70**:2178–2183.

Mkrtichyan M, Najjar YG, Raulfs EC, Liu L, Langerman S, Guittard G, Ozbun L, Khleif SN: **B7-DC-Ig enhances vaccine effect by a novel mechanism dependent on PD-1 expression level on T cell subsets.** *Journal of Immunology* 2012, **189**:2338–2347.

Mockridge CI, Potter KN, Wheatley I, Neville LA, Packham G, Stevenson FK: **Reversible anergy of sIgM-mediated signaling in the two subsets of CLL defined by VH-gene mutational status.** *Blood* 2007, **109**:4424–4431.

Muthumani K, Choo AY, Shedlock DJ, Laddy DJ, Sundaram SG, Hirao L, Wu L, Thieu KP, Chung CW, Lankaraman KM, Tebas P, Silvestri G, Weiner DB: **Human immunodeficiency virus type 1 Nef induces programmed death 1 expression through a p38 mitogen-activated protein kinase-dependent mechanism.** *Journal of Virology* 2008, **82**:11536–11544.

Naessens J, Chris J, Hopkins J: **Nomenclature and characterization of leukocyte differentiation antigens in ruminants.** *Immunology Today* 1997, **18**:365–368.

Nielsen C, Ohm-Laursen L, Barington T, Husby S, Lillevang ST: **Alternative splice variants of the human PD-1 gene.** *Cellular Immunology* 2005, **235**:109–116.

Niwa H, Yamamura K, Miyazaki J: **Efficient selection for high-expression transfectants with a novel eukaryotic vector.** *Gene* 1991, **108**:193–199.

Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, Nakamura S, Enomoto K, Yagita H, Azuma M, Nakajima Y: **Clinical significance and therapeutic**

potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clinical Cancer Research* 2007, **13**:2151–2157.

Orlik O, Splitter GA: **Progression to persistent lymphocytosis and tumor development in bovine leukemia virus (BLV) -infected cattle correlates with impaired proliferation of $CD4^+$ T cells in response to gag- and env-encoded BLV proteins.** *Journal of Virology* 1996, **70**:7584–7593.

Panei CJ, Takeshima S-N, Omori T, Nunoya T, Davis WC, Ishizaki H, Matoba K, Aida Y: **Estimation of bovine leukemia virus (BLV) proviral load harbored by lymphocyte subpopulations in BLV-infected cattle at the subclinical stage of enzootic bovine leucosis using BLV-CoCoMo-qPCR.** *BMC Veterinary Research* 2013, **9**:95.

Paterson AM, Brown KE, Keir ME, Vanguri VK, Riella LV, Chandraker A, Sayegh MH, Blazar BR, Freeman GJ, Sharpe AH: **The programmed death-1 ligand 1:B7-1 pathway restrains diabetogenic effector T cells in vivo.** *Journal of Immunology* 2011, **187**:1097–1105.

Pays E, Vanhamme L, Perez-Morga D: **Antigenic variation in Trypanosoma brucei: facts, challenges and mysteries.** *Current Opinion in Microbiology* 2004, **7**:369–374.

Peng J-M, Tian Z-J, Liu H-G, An T-Q, Zhou Y-J, Wang Y, Li D-Y, Chen J-Z, Yang Y-Q, Tong G-Z: **Cloning and identification of porcine programmed death 1.** *Veterinary Immunology and Immunopathology* 2010, **136**:157–162.

Powers MA, Radke K: **Activation of bovine leukemia virus transcription in lymphocytes from infected sheep : rapid transition through early to late gene expression.** *Journal of Virology* 1992, **66**:4769–4777.

Pulko V, Harris KJ, Liu X, Gibbons RM, Harrington SM, Krco CJ, Kwon ED, Dong H: **B7-H1 expressed by activated CD8 T cells is essential for their survival.** *Journal of Immunology* 2011, **187**:5606–5614.

Pyeon D, O'Reilly KL, Splitter GA: **Increased interleukin-10 mRNA expression in tumor-bearing or persistently lymphocytotic animals infected with bovine leukemia virus.** *Journal of Virology* 1996, **70**:5706–5710.

Pyeon D, Splitter GA: **Interleukin-12 p40 mRNA expression in bovine leukemia virus-infected animals: increase in alymphocytosis but decrease in persistent lymphocytosis.** *Journal of Virology* 1998, **72**:6917–6921.

Quach TD, Manjarrez-Orduno N, Adlowitz DG, Silver L, Yang H, Wei C, Milner ECB, Sanz I: **Anergic responses characterize a large fraction of human autoreactive naive B cells expressing low levels of surface IgM.** *Journal of Immunology* 2011, **186**:4640–4648.

Richter K, Agnellini P, Oxenius A: **On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection.** *International Immunology* 2009, **22**:13–23.

Rosignoli G, Lim CH, Bower M, Gotch F, Imami N: **Programmed death (PD)-1 molecule and its ligand PD-L1 distribution among memory CD4 and CD8 T cell subsets in human immunodeficiency virus-1-infected individuals.** *Clinical and Experimental Immunology* 2009, **157**:90–97.

Sagata N, Yasunaga T, Tsuzuku-Kawamura J, Ohishi K, Ogawa Y, Ikawa Y: **Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses.** *Proceedings of the National Academy of Sciences of the United States of America* 1985, **82**:677–681.

Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, Hill BJ, Noto A, Ancuta P, Peretz Y, Fonseca SG, Grevenynghe JV, Boulassel MR, Bruneau J, Shoukry NH, Routy J-P, Douek DC, Haddad EK, Sekaly R-P: **Programmed death-1-induced interleukin-10 production by monocytes impairs $CD4^+$ T cell activation during HIV infection.** *Nature Medicine* 2010, **16**:452–459.

Saito YD, Jensen AR, Salgia R, Posadas EM: **Fyn: a novel molecular target in cancer.** *Cancer* 2010, **116**:1629–1637.

Salama AD, Chitnis T, Imitola J, Ansari MJI, Akiba H, Tushima F, Azuma M, Yagita H, Sayegh MH, Khoury SJ: **Critical role of the programmed death-1 (PD-1) pathway in regulation of experimental autoimmune encephalomyelitis.** *Journal of Experimental Medicine* 2003, **198**:71–78.

Sasaki F, Okuno T, Saeki K, Min L, Onohara N, Kato H, Shimizu T, Yokomizo T: **A high-affinity monoclonal antibody against the FLAG tag useful for G-protein-coupled receptor study.** *Analytical Biochemistry* 2012, **425**:157–165.

Saudemont A, Jouy N, Hetuin D, Quesnel B: **NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells.** *Blood* 2005, **105**:2428–2435.

Schwartz I, Bensaid A, Polack B, Perrin B, Berthelemy M, Levy D: **In vivo leukocyte tropism of bovine leukemia virus in sheep and cattle.** *Journal of Virology* 1994, **68**:4589–4596.

Shields RL, Namenuk AK, Hong K, Meng YG, Rae J, Briggs J, Xie D, Lai J, Stadlen A, Li B, Fox JA, Presta LG: **High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R.** *Journal of Biological Chemistry* 2001, **276**:6591–6604.

Shimauchi T, Kabashima K, Nakashima D, Sugita K, Yamada Y, Hino R, Tokura Y: **Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4$^+$ T-cells in adult T-cell leukemia/lymphoma.** *International Journal of Cancer* 2007, **121**:2585–2590.

So L, Fruman DA: **PI3K signalling in B- and T-lymphocytes: new developments and therapeutic advances.** *Biochemical Journal* 2012, **442**:465–481.

Stanciu LA, Bellettato CM, Laza-Stanca V, Coyle AJ, Papi A, Johnston SL: **Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines.** *Journal of Infectious Diseases* 2006, **193**:404–412.

Streeck H, Brumme ZL, Anastario M, Cohen KW, Jolin JS, Meier A, Brumme CJ, Rosenberg ES, Alter G, Allen TM, Walker BD, Altfeld M: **Antigen load and viral sequence diversification determine the functional profile of HIV-1-specific CD8$^+$ T cells.** *PLoS Medicine* 2008, **5**:e100.

Subudhi S, Zhou P, Yerian L, Chin R, Lo J, Anders R, Sun Y, Chen L, Wang Y, Alegre M-L, Fu Y-X: **Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection.** *Journal of Clinical Investigation* 2004, **113**:694–700.

Tajima S, Takahashi M, Takeshima S, Konnai S, Yin SA, Watarai S, Tanaka Y, Onuma M, Okada K, Aida Y: **A Mutant Form of the Tax Protein of Bovine Leukemia Virus (BLV), with Enhanced Transactivation Activity , Increases Expression and Propagation of BLV In Vitro but Not In Vivo.** *Journal of Virology* 2003, **77**:1894–1903.

Tajima S, Aida Y: **Induction of expression of bovine leukemia virus (BLV) in blood taken from BLV-infected cows without removal of plasma.** *Microbes and Infection* 2005, **7**:1211–1216.

Taniguchi Y, Nosaka K, Yasunaga J, Maeda M, Mueller N, Okayama A, Matsuoka M: **Silencing of human T-cell leukemia virus type I gene transcription by epigenetic mechanisms.** *Retrovirology* 2005, **2**:64.

Teague BN, Pan Y, Mudd PA, Zhang Q, Szodoray P, Wilson PC, Farris AD: **Cutting Edge : Transitional T3 B cells do not give rise to mature B cells, have undergone selection, and are reduced in murine lupus.** *Journal of Immunology* 2007, **178**:7511–7515.

Trabattoni D, Saresella M, Biasin M, Boasso A, Piacentini L, Ferrante P, Dong H, Maserati R, Shearer GM, Chen L, Clerici M: **B7-H1 is up-regulated in HIV infection and is a novel surrogate marker of disease progression.** *Blood* 2003, **101**:2514–2520.

Trautmann L, Janbazian L, Chomont N, Said AE, Gimmig S, Bessette B, Boulassel M-R, Delwart E, Sepulveda H, Balderas RS, Routy J-P, Haddad EK, Sekaly R-P: **Upregulation of PD-1 expression on HIV-specific CD8$^+$ T cells leads to reversible immune dysfunction.** *Nature Medicine* 2006, **12**:1198–1202.

Trentin L, Cabrelle A, Facco M, Carollo D, Miorin M, Tosoni A, Pizzo P, Binotto G, Nicolardi L, Zambello R, Adami F, Agostini C, Semenzato G: **Homeostatic chemokines drive migration of malignant B cells in patients with non-Hodgkin lymphomas.** *Blood* 2004, **104**:502–508.

Tsushima F, Yao S, Shin T, Flies A, Flies S, Xu H, Tamada K, Pardoll DM, Chen L: **Interaction between B7-H1 and PD-1 determines initiation and reversal of T-cell anergy.** *Blood* 2007, **110**:180–185.

Twizere J, Kruys V, Lefebvre L, Vanderplasschen A, Collete D, Debacq C, Lai WS, Jauniaux J-C, Bernstein LR, Semmes OJ, Burny A, Blackshear PJ, Kettmann R, Willems L: **Interaction of retroviral Tax oncoproteins with tristetraprolin and regulation of tumor necrosis factor-expression.** *Journal of the National Cancer Institute* 2003, **95**:1846–1859.

Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, Ferrari C: **PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion.** *Journal of Virology* 2006, **80**:11398–11403.

Usui T, Konnai S, Ohashi K, Onuma M: **Interferon-γ expression associated with suppression of bovine leukemia virus at the early phase of infection in sheep.** *Veterinary Immunology and Immunopathology* 2007, **115**:17–23.

Velu V, Titanji K, Zhu B, Husain S, Pladevega A, Lai L, Vanderford TH, Chennareddi L, Silvestri G, Freeman GJ, Ahmed R, Amara RR: **Enhancing SIV-specific immunity in vivo by PD-1 blockade.** *Nature* 2009, **458**:206–210.

Wan B, Nie H, Liu A, Feng G: **Aberrant regulation of synovial T cell activation by soluble costimulatory molecules in rheumatoid arthritis.** *Journal of Immunology* 2006, **177**:8844–8850.

Wang X, Zhang Z, Zhang S, Fu J, Yao J, Jiao Y, Wu H, Wang F-S: **B7-H1 up-regulation impairs myeloid DC and correlates with disease progression in chronic HIV-1 infection.** *European Journal of Immunology* 2008, **38**:3226–3236.

Wherry EJ, Ha S-J, Kaech SM, Haining WN, Sarkar S, Kalia V, Subramaniam S, Blattman JN, Barber DL, Ahmed R: **Molecular signature of $CD8^+$ T cell exhaustion during chronic viral infection.** *Immunity* 2007, **27**:670–684.

Wherry EJ: **T cell exhaustion.** *Nature Immunology* 2011, **131**:492–499.

Willard-Gallo KE, Furtado M, Burny A, Wolinsky SM: **Down-modulation of TCR/CD3 surface complexes after HIV-1 infection is associated with differential expression of the viral regulatory genes.** *European Journal of Immunology* 2001, **31**:969–979.

Willems L, Kettmann R, Dequiedt F, Portetelle D, Voneche V, Cornil I, Kerkhofs P, Burny A, Mammerickx M: **In vivo infection of sheep by bovine leukemia virus mutants.** *Journal of Virology* 1993, **67**:4078–4085.

Willems L, Kerkhofs P, Dequiedt F, Portetelle D, Mammerickx M, Burny A, Kettmann R: **Attenuation of bovine leukemia virus by deletion of R3 and G4 open reading frames.** *Proceedings of the National Academy of Sciences of the United States of America* 1994, **91**:11532–11536.

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

Yasuda MY, Ennea CNJ, Ennedya LJK, Eynoldsa JDR: **The sheep and cattle Peyer's patch as a site of B-cell development.** *Veterinary Research* 2006, **37**:401–415.

Zajac AJ, Blattman JN, Murali-Krishna K, Sourdive DJ, Suresh M, Altman JD, Ahmed R: **Viral immune evasion due to persistence of activated T cells without effector function.** *Journal of Experimental Medicine* 1998, **188**:2205–2213.

Zhu Y, Yao S, Chen L: **Cell surface signaling molecules in the control of immune responses: a tide model.** *Immunity* 2011, **34**:466–478.

Zikherman J, Parameswaran R, Weiss A: **Endogenous antigen tunes the responsiveness of naive B cells but not T cells.** *Nature* 2012, **489**:160–164.

Zinselmeyer BH, Heydari S, Sacristán C, Nayak D, Cammer M, Herz J, Cheng X, Davis SJ, Dustin ML, McGavern DB: **PD-1 promotes immune exhaustion by inducing antiviral T cell motility paralysis.** *Journal of Experimental Medicine* 2013, **210**:757–774.

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^{-} 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。