Studies on T-cell exhaustion mediated by immunoinhibitory receptors in chronic infections in cattle

牛の慢性感染症における免疫抑制受容体を介した
T 細胞の疲弊化に関する研究

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CONTENTS

ABBREVIATIONS

ADCC	antibody-dependent cell-mediated cytotoxicity
AL	aleukemic
APC	allophycocyanin
BAMA	bovine anti-mouse antibody
BLV	bovine leukemia virus
BLV−	bovine leukemia virus-uninfected
B-PPD	purified protein derivative of bovine tuberculin
BSA	bovine serum albumin
CFSE	carboxyfluorescein diacetate succinimidyl ester
chAb(s)	chimeric antibody(antibodies)
CHO	Chinese hamster ovary
ConA	concanavalin A
CPM	counts per minute
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
Cy	cyanin
DC(s)	dendritic cell(s)
dhfr	dihydrofolate reductase
dpi	days post-inoculation(infection)
EBL	enzootic bovine leukosis
EBV	Epstein-Barr virus
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FLK	BLV-uninfected fetal lamb kidney
FLK-BLV	BLV-infected fetal lamb kidney
FSC	forward scatter
Gal-9	galectin 9
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HTLV-1	human T-cell leukemia virus type 1

IFN	interferon
Ig	immunoglobulin
IL	interleukin
J-PPD	Johnin purified protein derivative
LAG-3	lymphocyte activation gene 3
LCMV	lymphocytic choriomeningitis virus
LN(s)	lymph node(s)
LTR	long terminal repeat
mAb(s)	monoclonal antibody(antibodies)
Map	*Mycobacterium avium* subsp. *paratuberculosis*
MHC I	major histocompatibility complex class I
MHC II	major histocompatibility complex class II
mLNs	mesenteric lymph nodes
MSP	major surface protein
MTX	methotrexate
OM	outer membrane
OMP	outer membrane proteins
PBMC(s)	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing 0.05% Tween 20
PCR	polymerase chain reaction
PCV	packed cell volume
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PE	phycoerythrin
PerCp	peridinin-chlorophyll-protein complex
PL	persistent lymphocytosis
PMA	phorbol 12-myristate acetate
PPs	Peyer’s patches
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SI	stimulation index

SSC	side scatter
TCGF	T-cell growth factor
TCR	T-cell receptor
Th1	type 1 helper T (cell)
TIM-3	T-cell immunoglobulin and mucin domain 3
TNF	tumor necrosis factor
uRBC	uninfected bovine red blood cells
wpi	weeks post-inoculation(infection)

NOTES

The contents of Chapter I have been published in *Infection and Immunity*.

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The contents of Chapter II, III, and IV will be submitted for publication in peer-reviewed journals, and thus cannot be shown in the thesis published online at the present time.

PREFACE

Cell-mediated immunity is central for protection against infection with viruses, bacteria, and parasites. However, a variety of chronic pathogens evade the host immune response through multiple mechanisms (Finlay and McFadden, 2006). For example, *Trypanosoma brucei* is able to change the antigenic character of its 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 and Hammerschmidt, 2012), and high frequencies of circulating regulatory T cells are associated with the suppression of effector functions of $CD4^+$ and $CD8^+$ T cells in chronic hepatitis C virus (HCV) infection (immunosuppression) (Manigold and Racanelli, 2007). In addition, "T cell exhaustion" plays an essential role in the immune evasion during chronic infections (Wherry and Ahmed, 2004; Klenerman and Hill, 2005). In persistent viral infections, such as lymphocytic choriomeningitis virus (LCMV) and human immunodeficiency virus (HIV), virus-specific T cells are still present in infected hosts but are functionally impaired (Zajac et al., 1998; Barber et al., 2006; Trautmann et al., 2006). These cells have lost the ability to produce sufficient cytokines, to proliferate in response to specific antigens, and to exhibit cytotoxic T-cell activity and thus cannot effectively eliminate pathogens.

Dysfunction of antigen-specific T cells is well documented as "T-cell exhaustion", defined as the loss of effector functions during various types of chronic infections (Blackburn et al., 2009; Khaitan and Unutmaz, 2011; Wherry, 2011; Fig. 1A). Exhausted T cells are phenotypically characterized by the cell-surface expression of immunoinhibitory receptors, such as programmed death 1 (PD-1), lymphocyte activation gene 3 (LAG-3), and T cell immunoglobulin and mucin domain 3 (TIM-3) (Wherry, 2011; Fig. 1B). PD-1, LAG-3, and TIM-3 inhibit T-cell receptor (TCR) signaling and subsequent effector functions of T cells after binding to their ligand PD-ligand 1 (PD-L1), major histocompatibility complex class II (MHC II), and galectin-9 (Gal-9), respectively (Kamphorst and Ahmed, 2013; Wherry and Kurachi, 2015; Fig. 1B). More importantly, blockade of inhibitory pathways by antibodies specific for either receptors or ligands restores the function of exhausted T cells (Fig. 1B). In numerous models of chronic infection and tumors, treatments using anti-PD-1, anti-PD-L1, anti-LAG-3, and anti-TIM-3 antibodies have been shown to significantly enhance T-cell responses and improve clinical conditions (Blackburn et al., 2009;

Ngiow et al., 2011; Butler et al., 2012; Woo et al., 2012). For example, PD-L1 blockade increases the number and effector function of LCMV-specific $CD8^+$ T cells, and combined blockade of PD-L1 and LAG-3 synergistically improves T-cell responses and diminishes viral load in a mouse model (Blackburn et al., 2009).

Persistent and continuous antigenic stimulation is considered a key signal driving T-cell exhaustion in chronic infection (Wherry, 2011; Fig. 1A). Additionally, other types of signals also appear to be important, including those involving proinflammatory cytokines (such as type I interferon (IFN), interleukin (IL)-6, and IL-27), suppressive cytokines (such as IL-10 and transforming growth factor-β), other regulatory leukocytes (such as regulatory T cells), and tissue microenvironments (such as altered hypoxia, nutrients, and pH) (Wherry and Kurachi, 2015). Together with chronic TCR activation, these signals induce elevated and sustained expression of inhibitory receptors (PD-1, LAG-3, and TIM-3), changes in usage of key transcription factors (such as T-bet, Eomes, Blimp-1, and NFAT/AP-1), altered metabolism, and a transcription program distinct from that of functional memory T cells (Wherry and Kurachi, 2015). Thus, these signals lead to progressive loss of effector functions in exhausted T cells.

In cattle, suppression (exhaustion) of T cells has been documented in a variety of chronic infections, including *Mycobacterium avium* subsp. *paratuberculosis* (Stabel, 2006; Sohal et al., 2008), *Mycobacterium bovis* (Welsh and Cunningham, 2005), *Anaplasma marginale* (Brown, 2012), and bovine leukemia virus (BLV) (Orlik and Splitter, 1996; Kabeya et al. 2001; Frie and Coussens, 2015). However, the molecular mechanisms underlying T-cell exhaustion in livestock animals are poorly understood.

M. avium subsp. *paratuberculosis* (Map) is the bacteria that causes chronic granulomatous enteritis in cattle known as Johne's disease or paratuberculosis. This disease is characterized by untreatable chronic diarrhea, emaciation, and the eventual death of the animal. Calves under the age of six months are most susceptible to infection due to the incomplete development of their immune systems. For this reason, infection frequently occurs by ingestion of contaminated colostrum or milk from infected cows (Harris and Barletta, 2001). The disease manifestation is typically evident at 2–3 years post infection. Once clinical symptoms develop, no specific treatment cures the disease. The current prevalence of Johne's disease is low in Japan because of periodic screening and active eradication, but its prevalence is still high (10%–60%) in various countries, including Australia, New Zealand, and Europe (Momotani, 2012).

A. marginale is a tick-borne rickettsial pathogen found in the temperate and tropical regions of the world (Brown, 2012). This rickettsia is transmissible by more than 20 species of ticks, and rarely contaminated needles, and invades and replicates within mature erythrocytes in ruminants. The infection results in severe anemia and a high mortality rate of up to 30% in naive cattle (Brown, 2012). In addition, cattle surviving acute infection develop lifelong persistent infection mediated by several mechanisms of evasion from host adaptive immunity, such as antigenic variation of immunodominant surface proteins and $CD4^+$ T-cell exhaustion (Brown and Barbet, 2016). These evasion mechanisms pose problems for the development of effective vaccines.

BLV is a member of the genus *Deltaretrovirus* (subfamily *Orthoretrovirinae*, family *Retroviridae*) and is genetically related to human T-cell leukemia virus type 1 (HTLV-1) (Sagata et al., 1985). BLV infects B cells in cattle and is integrated into the host genome as a provirus (Schwartz et al., 1994; Mirsky et al., 1996). After a long latent period (normally 3–5 years), less than 5% of infected cattle develop B-cell lymphoma or lymphosarcoma within different lymphoid tissues (Gillet et al., 2007; Rodríguez et al., 2011). BLV is transmitted horizontally through blood and milk and by insect bites. In addition, vertical transmission, including perinatal infection *in utero* and in the birth canal and postnatal infection via colostrum and milk, is also frequently observed in the field (Rodríguez et al., 2011; Mekata et al., 2014). BLV infection is widespread in all parts of the world except in Western Europe (Rodríguez et al., 2011) and causes major economic losses in production of milk and beef (Ott et al., 2003; Gillet et al., 2007). The cases of BLV-induced lymphoma and BLV-infected cattle have been increasing in Japan (Murakami et al., 2011, 2013). Nevertheless, there is no effective treatment or vaccination to control BLV infection.

Previous studies revealed that the upregulation of bovine PD-1 and LAG-3 in T cells was closely associated with disease progression in cattle infected with BLV (Shirai et al., 2011; Ikebuchi et al., 2013; Konnai et al., 2013). In contrast, PD-L1 and MHC II, which are the respective ligands of PD-1 and LAG-3, were upregulated in B cells including BLV-infected B cells in BLV-infected cattle (Stone et al., 1995; Isaacson et al., 1998; Ikebuchi et al., 2011, 2013). Moreover, blockade of these inhibitory pathways restores T-cell function and induces antiviral responses *in vitro* (Ikebuchi et al., 2011, 2013; Konnai et al., 2013). However, it remains unclear whether the PD-1 and LAG-3 pathways are involved in the development of T-cell exhaustion in other chronic

infections of cattle.

Therefore, in this study, expression analyses and blockade assays of PD-1/PD-L1 and LAG-3/MHC II were performed in cattle with subclinical paratuberculosis in Chapter I and in cattle infected with *A. marginale* in Chapter II. In Chapter III, the expression levels of PD-1, LAG-3, and TIM-3 were analyzed in BLV-infected cattle with or without B-cell lymphoma, and *in vitro* blockade assays were performed using anti-PD-1, anti-PD-L1, and anti-LAG-3 monoclonal antibodies (mAbs) for determining the blockade effect in BLV infection. Furthermore, in Chapter IV, anti-PD-1 and anti-LAG-3 chimeric antibodies (chAbs) were established to apply as new therapeutic agents for chronic infections in cattle. Finally, a clinical trial of anti-PD-1 chAb was conducted to clarify the *in vivo* effect of the blocking antibody in BLV-infected cattle.

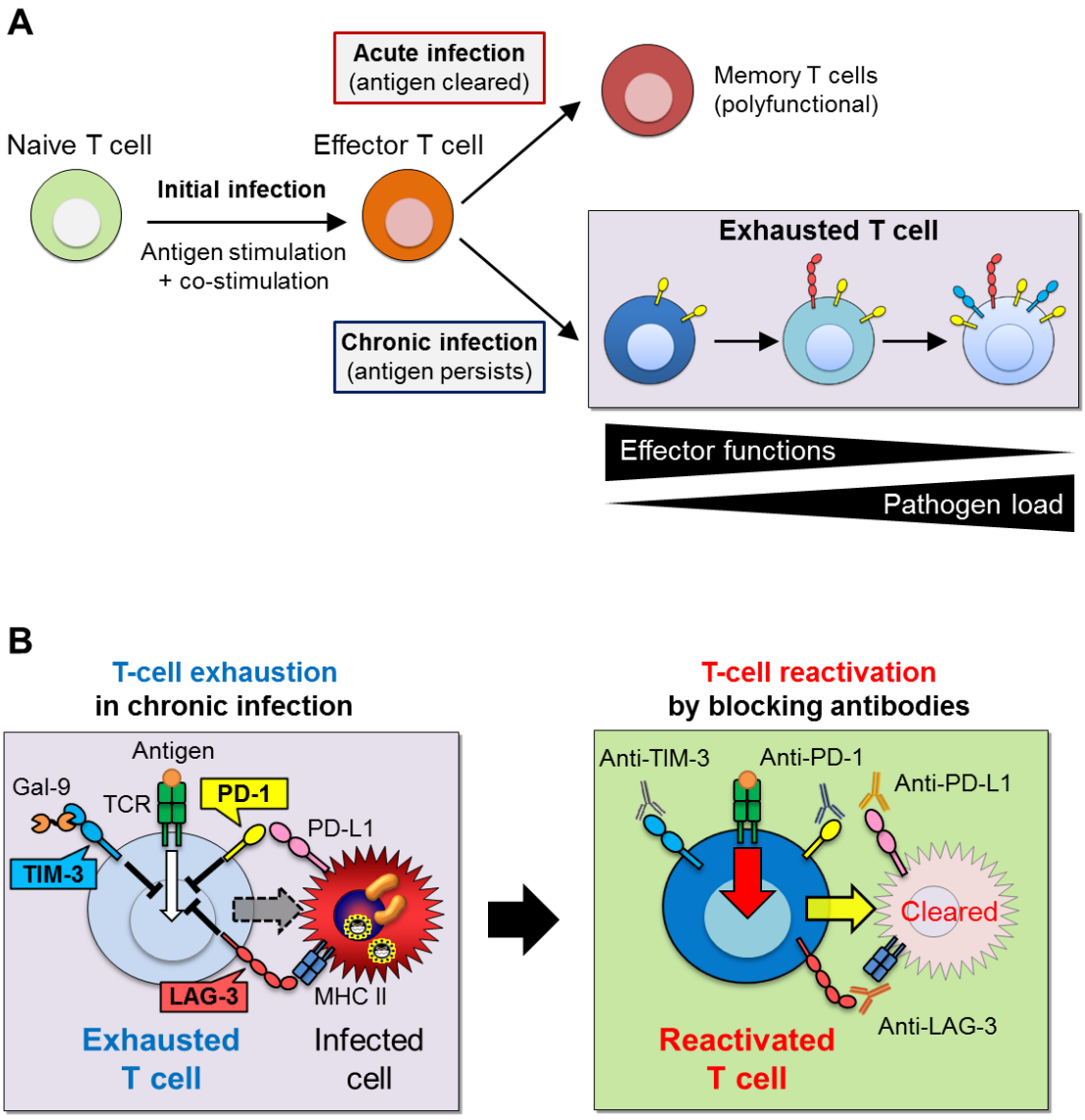

Figure 1. T-cell exhaustion during chronic infection.
(A) Hierarchical T-cell exhaustion during chronic infection. During initial infection (left), naive T cells are primed by antigen and costimulation and differentiate into effector T cells. In the case of acute infection (top), pathogen is cleared and these functional effector T cells further differentiate into polyfunctional memory T cells. During chronic infection (bottom), infection persists after the effector phase. As pathogen and antigen loads increase, T cells progress through stages of dysfunction (exhaustion), losing effector functions in a hierarchical manner. Hierarchy of T-cell exhaustion is regulated by a progressive increase in the amount and diversity of immunoinhibitory receptors expressed (Modified from Wherry, 2011). (B) Molecular mechanism of T-cell exhaustion and reactivation. Immunoinhibitory receptors such as PD-1, LAG-3, and TIM-3 are expressed on exhausted T cells, interact with their respective ligands expressed on infected cells, and impair T-cell effector functions by inhibition of the TCR signaling pathway (left). Antibody blockade of the inhibitory pathways reverses T-cell exhaustion and restores the effector functions, resulting in pathogen clearance (right).

CHAPTER I

Exhaustion of T-cell responses mediated by PD-1/PD-L1 and LAG-3/MHC II in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

INTRODUCTION

Map is the causative bacteria of Johne's disease (paratuberculosis), a chronic granulomatous enteritis that leads to diarrhea and severe weight loss in cattle. Once clinical symptoms develop, no specific treatment cures the disease; only supportive treatment can be given. Similar to other mycobacterial infections, Map elicits strong type 1 helper T cell (Th1)-mediated responses that are dominated by interferon-gamma (IFN-γ)-secreting $CD4^+$ T cells in the early stages of infection (Coussens, 2004; Stabel, 2006; Sohal et al., 2008). IFN-γ contributes to the activation of macrophages that eradicate intracellular mycobacteria during this phase (Coussens, 2004; Sohal et al., 2008). The Map-specific Th1 response is gradually suppressed during the late subclinical stage and is severely suppressed during the clinical stage (Bassey and Collins, 1997; Burrells et al., 1998; Weiss et al., 2006), facilitating bacterial growth, the formation of pathological lesions in the intestine, and the progression of clinical disease (Coussens, 2004; Sohal et al., 2008). Thus, Th1-mediated immunity is considered central to the control of this infection and disease progression. Further characterization of the mechanisms responsible for this supression of Map-specific T cells is required for establishing new treatments that enhance Th1 responses in animals with Johne's disease.

During a variety of chronic infections, PD-1/PD-L1 and LAG-3/MHC II interactions are closely associated with the inhibition of chronically activated, pathogen-specific T cells and the induction of T-cell exhaustion (Blackburn et al., 2009; Khaitan and Unutmaz, 2011; Bulter et al., 2012). Accordingly, the blockade of PD-1/PD-L1 and LAG-3/MHC II binding using antagonist antibodies inhibits these negative signals and reactivates T-cell responses such as proliferation, cytokine production, and cytotoxic activity in exhausted T cells (Sierro et al., 2011; Kamphorst and Ahmed, 2013).

Previous studies revealed that the upregulation of bovine PD-1 and LAG-3 in T cells was closely associated with disease progression in cattle infected with BLV (Shirai et al., 2011; Ikebuchi et al., 2013; Konnai et al., 2013). Moreover, the blockade of these inhibitory pathways restores exhausted T-cell function and induces antiviral responses *in vitro* (Ikebuchi et al., 2011, 2013; Konnai et al., 2013). However, it remains unclear whether the PD-1 and LAG-3 pathways are involved in the development of T-cell exhaustion in other chronic infections of cattle.

In the previous report, a polyclonal anti-human LAG-3 antibody was used for expression and functional analyses of bovine LAG-3 but showed only weak blockade activity toward T cells (Konnai et al., 2013). Therefore, in the study described in this chapter, anti-bovine LAG-3 mAbs were established to obtain antibodies with higher blockade activity. It was then determined whether the PD-1/PD-L1 and LAG-3/MHC II pathways downregulate Map-specific T-cell responses during late-stage Johne's disease. These analyses showed that increased expression of PD-1 and LAG-3 in T cells was associated with the suppression of Map-specific Th1 responses during the late subclinical stage. Additionally, PD-L1 and MHC II expressed on macrophages presumably interact with PD-1 and LAG-3 on T cells in Map-infected cattle. Furthermore, *in vitro* blockade using a novel anti-LAG-3 mAb restored IFN-γ production in Map-specific $CD4^+$ and $CD8^+$ T cells. The results of this study clarify the mechanisms that lead to exhaustion of Map-specific T-cell responses, and suggest that LAG-3 is a molecular target for the control of Map-specific T-cell responses.

MATERIALS AND METHODS

Generation of anti-bovine LAG-3 mAb

To establish an anti-bovine LAG-3 mAb that blocks LAG-3/MHC II binding, two antigen peptides were designed from an extracellular region of bovine LAG-3 that was expected to be essential for the interaction with bovine MHC II based on a previous report on MHC II binding site on murine LAG-3 (Huard et al., 1997). These peptides included peptides 71–99 (GSAAPTPRGPGPRRYTVLRLAPGGLRIGK) and 104–132 (PRVQLEEMGLQRGDFSLWLRPARRADAGE), and were synthesized and coupled with a keyhole limpet hemocyanin carrier protein at their NH_2 termini. Two rats were immunized into the footpad with about 250 μg of each peptide emulsion in TiterMax Gold Adjuvant (Sigma–Aldrich, St. Louis, MO, USA). Twenty-one days later, lymphocytes were isolated from iliac lymph nodes (LNs), fused with SP2 myeloma cells, and were grown into clonal colonies in methylcellulose-based semisolid medium. Rat immunization and hybridoma cultivation were performed at Cell Engineering Corporation (Osaka, Japan). Supernatants from hybridomas were screened using enzyme-linked immunosorbent assay (ELISA) with each immunized peptide and flow cytometry with COS-7 cells transfected with pEGFP-N2 (Clontech, Palo Alto, CA, USA) coding full-length bovine LAG-3. Anti-LAG-3 mAbs were later purified from culture supernatants of hybridomas. Purified mAbs were then analyzed using flow cytometry, and their reactivity was determined with COS-7 cells expressing enhanced green fluorescent protein (EGFP)-tagged LAG-3 and bovine peripheral blood mononuclear cells (PBMCs) that were either freshly isolated or stimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Sigma–Aldrich) for 48 h.

Western blotting

To confirm the specificity of the anti-LAG-3 mAb, western blot analyses were performed using COS-7 cells expressing LAG-3-EGFP. Cells (about 1×10^6) were lysed in 2 × sodium dodecyl sulfate (SDS) buffer containing 125 mM tris(hydroxymethyl)aminomethane hydrochloride (pH 6.8), 4% SDS, 10% 2-mercaptoethanol, and 20% glycerol and were boiled for 5 min. Samples were then separated on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Merck Millipore, Billerica, MA, USA). After blocking with 3% skim milk in phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20

(PBS-T), membranes were incubated at room temperature for 1 h with anti-LAG-3 mAb (71-2D8, 2 μg/ml), washed with PBS-T, and incubated with horseradish peroxidase (HRP)-conjugated anti-rat IgG (1:5,000 dilution; MP Biomedicals, Irvine, CA, USA). Membranes were also probed with anti-EGFP (0.5 μg/ml; Clontech) and anti-actin antibodies (1:1,000 dilution; Merck Millipore) as positive and loading controls, respectively. After washing with PBS-T, membranes were incubated with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore) to visualize signals, which were analyzed using a Fluor-S Multi Imager (Bio-Rad, Hercules, CA, USA).

Animals and experimental infection

Eight male Holstein calves between 1 and 6 weeks of age were orally inoculated with intestinal tissue homogenate from a clinically infected cow as described previously (Nagata et al., 2005, 2010). The total number of Map used in this experimental infection was 1.03×10^8–6.27×10^8 viable organisms per calf. Three age-matched male Holstein calves were maintained separate from the inoculated cattle as uninfected controls. All calves were kept in a biosafety level II animal facility at the National Institute of Animal Health (Tsukuba, Ibaraki, Japan). Peripheral blood and fecal samples were collected before infection and every 2–4 weeks post-inoculation (wpi). All animal experiments carried out in this study were approved by the National Institute of Animal Health Ethics Committee. In addition, ileum tissues were collected from two cows which are naturally infected with Map. These cattle showed clinical symptoms such as chronic weight loss and intermittent diarrhea and were confirmed Map infection by quantitative real-time PCR using fecal samples.

IFN-γ assays and detection of Map in feces

To examine the development of Th1 responses to Map, whole blood cells were incubated with 1 μg/ml of Johnin purified protein derivative (J-PPD) at 37°C under 5% CO_2 for 24 h. Collected culture supernatants were assayed for IFN-γ using ELISA as described previously (Nagata et al., 2005, 2010). The levels of fecal shedding of Map were determined using quantitative real-time PCR assay targeting the Map-specific gene IS*900* as described previously (Kawaji et al., 2014).

Cell preparation from blood and lymphoid tissues

Seven infected cattle, excluding animal 65, and all three age-matched uninfected controls were euthanized at 135–148 wpi. Heparinized peripheral blood, spleen, ileocecal LNs, ileal and jejunal mesenteric LNs (mLNs), ileum, and jejunum were collected from all cattle. PBMCs were purified from blood samples using density gradient centrifugation on Percoll (GE Healthcare, Buckinghamshire, UK). Peyer's patches (PPs) of ileum and jejunum were identified by gross morphological examination of the tissues. Spleen, LNs, and PPs of ileum and jejunum tissues were minced in PBS and passed through a 40-μm cell strainer (BD Biosciences, San Jose, CA, USA), and lymphocytes collected from the tissues were washed twice with PBS. Animal 65 was separately euthanized due to its severe diarrhea at 103 wpi and the ileum tissue sample was used only for immunohistological analysis.

Flow cytometric analysis of PD-1 and LAG-3

PBMCs, splenocytes, and lymphocytes were isolated from tissues and incubated in PBS containing 10% goat serum (Sigma–Aldrich) at room temperature for 15 min to prevent nonspecific reactions. Cells were then washed and stained with anti-PD-1 (5D2; rat IgG2a; Ikebuchi et al., 2013) or anti-LAG-3 (71-2D8; rat IgG1; this study) for 30 min at room temperature. After washing with PBS containing 10% goat serum, cells were stained with the following antibodies: anti-CD4-fluorescein isothiocyanate (FITC) (CC8; AbD Serotec, Oxford, UK), anti-CD8-peridinin-chlorophyll-protein complex (PerCp)/cyanin (Cy) 5.5 (CC63; AbD Serotec), anti-IgM-phycoerythrin (PE)/Cy7 (IL-A30; AbD Serotec), anti-CD3 (MM1A; VMRD, Pullman, WA, USA) pre-labeled with Zenon PE Mouse IgG1 Labeling Kit (Life Technologies, Carlsbad, CA, USA), and allophycocyanin (APC)-conjugated anti-rat Ig (Beckman Coulter, Fullerton, CA, USA) for 30 min at room temperature. CC63 and IL-A30 were conjugated with PerCp/Cy5.5 and PE/Cy7, respectively, using Lightning-Link Conjugation Kits (Innova Biosciences, Cambridge, UK). Cells were then washed and analyzed immediately using FACS Aria (BD Biosciences) and FCS Express 4 (De Novo Software, Glendale, CA, USA). The primary antibodies used in this study are shown in Table I-1.

Flow cytometric analysis of PD-L1 and MHC II

PBMCs, splenocytes, and lymphocytes were isolated from tissues and then blocked with PBS containing 10% goat serum, and washed and stained with anti-PD-L1 (4G12; rat IgG2a; Ikebuchi et al., 2014a) and anti-CD11b (CC126; mouse IgG2b, AbD

Serotec) for 30 min at room temperature. After washing with PBS containing 10% goat serum, cells were stained with the following antibodies: anti-CD14-APC/Cy7 (CAM36A; VMRD), anti-MHC II (CAT82A; VMRD) pre-labeled with Zenon PE Mouse IgG1 Labeling Kit (Life Technologies, Carlsbad, CA, USA), APC-conjugated anti-rat Ig (Beckman Coulter), and FITC-conjugated anti-mouse IgG2b (Beckman Coulter) for 30 min at room temperature. CAM36A was conjugated using Lightning-Link APC-Cy7 Tandem Conjugation Kit (Innova Biosciences). Cells were then washed and immediately analyzed using FACS Aria and FCS Express 4. The primary antibodies used in this study are shown in Table I-1.

Immunohistochemical analysis of PD-L1 and Ziehl–Neelsen staining

Tissue sections of ileum from cattle that were experimentally or naturally infected with Map were subjected to immunohistochemical study. Tissue samples of the experimentally infected animals were collected from animals 58 and 65, both of which showed Map shedding in feces and clinical symptoms of disease onset such as diarrhea. All of the naturally infected animals used in this study also showed clinical signs of Johne's disease. The ileum tissues were fixed by formalin, embedded into paraffin wax and then cut into 4-mm-thick sections. The dried sections were deparaffinized in xylene on the slide glass. Antigen retrieval was performed in citrate buffer (0.37 g/ml of citric acid and 2.4 g/ml of trisodium citrate dihydrate) by microwave heating for 10 min. Endogenous peroxidase activity was blocked by incubating the sections in methanol containing 0.3% hydrogen peroxide for 15 min. Primary antibody incubation was performed at room temperature for 30 min using anti-bovine PD-L1 mAb (6C11; rat IgG2a; 5 μg/ml; Ikebuchi et al., 2014a). Sections were washed twice with PBS and incubated with Histofine simple stain MAX PO (rat) (Nichirei, Tokyo, Japan) at room temperature for 30 min. Positive staining was visualized with 3-diaminobenzidine tetrahydrochloride. The sections were observed under an optical microscope. Additionally, a Ziehl–Neelsen staining was performed to detect acid-fast bacilli in ileum tissues.

Blockade assays in whole blood culture and IFN-γ ELISA

To investigate whether immunoinhibitory receptors alter Map-specific IFN-γ responses, heparinized whole blood cells were incubated with 10 μg/ml of the blocking mAbs anti-PD-1 (5D2; Ikebuchi et al., 2013), anti-PD-L1 (5A2; Ikebuchi et al., 2014a),

or anti-LAG-3 (71-2D8; this study) in the presence of 5 μg/ml J-PPD; rat IgG (Sigma–Aldrich) was used as a negative control antibody. Negative control antigens were PPD purified from *M. bovis* BCG strain (B-PPD; 5 μg/ml) or sterile PBS. All blood cultures were grown in 24-well plates (Corning Inc., Corning, NY, USA) at 37°C with 5% CO_2 for 24 h. Subsequently, plasma was harvested, and IFN-γ was determined using ELISA for bovine IFN-γ (Mabtech, Nacka Strand, Sweden) according to the manufacturer's protocol. Results were calculated based on a standard curve ranging from 7.8 to 500 pg/ml. Data are presented as the mean of duplicate samples.

PBMC blockade assays and flow cytometric analysis of IFN-γ-producing T cells

To examine the effects of blocking mAbs on Map-specific T-cell responses, *in vitro* blockade assays were performed using PBMCs cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS; Cansera International Inc., Ontario, Canada), 200 IU/ml penicillin, 200 μg/ml streptomycin, and 0.01% v/v L-glutamine (Life Technologies). All cell cultures were grown in 24-well plates (Corning Inc.) containing 2.5×10^6 PBMCs in 500 μl medium with 10 μg/ml of anti-PD-1, anti-PD-L1, anti-LAG-3, or rat IgG (Sigma–Aldrich), and J-PPD (10 μg/ml) at 37°C with 5% CO_2 for 19 h. B-PPD (5 μg/ml) or sterile PBS were used as negative control antigens. For a positive control, the cells were stimulated with 10 μg/ml of concanavalin A (ConA; Sigma–Aldrich). Brefeldin A (Sigma–Aldrich) was added at 10 μg/ml for the final 6 h to enhance intracellular cytokine staining signals by blocking transport processes.

Cultured PBMCs were collected and incubated in PBS containing 10% goat serum as described above. Cells were then washed and stained with anti-CD4-FITC (CC8; AbD Serotec), anti-CD8-PerCp/Cy5.5 (CC63; AbD Serotec), and anti-CD69-Alexa Fluor 647 (KTSN7A; Kingfisher Biotech, Saint Paul, MN, USA) for 30 min at 4°C. KTSN7A was pre-labeled using Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit (Life Technologies). After surface staining, cells were fixed and permeabilized using FOXP3 Fix/Perm kit (BioLegend, San Diego, CA, USA) according to the manufacturer's protocol. Cells were subsequently stained with anti-IFN-γ-PE (CC302; AbD Serotec), washed, and analyzed immediately using FACS Aria (BD Biosciences) and FCS Express 4 (De Novo Software). The primary antibodies used in this study are shown in Table I-1.

Statistics

Differences were identified using Welch's *t*-test and Tukey's test with the MEPHAS program (http://www.gen-info.osaka-u.ac.jp/MEPHAS/), and $P < 0.05$ was considered statistically significant.

Table I-1. Primary antibodies used in flow cytometric analysis of Chapter I

Antigen	Isotype	Clone	Source (reference)	Fluorochrome	Conjugation or labeling
Flow cytometric analysis of PD-1 and LAG-3					
CD4	Mouse IgG2a	CC8	AbD Serotec	FITC	(Conjugated primary antibody)
CD8	Mouse IgG2a	CC63	AbD Serotec	PerCp-Cy5.5	Lightning-Link PerCp/Cy5.5 Conjugation Kit (Innova Biosciences)
CD3	Mouse IgG1	MM1A	VMRD	PE	Zenon PE Mouse IgG1 Labeling Kit (Life Technologies)
IgM	Mouse IgG1	IL-A30	AbD Serotec	PE-Cy7	Lightning-Link PE/Cy7 Conjugation Kit (Innova Biosciences)
PD-1	Rat IgG2a	5D2	In house (Ikebuchi et al., 2013)	APC	APC-conjugated anti-rat Ig antibody (Beckman Coulter)
LAG-3	Rat IgG1	71-2D8	In house (this study)	APC	APC-conjugated anti-rat Ig antibody (Beckman Coulter)
Flow cytometric analysis of PD-L1 and MHC II					
CD14	Mouse IgG1	CAM36A	VMRD	APC-Cy7	Lightning-Link APC/Cy7 Conjugation Kit (Innova Biosciences)
CD11b	Mouse IgG2b	CC126	AbD Serotec	FITC	FITC-conjugated anti-mouse IgG2b antibody (Beckman Coulter)
MHC II	Mouse IgG1	CAT82A	VMRD	PE	Zenon PE Mouse IgG1 Labeling Kit (Life Technologies)
PD-L1	Rat IgG2a	4G12	In house (Ikebuchi et al., 2014a)	APC	APC-conjugated anti-rat Ig antibody (Beckman Coulter)
Flow cytometric analysis of IFN-γ-producing T cells (intracellular staining)					
CD4	Mouse IgG2a	CC8	AbD Serotec	FITC	(Conjugated primary antibody)
CD8	Mouse IgG2a	CC63	AbD Serotec	PerCp-Cy5.5	Lightning-Link PerCp/Cy5.5 Conjugation Kit (Innova Biosciences)
CD69	Mouse IgG1	KTSN7A	Kingfisher Biotech	Alexa Fluor 647	Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit (Life Technologies)
IFN-γ	Mouse IgG1	CC302	AbD Serotec	PE	(Conjugated primary antibody)

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCp, peridinin-chlorophyll-protein complex; APC, allophycocyanin; Cy, cyanin.

RESULTS

Establishment of anti-bovine LAG-3 mAbs

Supernatants containing antibody from 415 hybridoma clones were screened for binding activity with LAG-3 71–99 and 104–132 peptides using ELISA. In these experiments, 54 hybridomas produced mAbs that reacted with the immunizing peptide alone (Table I-2). To examine the binding of mAbs with LAG-3 on cell membranes, COS-7 cells expressing bovine LAG-3-EGFP were stained with supernatants or purified mAbs. Binding to LAG-3-EGFP-expressing COS-7 cells was observed for 14 supernatants (Table I-2), and the strongest fluorescence intensity in 8 purified anti-LAG-3 mAbs was observed in cells stained with 71-2D8 (Fig. I-1A). Therefore subsequent expression analyses of bovine LAG-3 were performed using anti-LAG-3 mAb 71-2D8. In addition, the 71-2D8 mAb recognized a heat-denatured LAG-3-EGFP protein of about 93 kD in western blot analysis (Fig. I-1B).

To confirm reactivity of anti-LAG-3 mAb 71-2D8 with naturally expressed LAG-3 on bovine lymphocytes, surface expression of LAG-3 was evaluated in freshly isolated $CD4^+$ and $CD8^+$ T cells or in those stimulated with PMA/ionomycin *in vitro*. The 71-2D8 mAb recognized LAG-3 expressed on the surface of $CD4^+$ and $CD8^+$ T cells (Fig. I-1C). LAG-3 was expressed mainly on $CD8^+$ T cells from freshly isolated PBMCs, whereas $CD4^+$ T cells expressed low levels of LAG-3 (Fig. I-1C). PMA/ionomycin stimulation strongly induced LAG-3 expression on both $CD4^+$ and $CD8^+$ T cells (Fig. I-1C).

Suppression of T-cell responses to J-PPD and fecal shedding of Map in Map-infected cattle

To characterize Th1 responses and disease progression in the present Map-infected cattle, IFN-γ responses from blood cells stimulated with J-PPD were determined and Map shedding was evaluated in feces every 2–4 weeks. Map DNA was detected in feces of all seven infected calves from 10 to 30 wpi (Fig. I-2), and with the exception of animal 58 and 65, shedding of Map subsequently declined and was intermittent for up to 2 years, when the study was terminated. Animals 58 and 65 resumed and sustained high levels of bacterial shedding and showed symptoms of clinical onset such as diarrhea. In particular, animal 65 showed the highest level of Map shedding (0.027–2.27 pg of Map DNA) in feces, from 92 to 100 wpi, just before the

necropsy (Fig. I-2). J-PPD-specific IFN-γ responses peaked during the acute phase of infection in infected animals 57, 58, 60, and 61 but peaked during the subclinical stage in the other three animals (62, 64, and 66; Fig. I-2). With the exception of animals 58 and 65, IFN-γ production was strongly suppressed by 120 wpi. The IFN-γ response of animal 58 declined until 93 wpi and increased again following bacterial growth in the intestine. In addition, nonspecific production of IFN-γ to J-PPD stimulation and fecal detection of Map in uninfected cattle is also shown as negative controls in Fig. I-2.

Upregulation of PD-1 and LAG-3 on T cells in Map-infected cattle at the late subclinical stage

Th1 responses to the J-PPD antigen were suppressed at the subclinical stage in the infected cattle. Thus, it was hypothesized that the exhaustion of Map antigen-specific T-cell responses was caused by upregulation of immunoinhibitory receptors on T cells. To test this hypothesis, the cell-surface expression of PD-1 and LAG-3 on $CD4^+$ and $CD8^+$ T cells was investigated by flow cytometric analysis of PBMCs and lymphocytes of the spleen, LNs, and PPs isolated from Map-infected cattle at 135–148 wpi. As shown in Fig. I-3A, $CD4^+$ and $CD8^+$ T cells were gated in IgM^-CD3^+ lymphocytes and were then further analyzed for PD-1 and LAG-3 expression. No significant differences in numbers of $PD\text{-}1^+CD4^+$ T cells were observed in tested tissues between infected and uninfected animals. However, $PD\text{-}1^+CD8^+$ T cells were significantly more abundant in ileal mLNs of infected animals (Fig. I-3B). In contrast, LAG-3 expression was upregulated on $CD4^+$ and $CD8^+$ T cells in peripheral blood from infected animals (Fig. I-3C), suggesting that upregulation of PD-1 and LAG-3 on T cells contributes to T-cell exhaustion in Map-infected cattle.

High expression of PD-L1 and MHC II on macrophages in Map-infected cattle during the late subclinical stage

To confirm the potential of macrophages from infected cattle to interact with T cells through immunoinhibitory ligands, the expression status of PD-L1 and MHC II on macrophages from blood, spleen, LNs and PPs was determined using flow cytometry. As shown in Fig. I-4A, $CD14^+CD11b^+$ macrophages were gated in monocytes from blood and were further analyzed for the expression of PD-L1 and MHC II. Large populations of $CD14^+CD11b^+$ macrophages were found in PBMCs and LNs, whereas those in ileal and jejunal PPs were few; expression analyses were therefore not

performed using monocytes of PPs (data not shown). Mean percentages of PD-L1-expressing $CD14^+CD11b^+$ macrophages were significantly higher in PBMCs of the Map-infected group, although two animals showed lower levels of PD-L1 expression than uninfected animals (Fig. I-4B). PD-L1 was expressed on the majority of $CD14^+CD11b^+$ macrophages from the spleen and LN tissues in both groups (Fig. I-4B). Moreover, mean frequencies of MHC II-expressing $CD14^+CD11b^+$ macrophages were 95.3% in PBMCs, 57.0% in spleen, and 74.6%–78.1% in LNs of the infected group (Fig. I-4B). Although the numbers of splenic MHC $II^+CD14^+CD11b^+$ macrophages from infected animals were significantly lower than in uninfected animals, no significant differences in the numbers of MHC $II^+CD14^+CD11b^+$ macrophages from PBMCs and LNs were observed between uninfected and infected animals (Fig. I-4C). Overall, macrophages from infected cattle express PD-L1 and MHC II and have the potential to interact with PD-1- and LAG-3-expressing T cells.

Expression of PD-L1 in Map-infected macrophages of the ileum

Previous reports have clarified the expression status of MHC II in the infected intestine during Johne's disease (Valheim et al., 2004; Lei et al., 2008), but that of PD-L1 in the intestine was still unknown. Therefore, the expression status of PD-L1 was examined in intestinal macrophages infected with Map using immunohistological staining with PD-L1 and Ziehl–Neelsen stain. $PD\text{-}L1^+$ cells were detected in the lamina propria of the ileum of experimentally infected cattle (58 and 65) with Map shedding in feces (Fig. I-5A). Map-infected cells were observed in the same lesion of animal 65, but not in those of animal 58 (Fig. I-5B), which is consistent with the lower level of bacterial shedding in animal 58 than animal 65 (Fig. I-2). Furthermore, Map-infected macrophages and epithelioid cells expressed PD-L1 in the ileum of naturally infected cattle with clinical diarrhea (Fig. I-5A and B).

Dual blockade of the PD-1 and LAG-3 pathways reactivates Map-specific IFN-γ production in blood from Map-infected cattle.

It remained unclear whether IFN-γ responses to Map antigens are restored by blockade of the PD-1/PD-L1 and LAG-3/MHC II pathways. To address this question, IFN-γ responses to J-PPD in whole blood were assessed in the presence of the blocking mAbs, anti-PD-1, anti-PD-L1, and anti-LAG-3. Although IFN-γ production in the blood of infected animals tended to be more strongly induced by J-PPD than B-PPD, this

difference (4.4-fold) was not significant (Fig. I-6). Nonetheless, LAG-3 blockade tended to elevate IFN-γ production in the presence of J-PPD compared with the control IgG (Fig. I-6). Furthermore, dual blockade using anti-PD-1 and anti-LAG-3 mAbs significantly enhanced the IFN-γ response to J-PPD relative to that in the presence of control IgG or anti-PD-1 mAb alone (Fig. I-6). These results suggest that combined blockade of the PD-1 and LAG-3 pathways reactivates Map-specific IFN-γ responses in Map-infected cattle.

LAG-3 blockade restores IFN-γ production from Map-specific T cells.

J-PPD is broadly recognized to induce IFN-γ-producing $CD4^+$ and $CD8^+$ T cells in Map-infected cattle (Stable et al., 2007; Plattner et al., 2011). Thus, it was investigated whether blockade of the PD-1 and LAG-3 pathways activates J-PPD-specific IFN-γ production in $CD4^+$ and $CD8^+$ T cells. As expected, $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells from Map-infected cattle produced IFN-γ in response to J-PPD (Fig. I-7A). Accordingly, $CD4^+CD69^+IFN\text{-}\gamma^+$ and $CD8^+CD69^+IFN\text{-}\gamma^+$ T cells induced by J-PPD were defined as J-PPD-specific T cells (Fig. I-7A). Interestingly, the numbers of J-PPD-specific T cells in total $CD4^+$ and $CD8^+$ T-cell populations were dramatically increased by LAG-3 blockade in infected animals (Fig. I-7A–C). Moreover, compared with control IgG treatments, LAG-3 blockade significantly enhanced IFN-γ production in J-PPD-specific $CD8^+$ T cells (Fig. I-7C), indicating that IFN-γ responses of J-PPD-specific $CD4^+$ and $CD8^+$ T cells can be reactivated by LAG-3 blockade in Map-infected cattle.

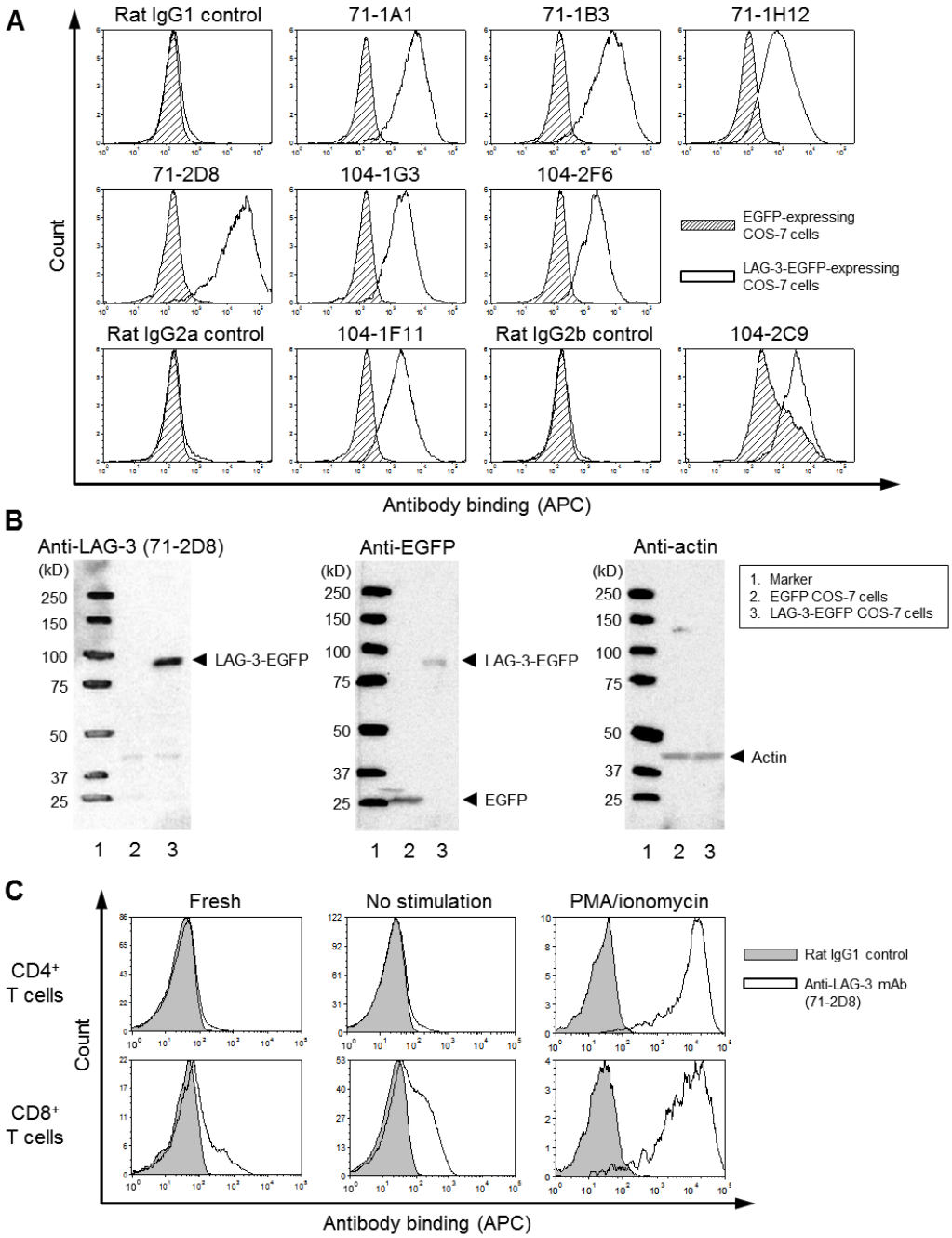

Figure I-1. Reactivity of the anti-LAG-3 mAb with LAG-3-expressing cells and bovine lymphocytes.

(A) Flow cytometric analysis of bovine LAG-3. COS-7 cells expressing LAG-3-EGFP (white area) and EGFP (shaded area) were stained using eight anti-LAG-3 mAb clones. Rat IgG1 (for 71-1A1, 71-1B3, 71-1H12, 71-2D8, 104-1G3, and 104-2F6), rat IgG2a (for 104-1F11), and rat IgG2b (for 104-2C9) were used as negative controls. (B) Western blots of bovine LAG-3 protein prepared from COS-7 cells using anti-LAG-3 mAb (71-2D8). Anti-EGFP and anti-actin antibodies were used as positive and loading controls, respectively. (C) Flow cytometric analysis of LAG-3 expression in $CD4^+$ and $CD8^+$ T cells. Freshly isolated bovine PBMCs were stained with anti-LAG-3:71-2D8 (white area), CD4, and CD8 mAbs. Rat IgG1 (gray area) was used as a negative control stain. PBMCs were cultured with PBS (no stimulation) or PMA/ionomycin for 48 h and were analyzed as described above.

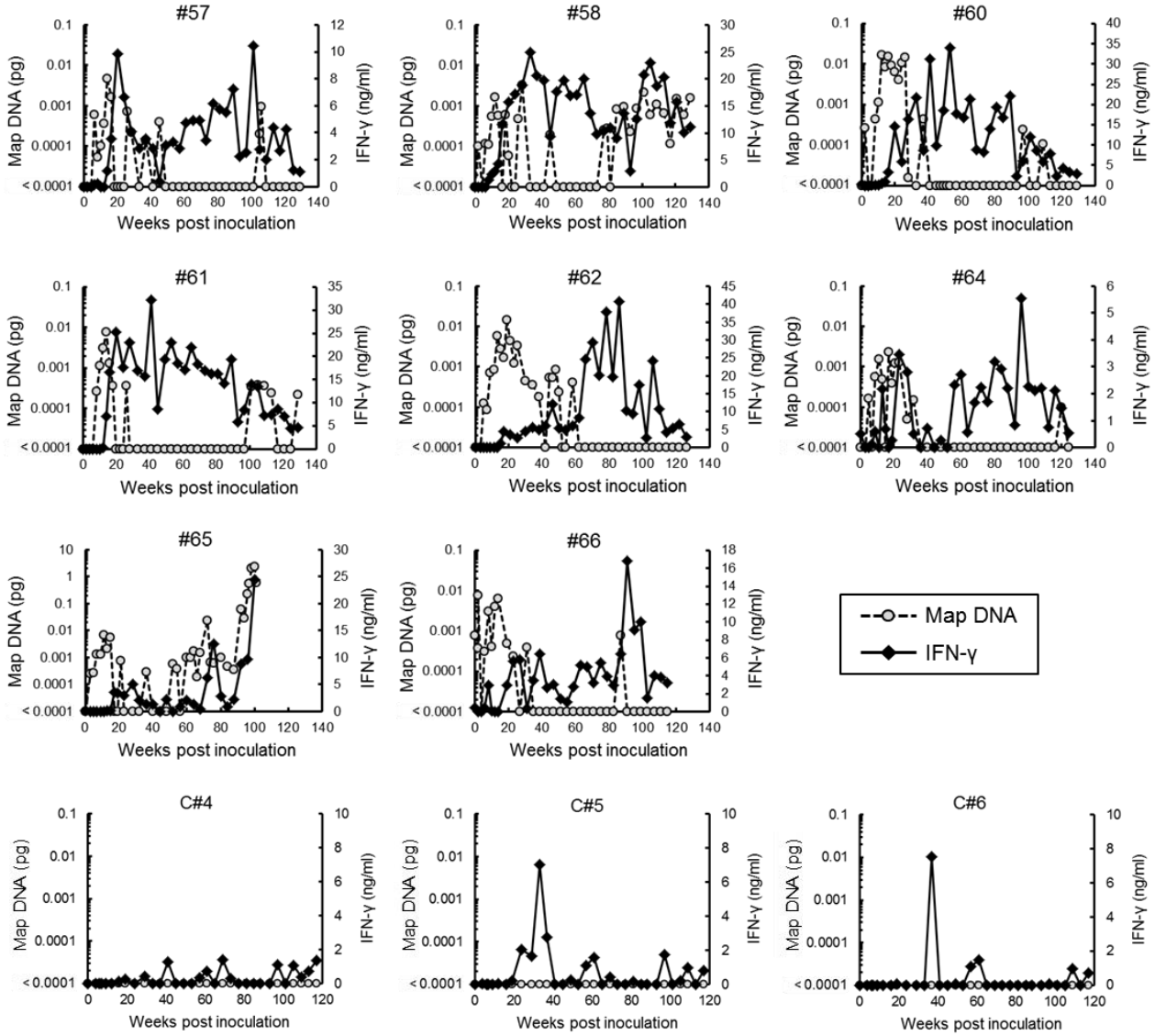

Figure I-2. IFN-γ responses to J-PPD in blood and fecal shedding of Map in cattle experimentally infected with Map.

Dates of sample collection are shown on the horizontal axis. The left vertical axis (gray circle) indicates Map DNA quantity in fecal samples which was determined using IS900 real-time PCR. The detection limit of this assay was 0.0001 pg/2.5 µl (Kawaji et al., 2014). Thus, the sample in which Map DNA was not detected is shown as "<0.0001 pg" on the graph. The right vertical axis (black diamond) shows IFN-γ productions in whole blood cultures stimulated with J-PPD.

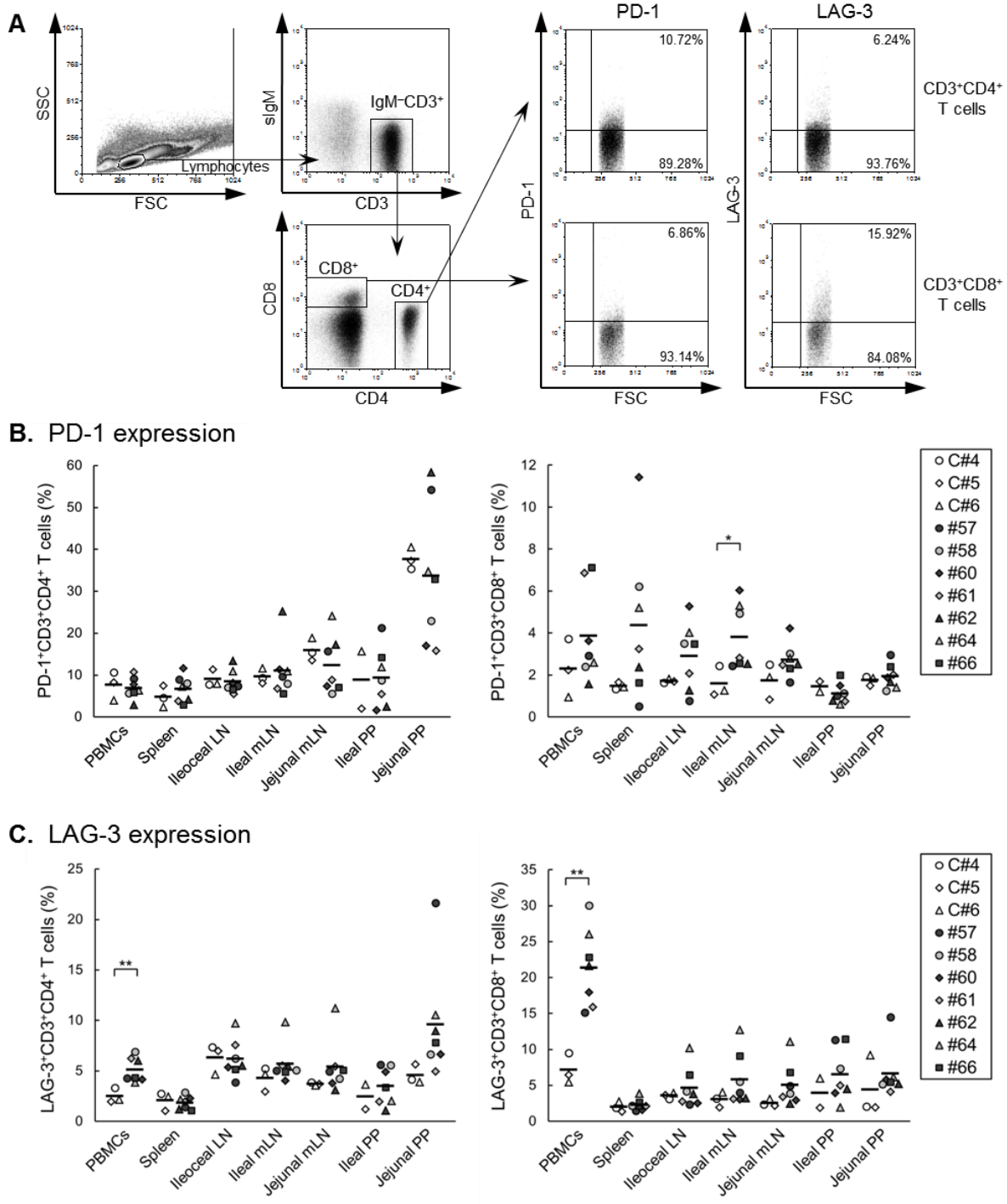

Figure I-3. Expression levels of PD-1 and LAG-3 on T cells in Map-infected cattle.
(A) Gating strategy and representative dot plots for expression analyses of PD-1 and LAG-3 on $IgM^-CD3^+CD4^+$ and $IgM^-CD3^+CD8^+$ T cells from the blood of Map-infected cattle. Values shown in the quadrant indicate percentages of cells. (B and C) Percentages of PD-1- (B) and LAG-3-expressing cells (C) in $IgM^-CD3^+CD4^+$ and $IgM^-CD3^+CD8^+$ T cells of peripheral blood, spleen, ileocecal LN, ileal and jejunal mLNs, and ileal and jejunal PPs isolated from uninfected cattle (n = 3) and Map-infected cattle (n = 7). Significant differences were determined using Welch's t-test. *, $P < 0.05$; **, $P < 0.01$ between the uninfected and infected groups.

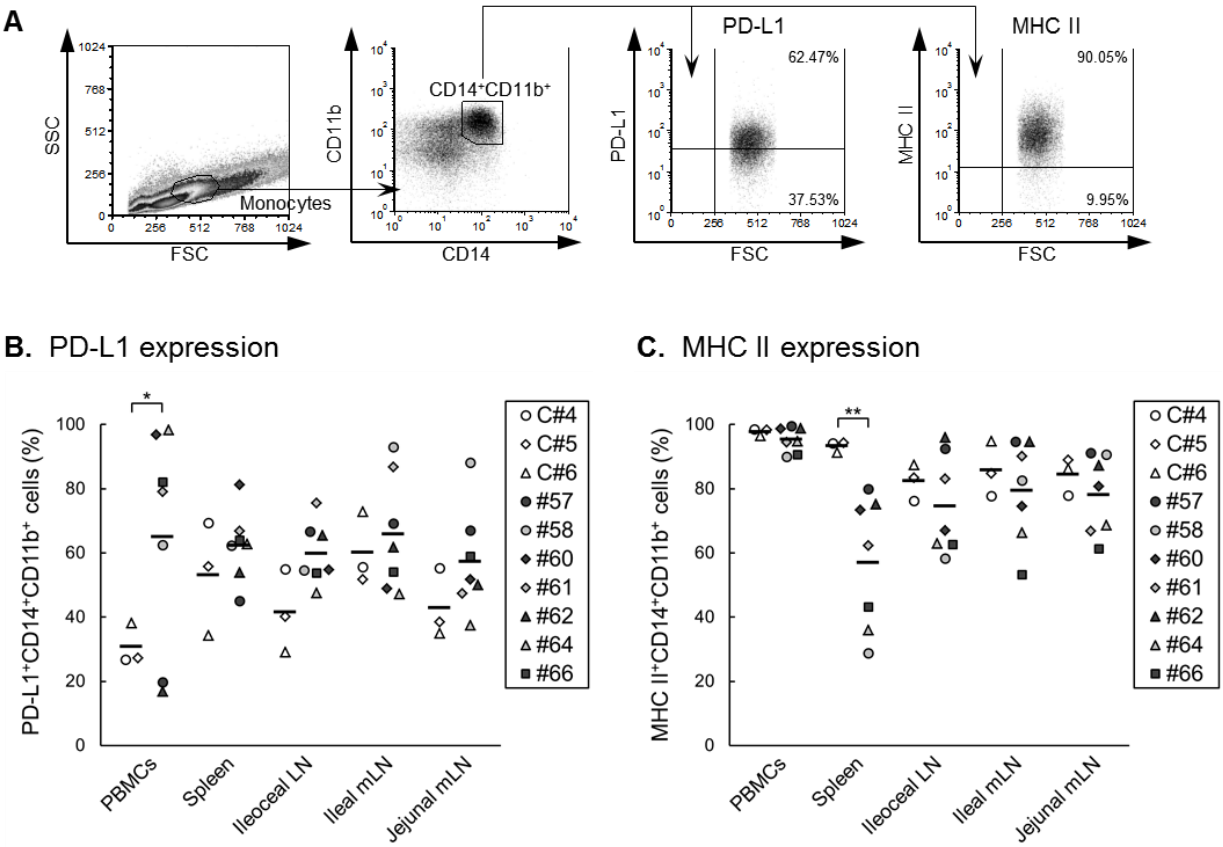

Figure I-4. Expression levels of PD-L1 and MHC II on macrophages in Map-infected cattle.

(A) Gating strategy and representative dot plots for expression analysis of PD-L1 and MHC II on $CD14^{+}CD11b^{+}$ macrophages from the blood of Map-infected cattle. Values shown in the quadrant indicate percentages of the cells. (B and C) Percentages of PD-L1- (B) and MHC II-expressing cells (C) among $CD14^{+}CD11b^{+}$ cells of peripheral blood, spleen, ileocecal LNs, and ileal and jejunal mLNs from uninfected cattle (n = 3) and Map-infected cattle (n = 7). Significant differences were determined using Welch's t-test. *, $P < 0.05$; **, $P < 0.01$ between the uninfected and infected groups.

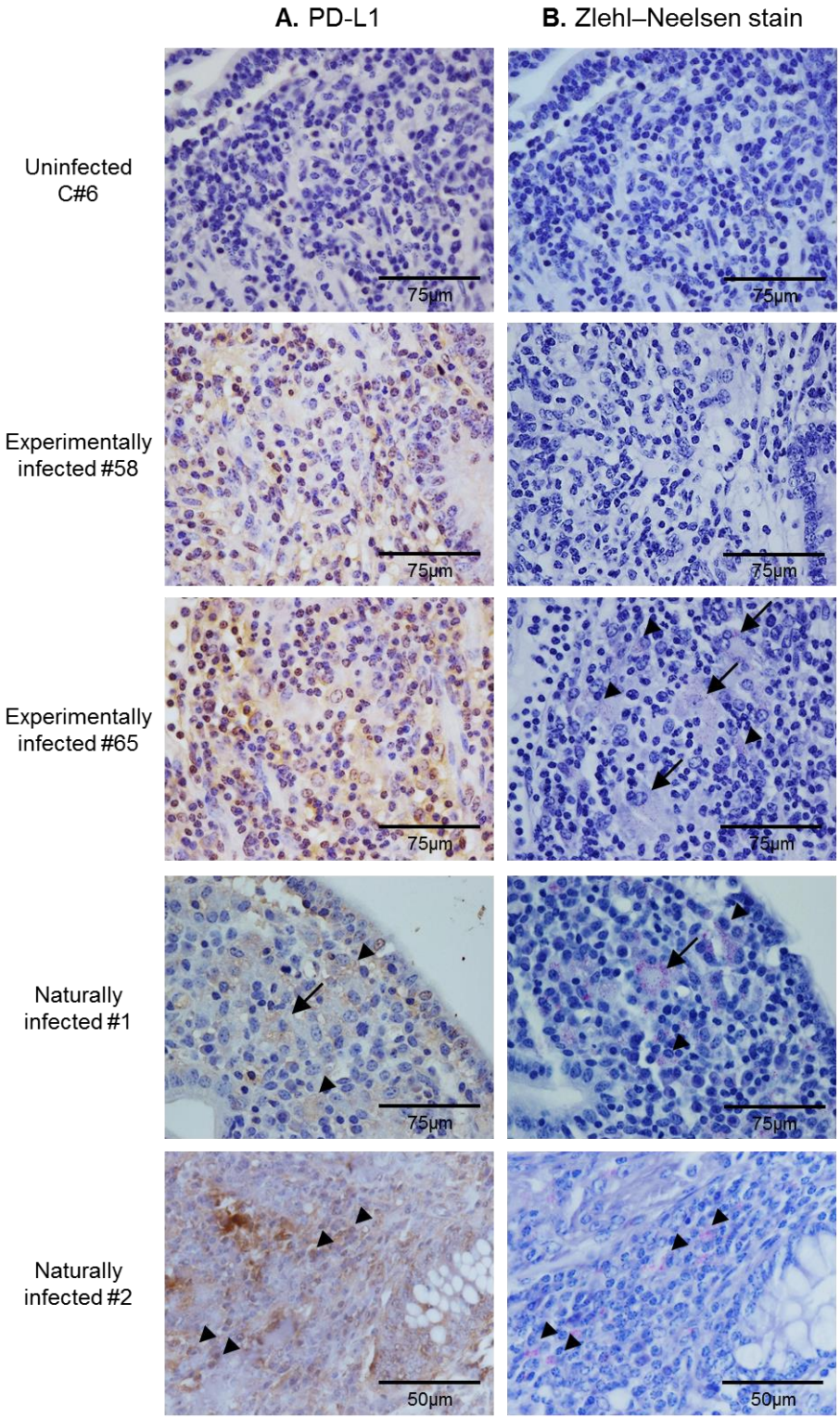

Figure I-5. PD-L1 expression and localization of Map in ileal mucosa of Map-infected cattle.

Immunohistochemical staining of PD-L1 (A) and Ziehl–Neelsen staining for acid fast bacilli (B) in ileum tissues. Immunohistochemical staining was performed using anti-PD-L1 mAb (6C11). Arrowheads and arrows indicate Map-infected macrophages and epithelioid cells, both of which express PD-L1, respectively.

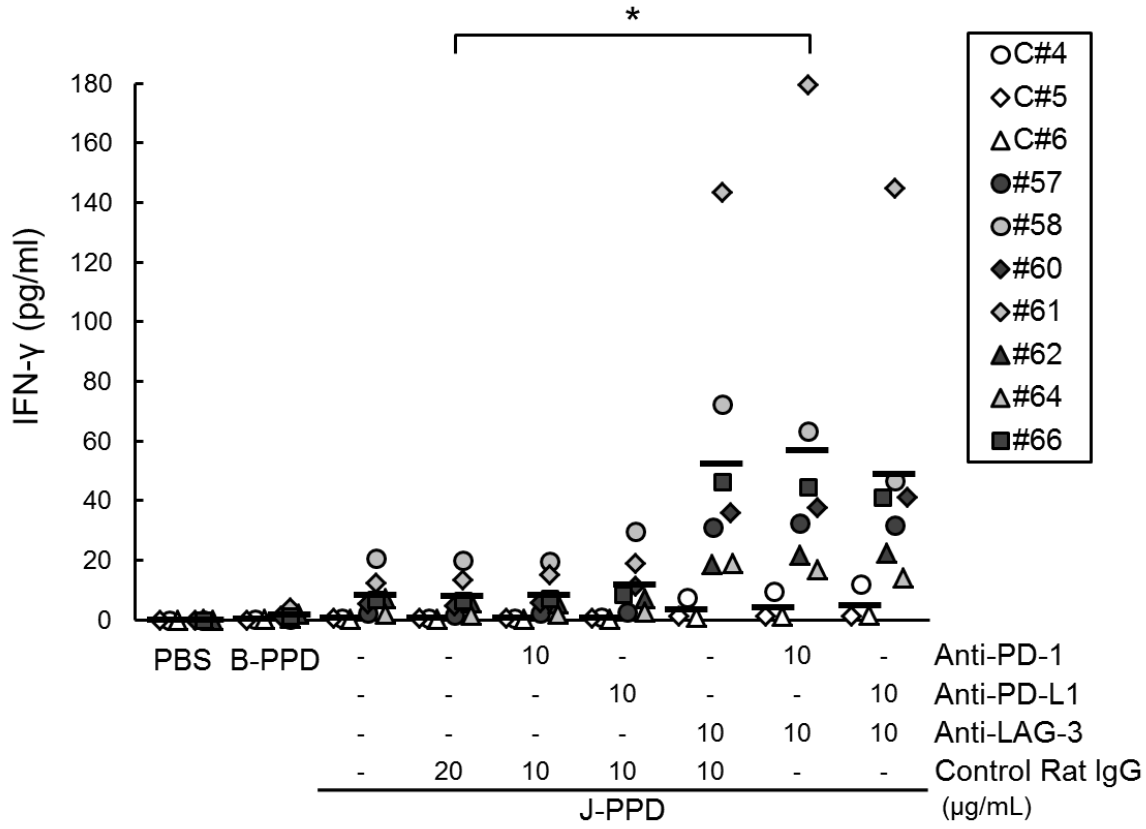

Figure I-6. Reactivation of J-PPD-specific IFN-γ responses by LAG-3 blockade.
Whole blood cells were cultured with blocking mAbs (anti-PD-1, anti-PD-L1, and anti-LAG-3 mAbs; 10 μg/ml) or rat IgG control in the presence of J-PPD (5 μg/ml). IFN-γ production in plasma was measured using ELISA (uninfected; $n = 3$, infected; $n = 7$). Significant differences between each group were determined using Tukey's multiple-comparison test. *, $P < 0.05$ between each stimulation.

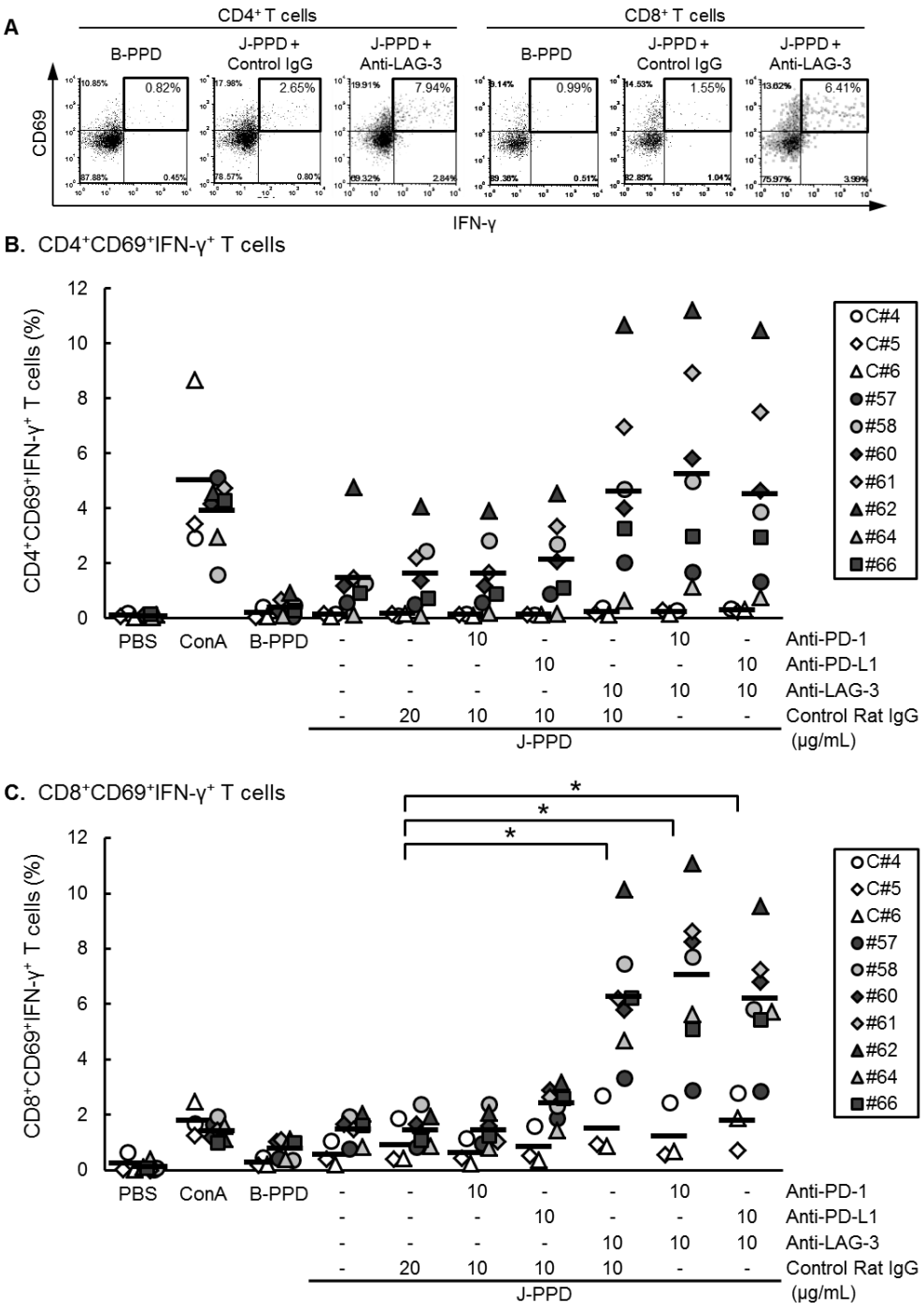

Figure I-7. Reactivation of J-PPD-specific T cells by LAG-3 blockade.

PBMCs from Map-infected cattle (n = 7) and uninfected cattle (n = 3) were cultured with blocking mAbs (anti-PD-1, anti-PD-L1, and anti-LAG-3 mAbs; 10 μg/ml) or rat IgG control in the presence of J-PPD (10 μg/ml). (A) Representative dot plots for $CD4^+CD69^+IFN\text{-}\gamma^+$ and $CD8^+CD69^+IFN\text{-}\gamma^+$ T cells of an infected animal following treatment with B-PPD (left panels) or J-PPD with and without LAG-3 blockade (right and central panels, respectively). Values in the quadrant indicate percentages of cells evaluated by flow cytometry. (B and C) Percentages of $CD69^+IFN\text{-}\gamma^+$ cells among $CD4^+$ (B) and $CD8^+$ T cells (C) after treatment with J-PPD and blocking mAbs. Significant differences between each group were determined using Tukey's multiple-comparison test. *, $P < 0.05$ between each stimulation.

DISCUSSION

In cattle with Johne's disease, intracellular Map infection of macrophages leads to presentation of bacterial antigens to Map-specific T cells, which elicit strong Th1 responses during the acute phase of infection (Stabel, 2006; Coussens, 2004; Sohal et al., 2008). When antigen presentation is extended beyond the T-cell expansion phase, Map-specific T cells are gradually exhausted and become unresponsive to stimulation with Map antigen (Bassey and Collins, 1997; Burrells et al., 1998; Weiss et al., 2006). Thus, to elucidate the associated molecular mechanisms, this study determined whether immunoinhibitory receptors mediate the development of exhausted T cells during subclinical Map infection in cattle.

The present study demonstrated that PD-1 was upregulated on $CD8^+$ T cells of ileal mLNs in Map-infected cattle with long-term suppression of Th1 responses at the late subclinical stage Consistent with this finding, PD-1 is reportedly upregulated on $CD4^+$ T cells in *M. bovis*-infected mice and in *M. tuberculosis*-infected patients, leading to $CD4^+$ T-cell exhaustion and bacterial persistence (Sakai et al., 2010; Singh et al., 2013). Thus, T-cell exhaustion via the PD-1 pathway appears to be common among mycobacterial infections. In addition, large populations of PD-1^+CD4^+ T cells (15%–58% of total $CD4^+$ T cells) were found in jejunal PP from both uninfected and infected animals, and no significant differences were observed between these groups (Fig. I-3B). Potentially, these cells act as follicular helper T cells, which reportedly play key roles in the development and maintenance of B cells in PPs (Kawamoto et al., 2012; Maruya et al., 2013), but differ from the present PD-1^+CD4^+ exhausted T cells.

Inhibitory signals of exhausted T cells are induced by cross-linking between immunoinhibitory receptors on T cells and their ligands on antigen-presenting cells (Kamphorst and Ahmed, 2013; Sierro et al., 2011). Map persistently infects macrophages of the ileum and of draining LNs and escapes host immune responses through various mechanisms (Coussens, 2004; Sohal et al., 2008). In the current study, PD-L1 was expressed in macrophages of mLNs and in Map-infected cells of the ileum, indicating that PD-1/PD-L1 interaction inhibits T-cell function in infected lesions. Additionally, PD-L1 was significantly upregulated on $CD14^+CD11b^+$ macrophages in PBMCs of the Map-infected cattle. Furthermore, the percentages of PD-L1-expressing $CD14^+CD11b^+$ macrophages tended to be elevated in the spleen and LNs of the infected

animals, although the difference was not significant due to the limited number of samples tested in this study.

LAG-3 was upregulated on $CD4^+$ and $CD8^+$ T cells in the peripheral blood of Map-infected cattle, indicating that LAG-3 plays a more dominant immunomodulatory role than PD-1 in circulating $CD4^+$ and $CD8^+$ T cells. In contrast, LAG-3 expression was not changed on $CD4^+$ and $CD8^+$ T cells in mLNs and PPs from the infected animals (Fig. I-3C), where T cells encounter the pathogen rather than peripheral blood. In PBMCs, there are a number of J-PPD-responding T cells, including "Map-specific T cells" (Fig. I-7). As to the significance of LAG-3 expression in PBMCs, it is hypothesized that LAG-3 would be upregulated on Map-specific T cells in the peripheral blood, causing loss of their effector functions. Further studies are required to clarify the mechanisms of LAG-3 upregulation in peripheral blood but not in lymphoid tissues. The MHC II molecule was reportedly expressed in the small intestinal mucosa of a goat with subclinical infection (Valheim et al., 2004), and in $CD11c^+$ dendritic cells from Map-induced cattle granulomas (Lei et al., 2008). Nonetheless, a previous report showed that *in vitro* Map infection of bovine macrophages resulted in the downregulation of MHC II (Weiss et al., 2001), and MHC II was downregulated on splenic $CD14^+CD11b^+$ macrophages in the current study. However, these molecules were not downregulated in blood and LNs, despite prevalent expression (95% and 74%–79% of macrophages in blood and LNs, respectively).

$CD8^+$ cytotoxic T cells play a central role in the killing of intracellular bacteria, including Map, in macrophages. In this study, the cell-surface expression of PD-1 and LAG-3 were upregulated in $CD8^+$ T cells of the infected animals, suggesting the functional exhaustion of $CD8^+$ cytotoxic T cells during subclinical Map infection. Such exhaustion might facilitate bacterial persistence and disease progression.

In chronic infections, expression of PD-1 and LAG-3 is induced by continuous antigen presentation and TCR stimulation (Wherry, 2011; Kamphorst and Ahmed, 2013; Sierro et al., 2011). Thus, PD-1 and LAG-3 were hypothesized to be upregulated on Map antigen-specific T cells, causing the loss of their effector functions in these cells. PD-1 and LAG-3 were upregulated in total $CD4^+$ and $CD8^+$ T-cell populations from Map-infected cattle. However, in this study, flow cytometric analysis of PD-1 and LAG-3 was not performed on Map antigen-specific T cells because of the lack of an MHC-peptide tetramer. The construction of a Map-specific MHC-peptide tetramer would enable further analysis of Map-specific $PD\text{-}1^+$ and $LAG\text{-}3^+$ exhausted T cells.

Nevertheless, the present broad expression analysis revealed the upregulation of PD-1 and LAG-3 on T cells originating from different sites in Map-infected cattle, suggesting that PD-1 and LAG-3 play immunomodulatory roles in various populations of T cells. These observations warrant further studies to determine whether PD-1 and LAG-3 are co-expressed to synergistically depress the function of exhausted T cells in Johne's disease. Furthermore, other immunoinhibitory receptors TIM-3 (Chapter III) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4; Suzuki et al., 2015) are also likely involved in the development of exhausted T cells in Johne's disease. Accordingly, a recent study showed that $CD4^+$ T cells in cattle upregulated their cell-surface expression of CTLA-4 during the subclinical stage of Map infection (Leite et al., 2015).

Previous studies show that Map infection inhibits phagocytic maturation of macrophages (Frehel et al., 2002), downregulates MHC class I and II molecules (Weiss et al., 2001), and induces IL-10 production (Balcewicz-Sablinska et al., 1999), resulting in immune evasion and bacterial persistence. Accordingly, PD-L1 was upregulated on circulating macrophages and was localized to Map-infected cells of the ileum in the Map-infected cattle from the present study. These observations suggest that Map evades the immune system by upregulating PD-L1 in infected macrophages.

In vitro blockade assays of whole blood cells and PBMCs showed that blockade with anti-LAG-3 mAb efficiently reactivated Map-specific T-cell responses, and this was consistent with the high-level expression of LAG-3 on T cells in PBMCs of the infected animals. In addition, $CD8^+$ T cells in PBMCs strongly expressed LAG-3 and responded to LAG-3 blockade with significant restoration of Map-specific IFN-γ production. Moreover, PD-1 was upregulated on $CD8^+$ T cells in ileal mLN tissues, indicating that the reactivation effect of PD-1/PD-L1 blockade may also occur in blockade assays of lymphocytes from mLN tissues. From the diagnostic point of view, enhancing Map-specific IFN-γ responses by LAG-3 blockade could be helpful to the diagnosis of Map using the IFN-γ test (Jungersen et al., 2002) in infected cattle with T-cell exhaustion.

This chapter confirms Map-specific IFN-γ production as a key response in Th1-mediated immunity and describes novel mechanisms of T-cell exhaustion mediated by immunoinhibitory receptors in Johne's disease. However, additional studies are required to determine multiple effects of blocking mAbs during rejuvenation of exhausted T cells. Specifically, measurements of other Th1 cytokines, such as tumor necrosis factor-alpha (TNF-α), IL-2, and IL-12, and T-cell proliferation and cytotoxic

activity, may reveal further aspects of T-cell exhaustion in Johne's disease. The present findings may contribute to the development of novel strategies for manipulating Map-specific T-cell responses to prevent disease progression.

SUMMARY

Johne's disease (paratuberculosis) is a chronic enteritis in cattle that is caused by intracellular infection with Map. This infection is characterized by the functional exhaustion of T-cell responses to Map antigens during late subclinical and clinical stages, presumably facilitating the persistence of this bacteria and the formation of clinical lesions. However, the mechanisms underlying T-cell exhaustion in Johne's disease are poorly understood. Thus, expression and functional analyses of the immunoinhibitory molecules PD-1/PD-L1 and LAG-3/MHC II were performed in Map-infected cattle during the late subclinical stage. Flow cytometric analyses revealed the upregulation of PD-1 and LAG-3 in T cells in infected animals, accompanied by progressive suppression of IFN-γ responses to the Map antigen. In addition, PD-L1 and MHC II were expressed on macrophages from infected animals, consistent with the fact that the PD-1 and LAG-3 pathways contribute to the suppression of IFN-γ responses during the subclinical stages of Map infection. Furthermore, dual blockade of PD-1 and LAG-3 by specific mAbs enhanced Map-specific IFN-γ responses in blood from infected animals, and *in vitro* LAG-3 blockade enhanced IFN-γ production from Map-specific $CD4^+$ and $CD8^+$ T cells. Taken together, the present data show that Map-specific T-cell exhaustion is in part mediated by PD-1/PD-L1 and LAG-3/MHC II interactions and that LAG-3 is a molecular target for the control of Map-specific T-cell responses.

CHAPTER II

Cooperation of PD-1 and LAG-3 contributes to T-cell exhaustion in *Anaplasma marginale*-infected cattle

The contents of this chapter will be submitted for publication in peer-reviewed journal, and thus cannot be shown in the thesis published online at the present time.

ABSTRACT

A. marginale is the most prevalent tick-borne pathogen in cattle worldwide. The infection results in severe anemia and a mortality rate of up to 30% in naive cattle. Previous studies have shown that infection with *A. marginale* induces a functional exhaustion of $CD4^+$ T-cell response to *A. marginale* OM immunogens, which presumably facilitates clinical symptoms and persistence of this rickettsia. In the present study, T-cell exhaustion following infection is hypothesized to be induced by the upregulation of immunoinhibitory receptors on T cells, such as PD-1 and LAG-3. Therefore OMP-specific T-cell responses and the kinetics of $PD\text{-}1^+LAG\text{-}3^+$ exhausted T cells were monitored in *A. marginale*-challenged cattle previously immunized with OM.

OMP-specific proliferation of PBMC and IFN-γ production were remarkably suppressed in all of the challenged animals after 5 wpi, which is consistent with previous studies. Additionally, rickettsemia and anemia also peaked in these animals at 5 wpi. Flow cytometric analysis showed that the number of $PD\text{-}1^+LAG\text{-}3^+$ exhausted T cells in the $CD4^+$ and $CD8^+$ T-cell populations gradually increased and also peaked at 5 wpi. In contrast, LAG-3, but not PD-1, was strongly upregulated in γδ T cells. These results suggest that the co-expression of PD-1 and LAG-3 on $CD4^+$ and $CD8^+$ T cells contributes to the rapid loss of T-cell response to *A. marginale* following the infection. Furthermore, OMP-specific PBMC proliferation and IFN-γ production were partially restored by *in vitro* blockade of the PD-1 and LAG-3 pathways using mAbs at 5 wpi, indicating that blocking antibodies against PD-1 and LAG-3 could be effective tools to regulate the T-cell response in *A. marginale*-infected cattle. The present study confirms a molecular mechanism underlying the exhaustion of *A. marginale*-specific T-cell response and the possibility of PD-1 and LAG-3 as therapeutic targets for the control of bovine anaplasmosis.

CHAPTER III

Roles of PD-1, LAG-3, and TIM-3 on T-cell exhaustion and disease progression in bovine leukemia virus-infected cattle

Some of the contents in this chapter will be submitted for publication in peer-reviewed journal, and thus cannot be shown in the thesis published online at the present time.

ABSTRACT

BLV is a retrovirus that infects B cells in cattle and causes bovine leukosis after a long latent period. Progressive exhaustion of T-cell functions is one of the characteristics of disease progression in BLV infection. PD-1, LAG-3, and TIM-3 are immunoinhibitory receptors that contribute to T-cell exhaustion caused by chronic infection. In this study, expression levels of PD-1, LAG-3, and TIM-3 were investigated in BLV-infected cattle in different stages of infection. To elucidate the kinetics of heavily exhausted T cells in BLV infection, the frequencies of PD-1^{+}LAG-3^{+} T cells were analyzed in each T-cell population of BLV-infected animals. In the blood of cattle with B-cell lymphoma, the population of PD-1^{+}LAG-3^{+} T cells amoung $CD4^{+}$ and $CD8^{+}$ T cells was increased over that of BLV-uninfected, AL, and PL cattle. Investigation of the expression levels of mRNAs encoding TIM-3 and its ligand Gal-9 by quantitative real-time RT-PCR revealed that *Tim-3* mRNA is mainly expressed in $CD4^{+}$ and $CD8^{+}$ T cells, while *Gal-9* mRNA is mainly expressed in $CD14^{+}$ monocytes and T cells. *Tim-3* mRNA expression in $CD4^{+}$ and $CD8^{+}$ T cells was upregulated during disease progression in BLV infection. PBMC blockade assays were performed to examine whether blockade of the PD-1 and LAG-3 pathways by antagonist mAbs could restore T-cell function during BLV infection. Dual blockade of PD-1 and LAG-3 enhanced IFN-γ production in PBMCs of BLV-infected cattle. In addition, blockade using anti-PD-L1 or anti-LAG-3 mAbs upregulated IFN-γ production in PBMCs in response to BLV antigen. These data indicate that T-cell exhaustion in BLV-infected cattle with progressed disease is mediated in part by PD-1, LAG-3, and TIM-3 on $CD4^{+}$ and $CD8^{+}$ T cells.

CHAPTER IV

Establishment and characterization of therapeutic anti-PD-1 and LAG-3 antibodies

The contents of this chapter will be submitted for publication in peer-reviewed journal, and thus cannot be shown in the thesis published online at the present time.

ABSTRACT

Blockade of immunoinhibitory receptors is a promising strategy for reinvigorating exhausted T cells and preventing disease progression during a variety of chronic infections. Application of this strategy to cattle requires therapeutic antibodies specific for bovine immunoinhibitory receptors. In this study, therapeutic anti-PD-1 and anti-LAG-3 antibodies were established and produced using mammalian expression systems. Purified therapeutic anti-PD-1 and anti-LAG-3 antibodies were capable of detecting cell-surface PD-1 and LAG-3 molecules expressed in flow cytometric analysis. In particular, the binding affinity of therapeutic anti-PD-1 antibody was significantly similar to that of the original anti-PD-1 rat mAb as determined by Biacore analysis. The therapeutic anti-PD-1 antibody was also capable of blocking PD-1/PD-L1 binding at the same level as the original rat mAb. The immunomodulatory and antiviral effects of therapeutic anti-PD-1 antibody were then evaluated by *in vivo* administration of the therapeutic antibody to a BLV-infected calf. Inoculation of therapeutic anti-PD-1 antibody resulted in reactivation of effector functions of T cells as well as BLV-specific $CD4^+$ T cells and a decrease in the proviral load of BLV in PBMCs. In conclusion, therapeutic anti-PD-1 antibody can be used as a novel agent for controlling BLV infection.

CONCLUSION

Cell-mediated immunity is essential for the clearance of pathogens such as intracellular bacteria and viruses. However, some types of chronic infection can induce functional exhaustion of T cells, resulting in impaired cell proliferation, cell survival, cytokine production, and cytotoxicity. Overexpression of immunoinhibitory receptors such as programmed death 1 (PD-1), lymphocyte activation gene 3 (LAG-3), and T cell immunoglobulin and mucin domain 3 (TIM-3), is a key driver of T-cell exhaustion. Remarkably, blocking of the interaction between inhibitory receptors and their ligands restores the effector functions of exhausted T cells. Therefore, blocking monoclonal antibodies (mAbs) can be used to treat chronic infections. In the present study, the immunomodulatory roles of bovine inhibitory receptors were investigated in several types of chronic infections in cattle. Additionally, therapeutic antibodies were established as a first step toward their clinical applications to the control of refractory chronic infections in cattle.

CHAPTER I: Cattle were inoculated with *Mycobacterium avium* subsp. *paratuberculosis* (Map) and developed the subclinical stage of paratuberculosis. In these Map-infected cattle, the population of PD-$1^{+}CD8^{+}$ T cells increased in mesenteric lymph nodes. In contrast, LAG-3 expression was upregulated in both $CD4^{+}$ and $CD8^{+}$ T cells in the blood of Map-infected animals. Moreover, the immunoinhibitory ligands, PD-ligand-1 (L1) and major histocompatibility complex class II molecules were expressed on macrophages in the infected animals. Remarkably, LAG-3 blockade enhanced IFN-γ production by Map-specific $CD4^{+}$ and $CD8^{+}$ T cells. These data shows that LAG-3 is essential for the functional exhaustion of Map-specific T cells and represents a potential novel therapeutic target for preventing clinical paratuberculosis.

CHAPTER II: The involvement of PD-1 and LAG-3 in rapid T-cell exhaustion following infection was clarified in *Anaplasma marginale*-challenged cattle previously immunized with the bacterial antigen. *A. marginale*-specific T-cell responses were remarkably suppressed in challenged cattle after five weeks post-infection (wpi). Clinical symptoms, such as rickettsemia and anemia, also peaked in these animals at 5 wpi. Consistently, the percentages of PD-1^{+}LAG-3^{+} exhausted T cells in $CD4^{+}$ and $CD8^{+}$ T-cell populations gradually increased and peaked at 5 wpi. Furthermore, dual blockade of the PD-1 and LAG-3 pathways by blocking monoclonal antibodies (mAbs) partially restored the functions of *A. marginale*-specific T cells *in vitro*. The present

study confirmed that co-expression of PD-1 and LAG-3 on $CD4^+$ and $CD8^+$ T cells contributes to the rapid loss of T-cell responses to *A. marginale* following infection.

CHAPTER III: The immunological roles of PD-1, LAG-3, and TIM-3 in the disease progression of bovine leukemia virus (BLV) infection and bovine leukosis were investigated. The frequency of $PD\text{-}1^+LAG\text{-}3^+$ T cells in $CD4^+$ and $CD8^+$ T-cell populations was higher in the peripheral blood of BLV-infected cattle with leukosis than in those without leukosis. In addition, quantitative real-time RT-PCR analysis revealed that *Tim-3* mRNA expression was mainly detected in $CD4^+$ and $CD8^+$ T cells of BLV-infected cattle. The expression level of *Tim-3* was upregulated in $CD4^+$ and $CD8^+$ T cells in accordance with disease progression in BLV infection. Furthermore, blockade of the PD-1 and LAG-3 pathways by mAbs restored the effector functions of T cells in BLV-infected PBMCs. These results suggest that PD-1, LAG-3, and TIM-3 partially mediate the exhaustion of $CD4^+$ and $CD8^+$ T cells during BLV infection, resulting in disease progression and tumor development.

CHAPTER IV: To develop an effective method for the control of chronic infections in cattle, therapeutic anti-bovine PD-1 antibody was established and investigated for therapeutic effects of PD-1 blockade *in vivo*. Therapeutic anti-bovine LAG-3 antibody was also prepared for future clinical applications. The binding affinity to PD-1 and blocking ability of PD-1/PD-L1 binding were conserved between therapeutic anti-PD-1 antibody and the original anti-PD-1 rat mAb. Administration of the therapeutic anti-PD-1 antibody to a BLV-infected calf reactivated the effector functions of T cells as well as BLV-specific $CD4^+$ T cells and decreased the proviral load of BLV remarkably. These observations show that therapeutic anti-PD-1 antibody is a candidate agent to prevent disease progression in BLV infection.

In conclusion, this study demonstrates that T-cell exhaustion is a common feature of chronic infections in cattle and is caused by the upregulation of immunoinhibitory receptors, mainly PD-1 and LAG-3, on T cells. T-cell exhaustion presumably facilitates the bacterial growth and disease progression in paratuberculosis, the acute clinical symptoms and bacterial persistence in anaplasmosis, and the tumor development in BLV infection. Furthermore, blockade of the PD-1 and LAG-3 pathways rejuvenates T-cell responses against specific antigens. Further engineering of these established antibodies is required for clinical applications of the blockade method. Therapeutic antibodies specific for immunoinhibitory receptors are a promising strategy for the control of disease progression in various types of infections in cattle.

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SUMMARY IN JAPANESE

和文要旨

細胞性免疫は、細胞内寄生菌やウイルスの排除に不可欠な免疫応答である。しかしながら慢性感染症においては、多くの病原体が細胞性免疫の中心を担う T 細胞を機能的に疲弊化させることで、宿主の免疫応答を回避している。T 細胞の疲弊化とは、細胞表面に免疫抑制受容体 programmed death 1 (PD-1)、lymphocyte activation gene 3 (LAG-3)、T cell immunoglobulin and mucin domain 3 (TIM-3) などが過剰発現することにより引き起こされ、結果として細胞増殖や生存、サイトカイン産生、細胞傷害性といった T 細胞の機能が抑制される。しかしその一方で、免疫抑制受容体とそのリガンドとの結合を特異的抗体によって阻害することで、疲弊化 T 細胞が再活性化され、本来発揮するべきエフェクター機能を回復できることも知られている。そのため様々な研究で、免疫抑制因子に対する特異抗体の慢性感染症治療への応用が試みられている。

本稿では、ヨーネ病、アナプラズマ症、牛白血病といったウシの慢性感染症を対象として、各感染症の病態進行に及ぼす免疫抑制受容体の免疫学的役割を解析した。さらに、ウシの慢性感染症に対する臨床応用を目指して、PD-1 と LAG-3 に特異的な治療用抗体を樹立し、その治療効果を検討した。

第 1 章: ヨーネ菌の実験感染牛では、持続感染後期において T 細胞応答が徐々に疲弊化していくことがひとつの特徴である。本章ではヨーネ菌の持続感染牛モデルを用いて、PD-1 と LAG-3 の発現解析および機能解析を行った。ヨーネ菌持続感染牛において、PD-1 は腸間膜リンパ節の $CD8^+$ T 細胞で、LAG-3 は末梢血中の $CD4^+$および $CD8^+$ T 細胞で、非感染群に比べて発現量が上昇していた。また、ヨーネ菌の主な感染細胞であるマクロファージにおいて、PD-1、LAG-3 のリガンドである PD-ligand 1 (PD-L1) および MHC クラス II 分子の発現が認められた。さらに、リンパ球に抗 LAG-3 抗体を添加しリガンドとの結合を阻害すると、ヨーネ菌特異的 $CD4^+$および $CD8^+$ T 細胞が活性化され、インターフェロン-γ (IFN-γ) 産生量が増強された。これらの結果より、LAG-3 が主にヨーネ菌特

異的 T 細胞の疲弊化には関与しており、ヨーネ病の発症を予防する新たな治療標的となりうることが示唆された。

第 2 章 : 以前の研究により、*Anaplasma marginale* 感染牛では感染後 4〜6 週までに抗原特異的 $CD4^+$ T 細胞が急激に疲弊化・枯渇し、急性感染期におけるアナプラズマの増殖と臨床症状を助長することが知られていた。そこで本章では、*A. marginale* の急性感染期における PD-1 および LAG-3 の発現動態に着目し、抗原特異的 T 細胞の疲弊化を引き起こす分子機序の解明を試みた。*A. marginale* 外膜タンパク質をウシに 4 回免疫し抗原特異的な T 細胞応答を誘導した上で、同菌株を接種した。すると以前の研究と同様に、アナプラズマ特異的な T 細胞応答が感染後 5 週以降は著しく抑制された。このとき同時に臨床症状 (菌血症および貧血) も顕著に認められ、急性感染が 5 週目でピークを迎えた。発現解析の結果、$CD4^+$ または $CD8^+$ T 細胞中の $PD\text{-}1^+LAG\text{-}3^+$ T 細胞 (重度に疲弊化した T 細胞) が感染後徐々に増加し、感染 5 週目でピークに達した。さらに、抗 PD-1 抗体、抗 PD-L1 抗体、および抗 LAG-3 抗体を添加し、リンパ球における結合阻害試験を行うと、アナプラズマ特異的な T 細胞応答が再活性化された。以上より、*A. marginale* の急性感染期では $CD4^+$ および $CD8^+$ T 細胞に PD-1 と LAG-3 が共発現することで、急激な特異的 T 細胞応答の疲弊化を引き起こすことが明らかになった。

第 3 章 : 牛白血病ウイルス (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、牛白血病の前段階であるリンパ球増多症（B 細胞の非腫瘍性増殖）を呈した牛は T 細胞が疲弊化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。そこで本章では BLV 感染症において、3 種類の免疫抑制受容体 PD-1、LAG-3、TIM-3 の発現解析を行い、それらの発現が病態進行に及ぼす影響を調べた。その結果、牛白血病発症牛の $CD4^+$ および $CD8^+$ T 細胞中には $PD\text{-}1^+LAG\text{-}3^+$ 疲弊化 T 細胞が多く認められた。また、*Tim-3* mRNA は BLV 感染に伴い $CD4^+$ および $CD8^+$ T 細胞での発現が上昇していた。さらに、リンパ球における PD-1 および LAG-3 の結合阻害を行うと、T 細胞からの IFN-γ 産生量が増強された。結果として、牛白血病発症牛では $PD\text{-}1^+LAG\text{-}3^+$ T 細胞が増加する

ことで、T 細胞応答が疲弊化することが示された。また、BLV 感染症の病態進行の過程には別の免疫抑制受容体 TIM-3 も関与することが示唆された。

第 4 章: 第 1〜3 章により、PD-1 や LAG-3 などの免疫抑制受容体がヨーネ病、アナプラズマ症、牛白血病の病態進行に関与することが示された。そこで本章では、ウシの慢性感染症に対する新規制御法を開発することを目的として、治療用抗 PD-1 抗体および治療用抗 LAG-3 抗体を新たに樹立した。樹立した治療用抗 PD-1 抗体は、抗 PD-1 ラット抗体と同程度の結合親和性と結合阻害活性を有していた。また、治療用抗 LAG-3 抗体についても抗 LAG-3 ラット抗体と同程度の結合性を示した。さらに、BLV 感染牛における治療用抗 PD-1 抗体の臨床試験を実施したところ、抗体投与後に BLV 特異的 $CD4^+$ T 細胞を中心とした T 細胞機能の活性化が認められ、結果としてリンパ球中の BLV プロウイルス量が顕著に低下した。この結果より、治療用抗 PD-1 抗体は、BLV 感染症の病態進行を遅らせる新規制御法となりうる可能性が示された。

本研究により、T 細胞の疲弊化はウシの慢性感染症に共通して認められる特徴であり、主に PD-1、LAG-3 の発現上昇により引き起こされることが明らかになった。T 細胞の疲弊化により、ヨーネ病では臨床症状の発症、アナプラズマ症では急性期の臨床症状と細菌の潜伏感染、BLV 感染症では白血病の発症が促進されていると考えられる。さらに、PD-1 および LAG-3 とリガンドとの結合を抗体によって阻害することにより各種抗原に対する T 細胞応答が再活性化された。治療用抗体のさらなる改良は不可欠だが、今後、本研究を基にした免疫抑制受容体に対する抗体医薬がウシの様々な感染症に対する新規制御法として応用されることが期待される。