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Studies on the mechanism of the immunosuppression caused by bovine mycoplasmosis and the development of its novel control method

牛マイコプラズマ感染症における免疫抑制機序の解明と新規治療法の開発

Shinya Goto

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ABBREVIATIONS

Ab(s)	antibody (antibodies)
APC	allophycocyanin
BALF	bronchoalveolar lavage fluid
BLV	bovine leukemia virus
BSA	bovine serum albumin
Boch4G12	bovine chimeric 4G12
chAb(s)	rat-bovine chimeric antibody (antibodies)
cAMP	cyclic adenosine monophosphate
COX	cyclooxygenase
Cy	cyanin
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EP	eicosanoid receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FSL-1	fibroblast-stimulating lipopeptide-1
IFN	interferon
IL	interleukin
Ig	immunoglobulin
i. v.	intravenously
mAb(s)	monoclonal antibody (antibodies)
MmmSC	*Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony
MOI	multiplicity of infection
NET	neutrophil extracellular trap
NF-κB	nuclear factor-kappa B
NK	natural killer
OD	optical density
PBMC(s)	peripheral blood mononuclear cell(s)

PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-1	programmed death 1
PD-L1	programmed death ligand 1
PE	phycoerythrin
PerCp	peridinin-chlorophyll-protein complex
PGE_2	prostaglandin E_2
s. c.	subcutaneously
STAT3	signal transducer and activator of transcription 3
TLR	toll-like receptor
TNF	tumor necrosis factor
TSB-T	PBS with 50 mM Tris, 0.1% BSA and 0.05 % Tween20
Th1	type 1 helper T (cell)
Th17	type 17 helper T (cell)
SsnB	sparstolonin B

NOTES

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PREFACE

Mycoplasma species belong to the class Mollicutes. These microorganisms are characterized by their lack of cell wall, low G + C content (23%–40%) and small genome size [Parker *et al*., 2018]. The first isolation of *Mycoplasma* species was *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (MmmSC), characterized as the causative agent of contagious bovine pleuropneumonia in 1898 [McAuliffe *et al*., 2007; Nocard and Roux, 1990]. After spreading worldwide in the 19th century, MmmSC was controlled in most continents and eradicated thanks to an efficient long-term protective vaccine [Roberts *et al.*, 1973]. However, so far, up to 30 species of mycoplasmas have been isolated from cattle, and 13 organisms have caused mycoplasmosis in cattle including mastitis, arthritis, pneumonia, otitis media and reproductive disorders [Maunsell *et al*., 2011]. These organisms include *M. bovis*, *M. bovigenitalium*, *M. californicum*, *M. arginini*, *M. bovirhinis*, *M. alkalescens*, *M. canadense*, *M. dispar*, *M. gallinarum*, *M. alvi*, *M. leachii*, *M. bovoculi* and *M. canis* [Ayling *et al*., 2004; Fox *et al*., 2005; Hirose *et al*., 2001; Mackie *et al*., 2000; Roy *et al*., 2008; Stipkovits *et al*., 2013]. Recently, *M. bovis* has been recognized as one of the most important and frequently isolated *Mycoplasma* species associated with disease in cattle worldwide, including in North America, Europe and Japan [Caswell *et al.*, 2010; Fox *et al.*, 2012; Hata *et al*., 2014; Higuchi *et al.*, 2011a; Janardhan *et al*., 2010; Parker *et al*., 2018].

M. bovis was first isolated in the United States in 1961 from a severe case of mastitis outbreak in a dairy herd [Hale *et al*., 1962; Parker *et al*., 2018]. *M. bovis* has emerged as an important cause of not only mastitis but also other disease manifestations including pneumonia, arthritis, tenosynovitis and otitis media in cattle [Caswell *et al*., 2010; Fox *et al*., 2012; Gagea *et al.*, 2006]. *M. bovis* is a highly contagious pathogen and *M. bovis*-associated diseases, particularly pneumonia, occur predominantly as herd enzootics, causing considerable economic losses because of calf mortality and weight loss in surviving calves [Caswell and Archambault, 2007; Caswell *et al*., 2010; Fox *et al*., 2012; Gagea *et al*., 2006]. Unfortunately, no effective vaccines have been developed against *M. bovis* infection. The treatment with antibiotics is potentially effective against *M. bovis,* but strains which are resistant to antibiotics have recently been emerging and spreading

[Amram *et al*., 2015; Lerner *et al*., 2014; Sulyok *et al*., 2017]. Therefore, a novel alternative strategy for the control of *M. bovis* infection is required.

In the case of pneumonia, *M. bovis* enters lung epithelial cells and colonizes when cattle inhale aerosol from contaminated air. *M. bovis* disseminates systemically via blood to other tissue, such as synovial membrane, liver and kidneys [Adegboye *et al*., 1995; Haines *et al*., 2001; Prysliak *et al*., 2011]. In addition, *M. bovis* infection is chronic and sometimes asymptomatically progresses, and *M. bovis*-infected cattle shed the bacteria for months to years intermittently [Bennett and Jasper, 1977; Punyapornwithaya *et al*., 2010]. Therefore, it is likely that *M. bovis* has strategy to escape from host immunity and establish persistent infection. A previous report demonstrated that *M. bovis* infects and persists in subsets of bovine peripheral blood mononuclear cells (PBMCs), including monocytes [van der Merwe *et al*., 2010]. Indeed, *M. bovis* antigen was localized in the cytoplasm of macrophages in lesions of fibrinosuppurative bronchopneumonia with foci of coagulation necrosis [Caswell and Archambault, 2007; Gagea *et al.*, 2006; Khodakaram-Tafti and Lopez, 2004]. These studies indicate that, because of invasion into host cells, *M. bovis* can evade humoral immunity and be transported from the lung to other sites. On the other hand, several studies reported that *M. bovis* has immunosuppressive effects *in vitro.* The incubation of bovine PBMCs with *M. bovis* resulted in a significant reduction in their proliferative responses [Vanden Bush *et al*., 2002; van der Merwe *et al*., 2010]. In addition, *in vitro* infection of bovine blood monocytes with *M. bovis* delayed/suppressed the production of type 1 helper T cell (Th1) cytokine, interferon (IFN)-γ and tumor necrosis factor (TNF)-α from monocytes [Mulongo *et al*., 2014]. These characteristics of *M. bovis* could be also associated with the chronic disease progression. However, the mechanisms of immunosuppression caused by *M. bovis* infection have not yet been fully elucidated.

Several reports have demonstrated that upregulated the expression of immunoinhibitory molecules are one of the mechanisms of immunosuppression and contribute to the disease progression during various human chronic infections and cancers [Day *et al.*, 2006; Inman *et al*., 2007; Nakanishi *et al*., 2007; Thompson *et al*., 2004; Wu *et al*., 2006]. Cell surface immunoinhibitory receptors, such as programmed death 1 (PD-1), are negative regulators of T cell activation. When PD-1 binds to its ligand, PD-ligand

1 (PD-L1), PD-1 suppresses the activation signaling mediated by T cell receptor and inhibits the effector functions of T cells such as cytokine production and cell proliferation [Wherry, 2011; Fig. 1]. This dysfunction of T cells is called T cell exhaustion. Interestingly, the blockade of the PD-1/PD-L1 pathway by specific antibody (Ab) against either the receptor or ligand restores the function of exhausted T cells (Fig. 1).Thus, the targeting PD-1/PD-L1 molecules can be therapeutic strategy for human chronic infections and cancers.

In the veterinary field, during bovine chronic infections, such as *Mycobacterium avium* subsp. *paratuberculosis* infection (Johne's disease), bovine leukemia virus (BLV) infection and bovine anaplasmosis, the expression of PD-1 is upregulated on $CD4^+$ and $CD8^+$ T cells and PD-L1 is also upregulated on immune cells in line with disease progression [Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2013; Okagawa *et al*., 2016a; Okagawa *et al*., 2016b; Sajiki *et al*., 2018; Sajiki *et al*., 2019]. In addition, the blockade of the PD-1/PD-L1 pathway by specific Ab effectively improved antigen-specific immune reactions in these bovine chronic diseases. This suggests that causative pathogens of these diseases utilize T cell exhaustion to escape from host immune responses. Therefore, the PD-1/PD-L1 pathway appears to be potential targets for the control of bovine chronic infections.

Anit-PD-1/PD-L1 Abs are recognized as candidates for the treatment of several human cancers. Fully humanized monoclonal antibodies (mAbs) targeting PD-1 or PD-L1 have shown therapeutic efficacy in patients with melanoma and other cancers [Brahmer *et al*., 2012]. These antibody drugs were approved for the novel treatment as immunotherapy. In veterinary field, previous studies reported the establishment of anti-bovine PD-1 rat-bovine chimeric Ab (chAb) and anti-bovine PD-L1 chAb, and clinical studies were conducted in BLV-infected cattle [Nishimori *et al*., 2017; Okagawa *et al*., 2017]. Inoculation of cattle with the anti-PD-1 or PD-L1 chAb restored T cell functions, such as IFN-γ response and the proliferation of BLV-specific $CD4^+$ T cells. Moreover, the chAb treatment significantly reduced BLV provirus loads, clearly demonstrating that this treatment induced antiviral activities [Nishimori *et al*., 2017; Okagawa *et al*., 2017; Sajiki *et al*., 2019]. Therefore, the blockade of the PD-1/PD-L1 pathway expected to be a therapeutic strategy for bovine chronic infection.

Currently, prostaglandin E_2 (PGE_2), which has a relationship with the PD-1/PD-L1

pathway [Botti *et al*., 2017; Prima *et al*., 2017], is focused on as another immune inhibitory factor in chronic infections and cancers. PGE_2 is one of the lipid mediators which is synthesized from arachidonic acid by cyclooxygenase (COX) isoenzymes, COX-1 and COX-2 [Morita, 2002; Phipps *et al*., 1991; Fig. 2]. PGE_2 is known to promote the immunosuppression associated with several tumors and chronic inflammation [Martínez-Colón and Moore, 2018]. PGE_2 suppresses Th1 immune responses, limiting the functions of natural killer (NK) cells, $CD4^+$ T cells and cytotoxic T cells via eicosanoid receptor 2 (EP2) and 4 (EP4) [Martínez-Colón and Moore, 2018; Fig. 2]. After its binding to EP2 or EP4, PGE_2 induces anti-inflammatory effects by activating the cyclic adenosine monophosphate (cAMP)/protein kinase A/cAMP response element binding protein pathway [Martínez-Colón and Moore, 2018]. In the veterinary field, previous study demonstrated that PGE_2 suppresses the Th1 response in cattle and upregulated PD-L1 expression in bovine PBMCs *in vitro* [Sajiki *et al*., 2018; Fig. 2]. In addition, PGE_2 has immunosuppressive effects in Johne's disease and BLV infection [Sajiki *et al*. 2018; Sajiki *et al*., 2019]. Importantly, the dual blockade of PGE_2 and the PD-1/PD-L1 pathway substantially enhanced the antigen-specific T cell reaction in cattle with chronic infection. Moreover, the combined treatment of anti-bovine PD-L1 chAb with a COX-2 inhibitor in cattle with BLV infection significantly decreased BLV provirus loads [Sajiki *et al*., 2019]. Therefore, PGE_2 could be one of the immunosuppressive factors, and the combination therapy, which targets both the PD-1/PD-L1 pathway and PGE_2, can provide a novel control method against bovine chronic infection.

In this study, to elucidate the mechanism of immunosuppression in *M. bovis* infection and to develop the novel strategies for the control of *M. bovis* infection, the roles of the PD-1/PD-L1 pathway and PGE_2 in the *M. bovis* infection were investigated. In Chapter I, the expression analysis and blockade assays of the PD-1/PD-L1 pathway were performed in cattle infected with *M. bovis*. In Chapter II, to elucidate the mechanisms of upregulation of PD-L1 expression in *M. bovis* infection, the function analysis of PGE_2 in cattle infected with *M. bovis* were performed. In addition, the effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 was evaluated *in vitro* to assess the therapeutic potentials of this treatment. Finally, in Chapter III, the efficacy of combined treatment of anti-bovine PD-L1 chAb with a COX-2 inhibitor was examined in *M. bovis*-infected

cattle *in vivo*.

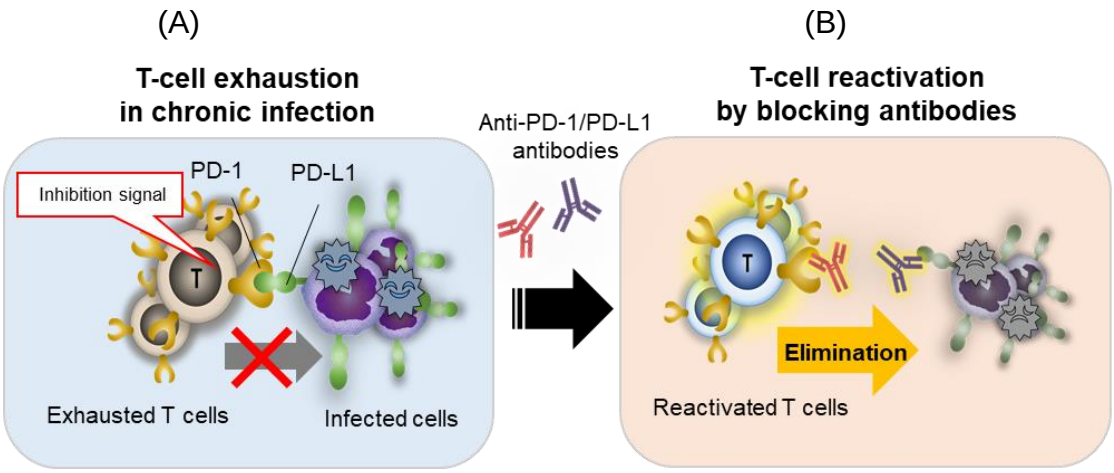

Figure 1. The PD-1/PD-L1 pathway in chronic infection. (A) The PD-1/PD-L1 pathway as a mechanism of immune evasion during chronic infection. PD-1, an inhibitory receptor expressed on T cells (exhausted T cells), interacts with its ligand, PD-L1, which is expressed on infected cells. The PD-1/PD-L1 axis suppresses effector functions of T cells. (B) Reactivation of T cells by the PD-1/PD-L1 blockade. Antibody blockade of the PD-1/PD-L1 pathway restores the effector functions, resulting in the clearance of pathogens.

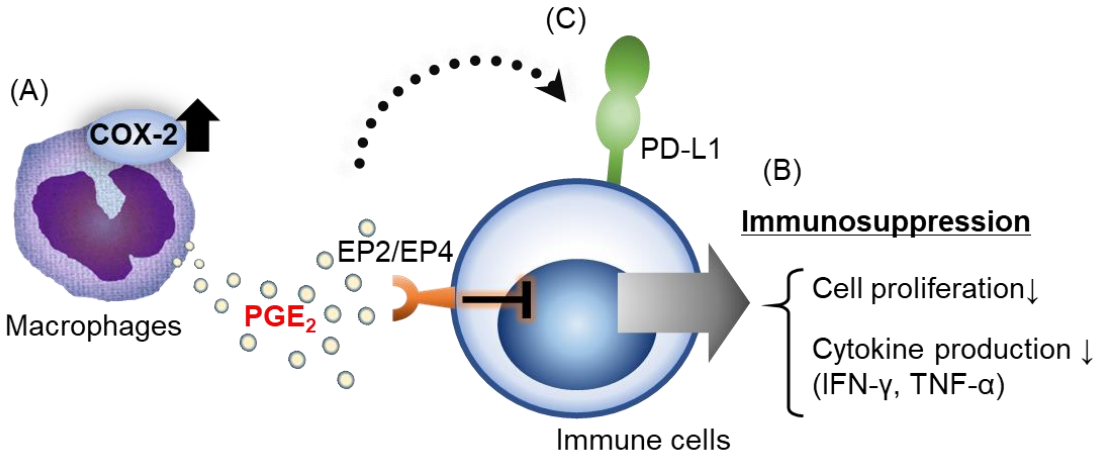

Figure 2. The immunosuppression caused by PGE_2. A mechanism of immune evasion by PGE_2 during chronic infection is shown. (A) PGE_2 is one of the lipid mediators which is synthesized from arachidonic acid by COX and produced from several cells including macrophages. (B) PGE_2 induces the suppression of several immune cells via EP2 or EP4. (C) PGE_2 is one of the inducers for PD-L1 expression in cancers and chronic infections.

CHAPTER I

Analysis of PD-1/PD-L1 expression and blockade assay of the PD-1/PD-L1 pathway in *Mycoplasma bovis* infection *in vitro*

INTRODUCTION

Bovine mycoplasmosis caused by *M. bovis* is a disease of cattle characterized by chronic pneumonia, therapy-resistant mastitis, otitis media and arthritis. Once clinical symptoms develop, it is difficult to completely cure the disease, resulting in calf mortality, weight loss in surviving calves and decreased milk production in dairy cows [Caswell *et al*., 2010; Fox *et al*., 2012; Gagea *et al*., 2006]. *M. bovis* has immunosuppressive effects, such as the inhibition of bovine PBMC proliferation, the induction of bovine lymphocyte apoptosis and delay of bovine monocyte apoptosis, along with the suppression of IFN-γ and TNF-α production [Mulongo *et al*., 2014; Vanden Bush *et al*., 2002; van der Merwe *et al*., 2010]. These characteristics could also be associated with the chronic progression of *M. bovis* infection. However, the mechanisms of immunosuppression caused by *M. bovis* have not yet been fully elucidated.

Dysfunction of antigen-specific T cells, called T cell exhaustion, has been shown to be involved in immunosuppression during various chronic infections [Blackburn *et al*., 2009; Khaitan and Unutmaz, 2011; Wherry *et al*., 2011]. Exhausted T cells lose their effector functions and are phenotypically characterized by the surface expression of immunoinhibitory receptors such as PD-1 [Wherry *et al*., 2011]. After its binding of PD-L1, PD-1 induces T cell dysfunction by inhibiting T cell receptor signaling [Kamphorst and Ahmed, 2013; Sierro *et al*., 2011]. The PD-1/PD-L1 interaction is closely associated with the inhibition of chronically activated pathogen-specific T cells and induction of T cell exhaustion [Kamphorst and Ahmed, 2013; Wherry *et al*., 2011].

Previous studies on chronic bovine infections, such as BLV infection, Johne's disease and bovine anaplasmosis, revealed that the upregulation of PD-1 and other immunoinhibitory receptors on T cells was closely associated with T cell exhaustion and disease progression [Ikebuchi *et al*., 2013; Konnai *et al*., 2013; Okagawa *et al*., 2016a; Okagawa *et al*., 2016b]. Moreover, the blockade of the PD-1/PD-L1 pathway reactivated T cell functions such as proliferation and cytokine production of T cells *in vitro* [Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2013; Ikebuchi *et al*., 2014; Okagawa *et al*., 2016a; Okagawa *et al*., 2016b]. However, the expression of PD-1/PD-L1 and their functions during *M. bovis* infection have not been investigated.

In this chapter, whether the PD-1/PD-L1 pathway downregulates *M. bovis*-specific T cell responses during *M. bovis* infection was determined. This analysis showed that the proportion of PD-1^+/PD-$L1^+$ immune cells was increased in the peripheral blood of *M. bovis*-infected cattle. This increase in the proportion of PD-1^+ T cells was associated with decreased IFN-γ production from bovine PBMCs. Furthermore, the blockade using either anti-bovine PD-1 mAb or anti-bovine PD-L1 mAb *in vitro* restored IFN-γ production from PBMCs stimulated by *M. bovis* antigens. The present data showed that exhausted T cells induced by the PD-1/PD-L1 interaction could contribute to the immunosuppression during *M. bovis* infection, and suggested that PD-1 and PD-L1 are candidate targets for the control of *M. bovis* infection.

MATERIALS AND METHODS

Samples and cell preparation

Peripheral blood samples of cattle with clinical symptoms of bovine mycoplasmosis were obtained from adult Holstein-breed cattle in Hokkaido, Japan. Cattle infected with *M. bovis* were clinically and microbiologically diagnosed at the Animal Medical Center, School of Veterinary Medicine, Rakuno Gakuen University, in 2016. The symptoms of the infected cattle were pneumonia, arthritis or otitis media. *M. bovis* infection was confirmed by polymerase chain reaction (PCR) using clinical samples as previously described [Higuchi *et al*., 2011b]. Control blood samples were collected from clinically healthy cattle with no history of *M. bovis* infection maintained at the Field Science Center for Northern Biosphere, Hokkaido University or at dairy farms in Hokkaido. All of control cattle were serologically negative for *M. bovis* infection determined by enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottom 96-well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 100 µl of solubilized *M. bovis* (PG45, ATCC 25523, 50 µg/mL in carbonate buffer) as the target antigen at 37°C for 17 h. After incubation at 37°C for 1 h, plates were washed 3 times with PBS with 50 mM Tris, 0.1% BSA and 0.05 % Tween20 (TSB-T) and incubated with skim milk (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) as a protein blocker at 37°C for 2 h. After washing 4 times with phosphate-buffered saline (PBS, pH 7.2) with 0.1% Tween20, 100 µl of serum samples were added on each plate. After washing 3 times with TSB-T, protein G-conjugated horseradish peroxidase (Rockland Immunochemicals, Pottstown, PA, USA) were added to the wells and incubated at 37°C for 1h. After washing 3 times with TSB-T, 3-ethylbenzothiazolin-6-sulfonic Acid (ABTS; Sera care, Milford, MA, USA) were added to the wells and optical density (OD) was measured at 415 nm using a plate reader (iMark Microplate Absorbance Reader, Bio-Rad, Hercules, CA, USA).

Bovine PBMCs were purified from blood samples by density gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, England, UK).

All experimental procedures were conducted following approval from the local committee for animal studies according to the Hokkaido University (17-0024). Informed consent was obtained from all owners of cattle sampled in this study.

IFN-γ assay

To examine the decrease in IFN-γ production in *M. bovis* infection, purified PBMCs were incubated with anti-bovine CD3 mAb (2 μg/mL; MM1A, Washington State University Monoclonal Antibody Center, Pullman, WA, USA) and anti-bovine CD28 mAb (2 μg/mL; CC220, Bio-Rad) in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (FCS; Thermo Fisher Scientific) and 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (Thermo Fisher Scientific) at 37°C under 5% CO_2 for 5 days. Collected culture supernatants were assayed for IFN-γ using a commercial ELISA kit (Mabtech, Nacka Strand, Sweden), in accordance with the manufacturer's instructions. Data are presented as means of duplicate samples.

Flow cytometric analysis of PD-1 and PD-L1

To examine the expression levels of PD-1 and PD-L1 during *M. bovis* infection, purified PBMCs were analyzed by flow cytometry. To detect PD-1^+ cells, PBMCs were and incubated in PBS containing 10% goat serum (Thermo Fisher Scientific) at room temperature for 15 min to prevent nonspecific binding of the staining Abs. Cells were then washed and stained with anti- bovine PD-1 mAb (5D2, rat IgG_{2a}; Ikebuchi *et al*., 2013) or rat IgG_{2a} isotype control (R35-95, BD Biosciences, San Jose, CA, USA) at room temperature for 30 min. After being washed with PBS containing 1% bovine serum albumin (BSA; Sigma–Aldrich), cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-bovine CD4 mAb (CC8, Bio-Rad), R-phycoerythrin (PE)-conjugated anti-bovine CD8 mAb (CC63, Bio-Rad), peridinin-chlorophyll-protein complex/cyanin 5.5 (PerCp/Cy5.5)-conjugated anti-bovine CD3 mAb (MM1A, Washington State University Monoclonal Antibody Center), PE/Cy7-conjugated anti-IgM mAb (IL-A30, Bio-Rad) and allophycocyanin (APC)-conjugated anti-rat immunoglobulin antibody (Southern Biotech, Birmingham, AL, USA) at room temperature for 30 min. Before the staining, MM1A and IL-A30 were conjugated with PerCp/Cy5.5 and PE/Cy7, respectively, using the Lightning-Link conjugation kits (Innova Biosciences, Cambridge, England, UK). Stained cells were then washed and immediately analyzed using FACS Verse (BD Biosciences) and FCS Express 4 (De Novo

Software, Glendale, CA, USA).

To detect PD-L1^{+} cells, PBMCs were incubated with PBS containing 10% goat serum and washed, followed by staining with anti-bovine PD-L1 mAb (4G12, rat IgG_{2a}; Ikebuchi *et al.*, 2014) or rat IgG_{2a} isotype control (R35-95, BD Biosciences) in the presence of anti-bovine CD11b mAb (CC126, mouse IgG_{2b}, Bio-Rad) at room temperature for 30 min. After washing with PBS containing 1% BSA, cells were stained with PerCp/Cy5.5-conjugated anti-bovine CD14 mAb (CAM36A: Washington State University Monoclonal Antibody Center), APC-conjugated anti-rat immunoglobulin antibody (Southern Biotech) and FITC-conjugated anti-mouse IgG_{2b} antibody (Beckman Coulter, Fullerton, CA, USA) and Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA, USA) at room temperature for 30 min. CAM36A was conjugated with PerCp/Cy5.5 using the Lightning-Link PerCp/Cy5.5 Antibody Labeling Kit (Innova Biosciences). Cells were then washed and immediately analyzed using FACS Verse (BD Biosciences) and FCS Express 4 (De Novo Software).

Blockade assay

To investigate the effect of the PD-1/PD-L1 blockade, PBMCs were incubated with 10 μg/mL anti-bovine PD-1 mAb (5D2; Ikebuchi *et al*., 2013) or anti-bovine PD-L1 mAb (4G12; Ikebuchi *et al*., 2014) in the presence of 1.5 μg/mL heat-killed *M. bovis* (PG45, ATCC 25523). Rat IgG (Sigma–Aldrich) was used as a control IgG. All antibodies used in this blockade assay were suspended in endotoxin-free sterile PBS (FUJIFILM Wako Pure Chemical Corporation). PBMC cultures were grown in 96-well plates (Corning, Inc., Corning, NY, USA) at 37°C with 5% CO_2 for 5 days. Subsequently, culture supernatants were harvested from individual wells and were tested by a bovine IFN-γ ELISA (Mabtech) as described above.

Statistics

Statistical differences were identified using Wilcoxon's matched pairs test and Spearman's rank test. Steel-Dwass test was used to compare the expression of PD-1 and PD-L1 among types of mycoplasmosis with different clinical symptoms. All statistical tests were performed using the statistical analysis program MEPHAS (*http://www.gen-*

info.osaka-u.ac.jp/MEPHAS/). p values of < 0.05 were considered significant.

RESULTS

Reduction in the IFN-γ production in cattle with bovine mycoplasmosis

To determine whether *M. bovis* infection is accompanied by immunosuppression, IFN-γ production from PBMCs of cattle with *M. bovis* infection and clinically healthy cattle was measured by ELISA. IFN-γ production from PBMCs stimulated with anti-bovine CD3 and anti-bovine CD28 antibodies was significantly lower in the cattle infected with *M. bovis* (Fig. I-1). No significant differences were observed in IFN-γ production among the different clinical symptoms (pneumonia, arthritis and otitis media) in the *M. bovis*-infected cattle (data not shown).

Upregulation of PD-1/PD-L1 expression in *M. bovis* infection

Because IFN-γ response was suppressed during *M. bovis* infection, it was hypothesized that the exhaustion of *M. bovis*-specific T cell responses was caused by the upregulation of immunoinhibitory molecules, PD-1/PD-L1, in infected cattle. To test this possibility, the expression of PD-1 on $CD4^+$ and $CD8^+$ T cells was investigated by flow cytometric analysis of PBMCs from *M. bovis*-infected cattle with pneumonia, arthritis or otitis media. As shown in Fig. I-2, the proportions of PD-1^+CD4^+ (Fig. I-2A) and PD-1^+CD8^+ T cells (Fig. I-2B) in PBMCs isolated from *M. bovis*-infected cattle were higher than those from healthy control cattle. Similarly, the proportion of PD-$L1^+CD11b^+CD14^+$ monocytes was higher in *M. bovis*-infected cattle than in healthy cattle ($p < 0.05$) (Fig. I-2C). No significant differences were observed in PD-1 and PD-L1 expressions among the types of mycoplasmosis with different clinical symptoms in *M. bovis*-infected cattle, although all of the median proportions of PD-1^+CD4^+ (Fig. I-3A), PD-1^+CD8^+ (Fig. I-3B) and PD-$L1^+CD11b^+CD14^+$ cells (Fig. I-3C) were higher than those of healthy control cattle. Interestingly, the increases in the proportion of PD-1^+CD4^+ and PD-1^+CD8^+ T cells were negatively correlated with the level of IFN-γ production from PBMCs in infected cattle (Figs. 4A and B).

Reactivation of IFN-γ production by the PD-1/PD-L1 blockade.

To test whether *M. bovis*-specific IFN-γ response was restored by the PD-1/PD-L1

blockade, PBMCs from *M. bovis*-infected cattle were cultivated in the presence of heat-killed *M. bovis* together with anti-bovine PD-1, anti-bovine PD-L1 mAb or an isotype control antibody. IFN-γ production was found to be significantly augmented in cells treated either with the anti-PD-1 or PD-L1 mAb compared to those treated with the control antibody (Fig. I-5).

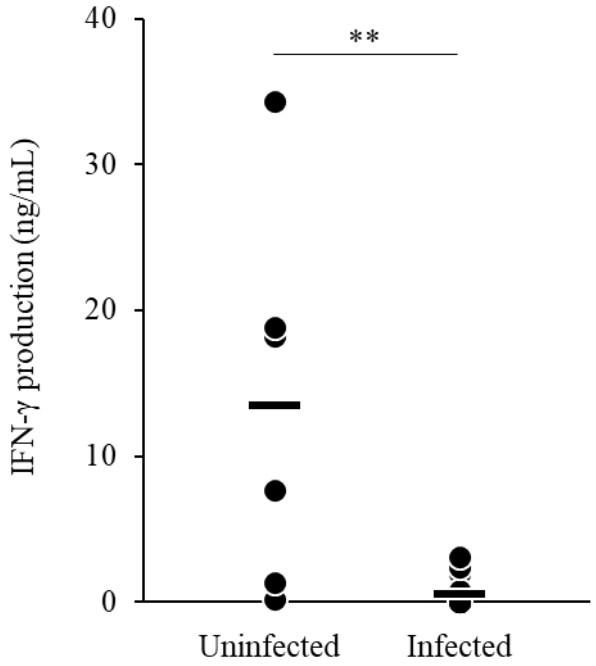

Figure I-1. Decreased IFN-γ response in *M. bovis* infection. IFN-γ production was determined in supernatant of the cultured PBMCs obtained from *M. bovis*-infected (n = 13) and healthy control Holstein cattle (n = 6) by ELISA assay. PBMCs were incubated with the combination of anti-bovine CD3 mAb and anti-bovine CD28 mAb. The bars are the median of the values. Statistical significance was determined by the Mann–Whitney U test. $^{**}p < 0.01$.

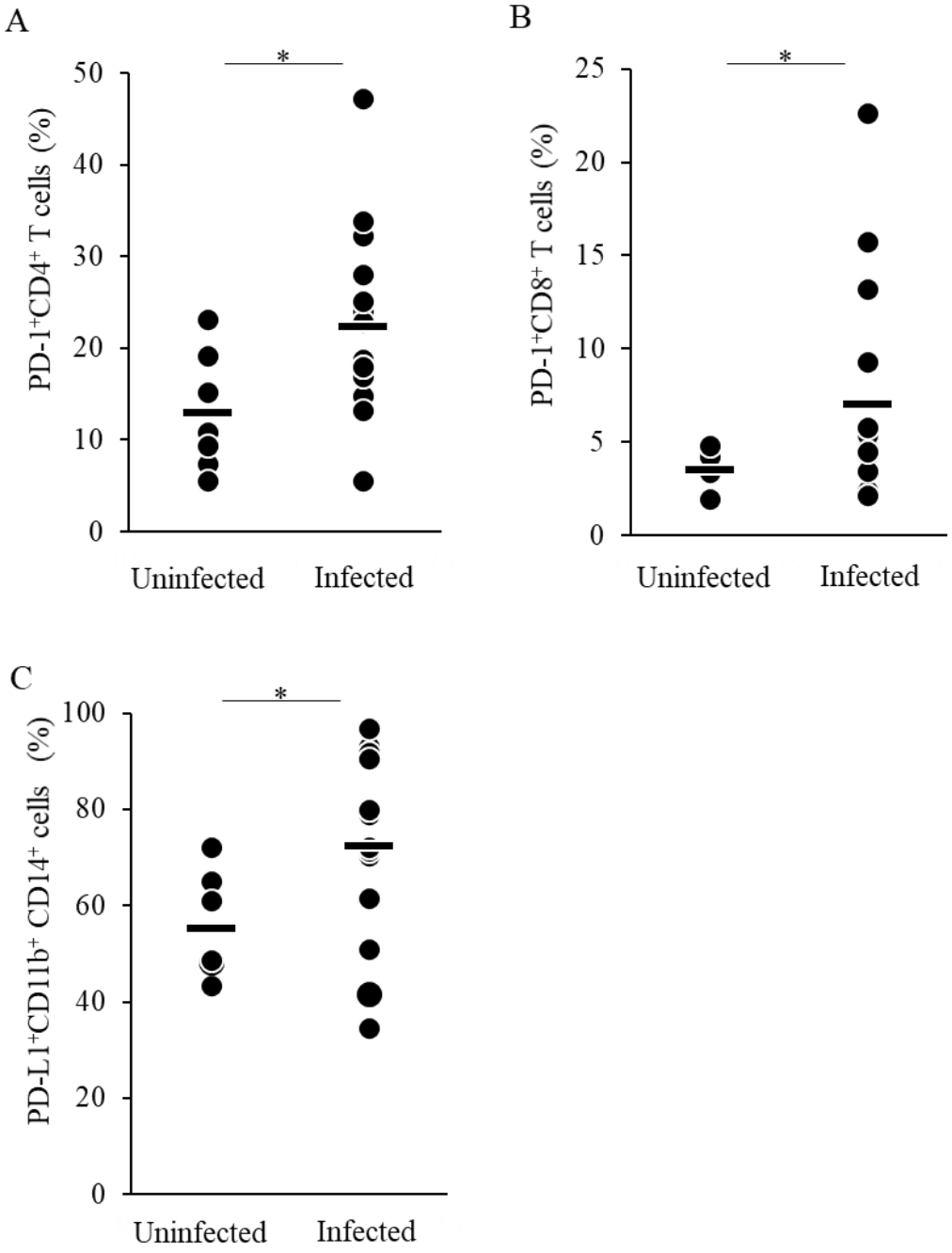

Figure I-2. Increased proportion of PD-1/PD-L1 expressing cells in *M. bovis* infection. Flow cytometric analysis of the PD-1 expression on $CD4^+$ T cells (A), $CD8^+$ T cells (B) and PD-L1 expression on $CD11b^+CD14^+$ monocytes (C) in PBMCs from cattle with *M. bovis* infection (n = 26) and healthy control cattle (n = 7). The bars are the median of the values. Statistical significance was determined by the Mann–Whitney U test. *$p < 0.05$.

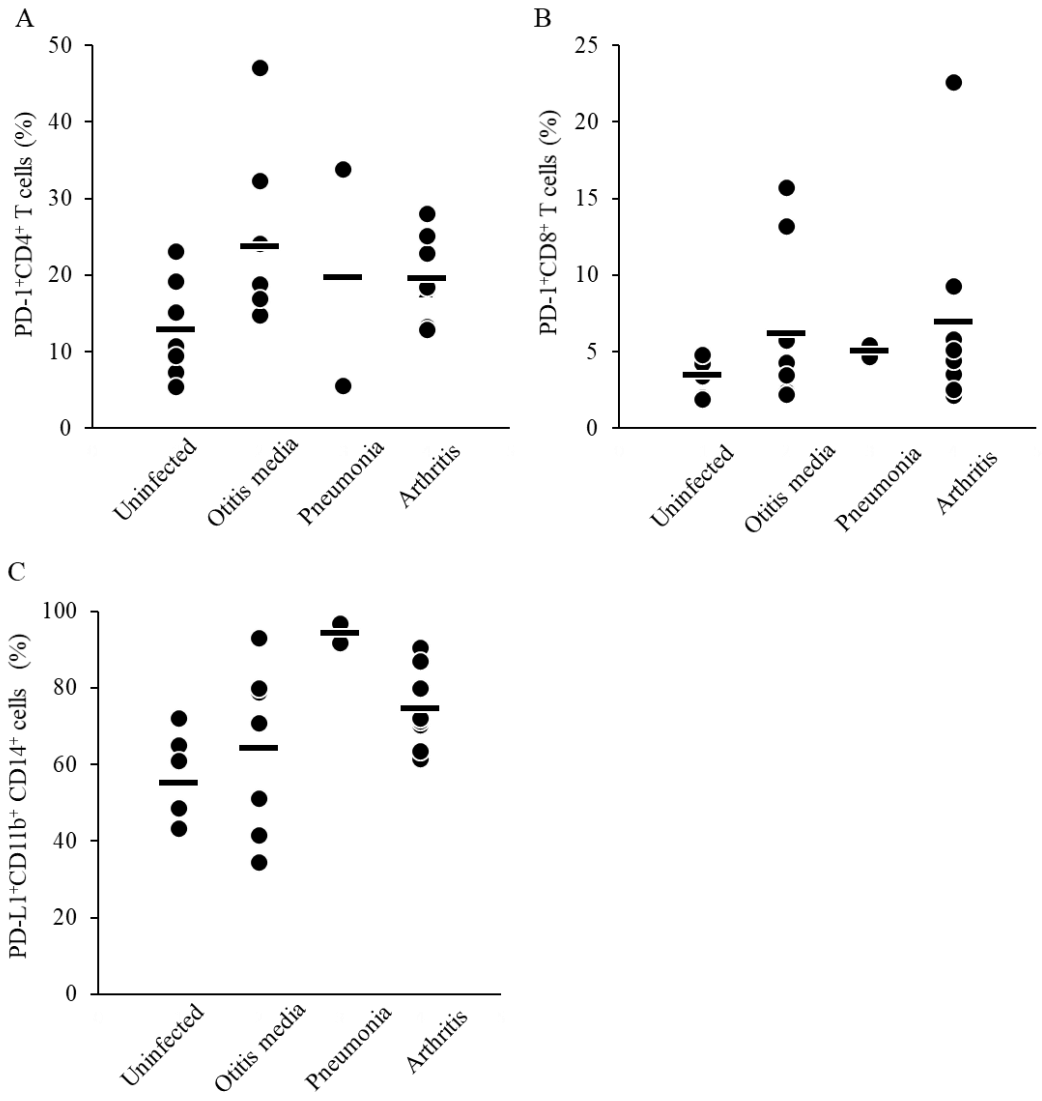

Figure I-3. Comparative analysis of PD-1 and PD-L1 expression among bovine mycoplasmosis cases with different symptoms. PBMCs from *M. bovis*-infected cattle with otitis media (n = 7), pneumonia (n = 2) and arthritis (n = 8) were analyzed. The bars are the median of the values (A–C).

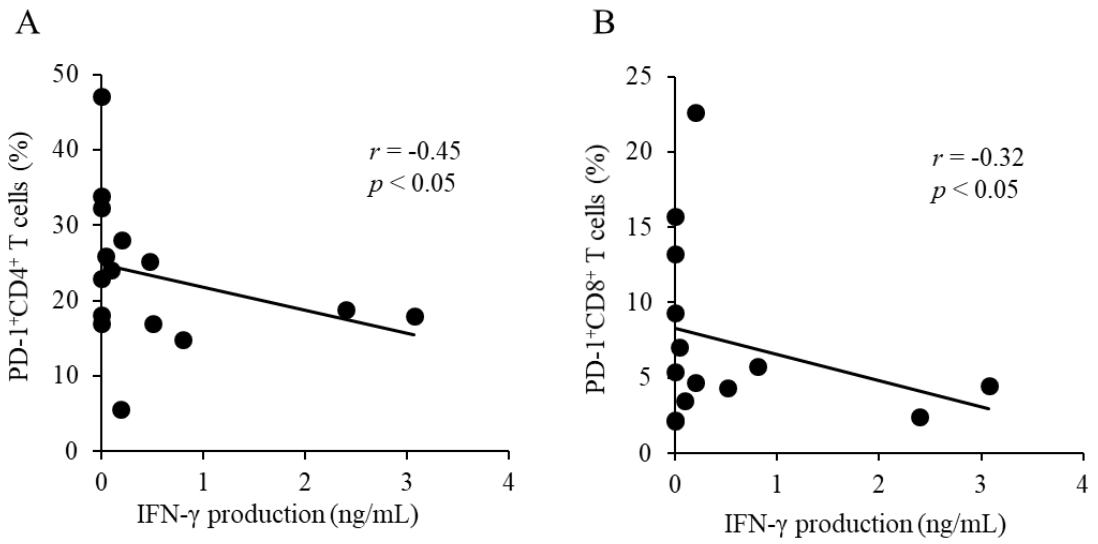

Figure I-4. Negative correlation between the proportion of PD-1$^+$ T cells and IFN-γ production. Correlation between IFN-γ production from PBMCs stimulated with anti-bovine CD3 mAb and anti-bovine CD28 mAb, and the proportion of PD-1$^+$ cells was analyzed (n = 15). Correlation statistics were analyzed using Spearman's correlation.

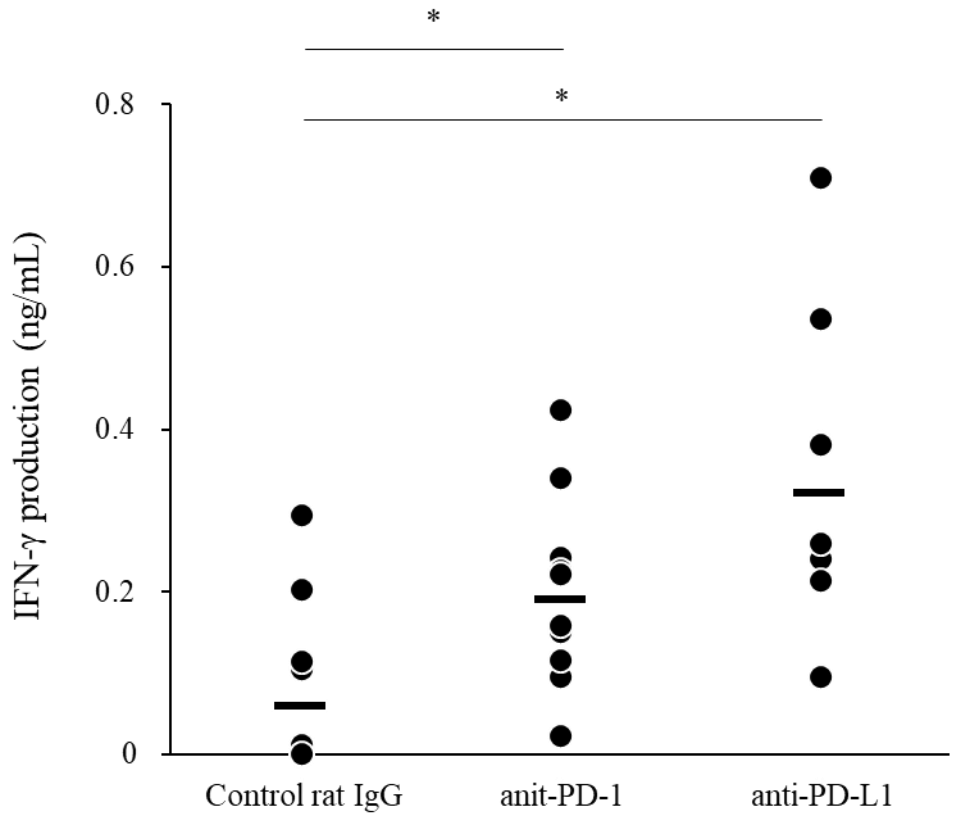

Figure I-5. Enhancement of IFN-γ production by anti-PD-1 or anti-PD-L1 mAb in PBMCs from cattle with mycoplasmosis. PBMCs were cultivated with control rat IgG, anti-bovine PD-1 mAb or anti-bovine PD-L1 mAb (n = 10) in the presence of heat-killed *M. bovis*. IFN-γ production was measured by ELISA assay. Statistical comparisons between control IgG and blocking mAbs were performed using Steel-Dwass test. *$p < 0.05$.

DISCUSSION

In bovine mycoplasmosis, it is well known that pathogens, chiefly *M. bovis*, exert immunosuppressive effects *in vitro* [Mulongo *et al*., 2014; Vanden Bush *et al*., 2002, van der Merwe *et al*., 2010]. Possibly because of these characteristics, this disease is suspected to chronically progress in most cases and, especially during lung infection, to allow the establishment of co-infection with other bacteria and other pathogens such as viruses [Caswell *et al*., 2010]. However, the mechanisms of the immunosuppression during *M. bovis* infection have not yet been fully elucidated.

During chronic infection, pathogens evade host immune responses and persist after the effector phase [Finlay *et al*., 2006; Higuchi *et al*., 2013], leading to persistent antigen stimulation and progressive T cell dysfunction known as T cell exhaustion [Wherry *et al*., 2011]. Exhausted T cells are controlled by the immunoinhibitory receptor PD-1 on the cell surface via T cell inhibitory signals that are transmitted upon binding to the ligand PD-L1 [Kamphorst and Ahmed, 2013]. Previous studies revealed that the expression of PD-1 was upregulated on exhausted T cells in various bovine chronic infections such as BLV infection, Johne's disease and bovine anaplasmosis [Ikebuchi *et al*., 2013; Konnai *et al*., 2013; Okagawa *et al*., 2016a; Okagawa *et al*., 2016b]. T cells expressing these receptors show low effector functions and allow chronic pathogens to establish persistent infection. Therefore, T cell exhaustion is regarded as a common mechanism of immune evasion in chronic infections. Thus, in this chapter, the roles of the PD-1/PD-L1 pathway with regard to the immunosuppression in cattle infected with *M. bovis* was investigated.

In the present study, to evaluate the immune function in *M.bovis* infection, IFN-γ response in cattle infected with *M. bovis* was investigated. IFN-γ is a Th1 cytokine mediating a cellular immune response partly by promoting the activation and proliferation of macrophages. In contagious bovine pleuropneumonia caused by other bovine mycoplasma, MmmSC, a correlation between the number of MmmSC-specific IFN-γ-secreting $CD4^+$ T cells and recovery from the disease was identified [Dedieu *et al*., 2005; Dedieu *et al.*, 2006]. Therefore, IFN-γ production might be important for the protection against bovine mycoplasmosis. The present study demonstrated that IFN-γ production from PBMCs against T cell stimulator (combination of anti-bovine CD3 mAb and anti-

bovine CD28 mAb) was decreased in infected cattle compared to that in healthy cattle (Fig. I-1). It suggested that Th1 response was decreased and this immunosuppression might be associated with chronic progression of *M. bovis* infection.

The proportions of $CD4^+$ and $CD8^+$ T cells expressing PD-1 and $CD14^+CD11b^+$ monocytes expressing PD-L1 were found to be upregulated during *M. bovis* infection (Fig. I-2). Moreover, lower proportions of circulating PD-1^+ cells were strongly correlated with increased IFN-γ production (Fig. I-4). This result indicates that decreased production of IFN-γ during *M. bovis* infection could be due to the increased number of PD-1^+ cells. In chronic infections, continuous antigen presentation and T cell receptor stimulation induce the expression of PD-1 [Kamphorst and Ahmed, 2013; Wherry *et al*., 2011]. Thus, it was hypothesized that PD-1 is upregulated on *M. bovis* antigen-specific T cells and causes effector function loss in these cells. The mechanism of PD-L1 upregulation during *M. bovis* infection remains unknown. In the models of human immunodeficiency virus infection, the cytokine microenvironment was proposed as one of the mechanisms by which PD-L1 expression is elevated [Kinter *et al*., 2008]. This hypothesis might contribute to understanding the mechanism of PD-L1 upregulation during mycoplasmosis, considering the changes in the cytokine microenvironment [Gondaira *et al*., 2015, Rodríguez *et al*., 2015a; Rodríguez *et al*., 2015b]. Nevertheless, further elucidation of the mechanism underlying the elevation of PD-L1 expression is warranted to obtain a comprehensive understanding of cell signaling pathways involved in the modulation of host immune responses.

The blockade assay of PD-1/PD-L1 in PBMCs *in vitro* showed that the blockade with anti-bovine PD-1 or anti-bovine PD-L1 mAbs efficiently reactivated the *M. bovis*-specific IFN-γ response (Fig. I-5). The results obtained in this study were consistent with those in other bovine diseases, such as BLV infection, Johne's disease and bovine anaplasmosis [Ikebuchi *et al*., 2013; Okagawa *et al*., 2016a; Okagawa *et al*., 2016b]. The results suggest that T cell exhaustion caused by the PD-1/PD-L1 pathway is associated with immunosuppression in *M. bovis* infection and the blockade of the PD-1/PD-L1 pathway could be an effective method for controlling *M. bovis* infection.

In this chapter, *M. bovis*-specific IFN-γ production was examined as a key response of Th1-mediated immunity, and novel mechanisms of T cell exhaustion mediated by

immunoinhibitory receptors in *M. bovis* infection was described. The present findings could contribute to the development of novel strategies for preventing the disease progression of *M. bovis* infection by manipulating *M. bovis*-specific T cell responses.. However, additional studies are required to determine multiple effects of blocking mAbs during the rejuvenation of T cell exhaustion. Specifically, the measurements of other Th1 cytokines, such as TNF-α, interleukin (IL)-2 and IL-12, and of T cell proliferation and cytotoxic activity may reveal further aspects of T cell exhaustion in bovine mycoplasmosis, including *M. bovi*s infection.

SUMMARY

Bovine mycoplasma, mainly *M. bovis*, is a pathogen that causes pneumonia, mastitis, arthritis and otitis media in cattle. This pathogen exerts immunosuppressive effects, such as the inhibition of interferon production. However, the mechanisms of the immunosuppression during bovine mycoplasmosis have not been fully elucidated. In this chapter, the roles of the PD-1/PD-L1 pathway in the immunosuppression during *M. bovis* infection were investigated.

IFN-γ production against T cell stimulator was measured by ELISA using PBMCs isolated from cattle with *M. bovis* infection. IFN-γ production in *M. bovis*-infected cattle was significantly decreased compared to that in uninfected cattle. Concomitantly, flow cytometric analysis revealed that the proportions of PD-$1^+$$CD4^+$ and PD-$1^+$$CD8^+$ T cells as well as PD-$L1^+$$CD14^+$ monocytes were increased in peripheral blood of *M. bovis*-infected cattle. Interestingly, the increases in the proportions of PD-$1^+$$CD4^+$ and PD-$1^+$$CD8^+$ T cells were negatively correlated with IFN-γ production from PBMCs during *M. bovis* infection. On the other hand, the blockade of the PD-1/PD-L1 pathway *in vitro* by anti-bovine PD-1 or anti-bovine PD-L1 mAbs significantly upregulated the IFN-γ production from *M. bovis*-specific cells. These results suggest that the PD-1/PD-L1 pathway could be involved in immune exhaustion of *M. bovis*-specific T cells. In conclusion, this study opens up a new perspective in the therapeutic strategy for *M. bovis* infection by targeting the immunoinhibitory receptor pathways.

CHAPTER II

Analysis of the mechanism of PD-L1 upregulation and dual blockade assay of the PD-1 PD-L1 pathway and PGE_2 in *M. bovis* infection *in vitro*

INTORDUCTION

In Chapter I, upregulation of PD-1^+ T cells and PD-$L1^+$ monocytes was found in *M. bovis*-infected cattle and PD-1/PD-L1 blockade by anti-bovine PD-1/PD-L1 mAb enhanced IFN-γ production against *M. bovis*. It suggested that T cell exhaustion caused by the PD-1/PD-L1 pathway could be involved in the disease progression during *M. bovis* infection. However, the mechanism of PD-L1 upregulation during *M. bovis* infection remains unknown. Therefore, in this chapter, the inducer of PD-L1 expression in *M. bovis* infection and its potential for the target of treatment for this disease were investigated.

In this study, as an inducer of PD-L1 expression, PGE_2, which is synthesized from arachidonic acids by COX-1 and COX-2, is focused. PGE_2 is one of the lipid mediators which promotes the immunosuppression associated with several tumors and chronic inflammation [Martínez-Colón and Moore, 2018; Wang and Dubois, 2010]. Interestingly, recent reports on human and murine cancers have shown a relationship between PGE_2 and the PD-1/PD-L1 pathway [Botti *et al*., 2017; Prima *et al*., 2017]. In human melanoma cells, COX-2 was positively correlated with PD-L1 expression [Botti *et al*., 2017]. In a mouse model of bladder tumor, tumor-infiltrating PD-$L1^+$ cells showed high levels of the PGE_2-forming enzymes, namely microsomal PGE_2 synthase 1 and COX-2 [Prima *et al*., 2017]. Therefore, PGE_2 was recently implicated in the induction of PD-L1 expression. In veterinary field, the previous study has demonstrated that PGE_2 suppressed the Th1 response in healthy cattle and upregulated PD-L1 expression in bovine PBMCs *in vitro* [Sajiki *et al*., 2018]. Indeed, PGE_2 production was significantly upregulated in bovine chronic diseases, such as Johne's disease [Sajiki *et al*., 2018] and BLV infection [Sajiki *et al*., 2019]. Furthermore, the dual blockade of the PD-1/PD-L1 pathway and PGE_2 significantly enhanced the antigen-specific T cell reaction in cattle with these diseases. Therefore, PGE_2 could be a potential therapeutic target to control bovine chronic infections. However, the involvement of PGE_2 in the immunosuppression during *M. bovis* infection has not yet been fully investigated.

To elucidate the function of PGE_2 underlying the T cell exhaustion observed in *M. bovis* infection as shown in Chapter I, in this chapter, the role of PGE_2 in the immunosuppression and the relationship between PGE_2 and the PD-1/PD-L1 pathway in

M. bovis infection were investigated. In addition, the effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 on *M. bovis*-specific immune response was evaluated *in vitro*.

MATERIAL AND METHODS

Bacterial strain

An *M. bovis* strain PG45 (ATCC25523) was cultured in NK broth (Miyarisan Pharmaceutical, Tokyo, Japan) at 37°C for 72 h, and collected by centrifugation. After washing 3 times with PBS, the bacteria was resuspended in RPMI medium (Sigma–Aldrich) containing 10% heat-inactivated FCS (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific) and stored at −80°C until use. The number of colony-forming units were counted using the NK agar plate (Miyarisan Pharmaceutical) by the dilution method.

Ethical approval

All experimental procedures were conducted following approval from the local committee for animal studies according to the Hokkaido University (17-0024). Informed consent was obtained from all owners of cattle sampled in this study.

Bovine samples

Peripheral blood samples of cattle were obtained from adult Holstein-breed cattle in Hokkaido, Japan. Cattle infected with *M. bovis* were diagnosed clinically and microbiologically at Rakuno Gakuen University and Hokkaido University. *M. bovis* infection was confirmed with PCR by using clinical samples as described in Chapter I. The symptoms of infected cattle included pneumonia, arthritis and otitis media. Negative control cattle (uninfected) were serologically negative for *M. bovis* infection according to ELISA as described in Chapter I.

Cell preparation and culture

Bovine PBMCs were purified from blood samples by density gradient centrifugation using Percoll (GE Healthcare). $CD14^+$ cells were freshly isolated from bovine PBMCs using the autoMACS Pro System (Miltenyi Biotec, Bergisch Gladbach, Germany) wtih anti-bovine CD14 mAb (CAM36A, Washington State University Monoclonal Antibody Center) and anti-mouse IgG_1 MicroBeads (Miltenyi Biotec).

$CD14^-$ cells were prepared from negative fractions of $CD14^+$ cell sorting after confirmation of their purity. The purity of cell populations was confirmed using FACS Verse (BD Biosciences). Only highly pure populations (>90%) were used for the experiments.

PBMCs, $CD14^+$ cells or $CD14^-$ cells (1×10^6 cells) from uninfected cattle were seeded into each well of a 48-well flat-bottom plate (Corning Inc.) with RPMI medium (Sigma–Aldrich) containing 10% heat-inactivated FCS (Thermo Fisher Scientific), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific), and were cultured in the presence of live *M. bovis* at a multiplicity of infection (MOI) of 0.1:1, 1:1 or 10:1, 1.5 ng/mL of heat-killed *M. bovis*, 2.5 μM of PGE_2 (Cayman Chemical, Ann Arbor, MI, USA) or 100 ng/mL of fibroblast-stimulating lipopeptide-1 (FSL-1; Adipogen Life Sciences, San Diego, CA, USA) at 37°C under 5 % CO_2 for 24 h. Heat-killed *M. bovis* was prepared by heating the bacteria at 70°C for 10 min.

To investigate the effects of the blockade of Toll-like receptor (TLR) 2 signaling, PBMCs (1×10^6 cells) from uninfected cattle were incubated with sparstolonin B (SsnB; Sigma–Aldrich) in the presence of 100 ng/mL of FSL-1 or 1.5 ng/mL of heat-killed *M. bovis* in a 48-well flat-bottom plate (Corning Inc.) at 37°C under 5 % CO_2 for 24 h. dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) was used as negative control.

To assess the effect of soluble factors on PD-L1 expression, 24-h culture supernatants of PBMCs incubated with live *M. bovis* were collected and fresh PBMCs were cultured in these culture supernatants. Briefly, to prepare 24-h culture supernatants, PBMCs (1×10^6 cells) from uninfected cattle were seeded were cultured with or without live *M. bovis* at a MOI of 0.1:1, 1:1 or 10:1 as described above. After centrifugation (300 ×*g* for 3 min), the culture supernatants of PBMCs were collected. Bacteria-free and cell-free supernatants were obtained by filtration through a 0.2-μm filter (Pall Life Sciences, Washington, NY, USA). To confirm that the supernatants were not contaminated with live *M. bovis*, each supernatant was incubated in NK broth (Miyarisan Pharmaceutical) for 72 h and the broth samples were tested by PCR targeting *M. bovis*-specific gene [Higuchi *et al*., 2011b]. Then, fresh PBMCs were isolated from the same individual cattle and cultured in the 24-h culture supernatants at 37°C under 5% CO_2 for 24 h.

Quantitation of PGE_2 by ELISA

To examine whether *M. bovis* antigen and TLR2 signaling promote PGE_2 production, PBMCs, $CD14^+$ cells or $CD14^-$ cells (1×10^6 cells) from uninfected cattle were incubated with live *M. bovis*, heat-killed *M. bovis* or FSL-1 (Adipogen Life Sciences) as described above. PGE_2 levels in culture supernatants were measured using the Prostaglandin E_2 Express ELISA kit (Cayman Chemical), according to the manufacturer's instructions. The optical density was measured at 450 nm in a microplate reader (Corona Electronics, Tokyo, Japan). To compare PGE_2 levels in peripheral blood between infected and uninfected cattle, PGE_2 concentrations in serum or plasma were measured using ELISA as described above.

Flow cytometric analysis of PD-L1

Flow cytometric analysis of PD-L1 was performed as described in Chapter I.

IFN-γ assay

To evaluate *M. bovis*-specific IFN-γ response in infected cattle, PBMCs (4×10^5 cells) from infected cattle were incubated with 1.5 ng/mL of heat-killed *M. bovis* in 96-well plates (Corning Inc.) at 37°C under 5 % CO_2 for 5 days. Subsequently, culture supernatants were obtained from individual wells and were tested for bovine IFN-γ using the ELISA kit (Mabtech) as described in Chapter I.

Immunohistochemical assays of PD-L1 and PGE_2

Tissue sections of lung from *M. bovis*-infected cattle with pneumonia (Japanese Black, male, 2 months old) and *M. bovis*-uninfected cattle (Holstein, male, 0 months old) were subjected to immunohistochemical study. *M. bovis* infection was confirmed with the loop-mediated isothermal amplification assay at Miyazaki University [Higa *et al*., 2016]. Then, sections of the lung lesions were immunohistochemically stained for PGE_2 and PD-L1 using anti-PGE_2 polyclonal Ab (ab2318, Abcam) and anti-bovine PD-L1 mAb (6C11-3A11, Rat IgG_{2a}; Sajiki *et al.*, 2018).

Functional analysis of dual blockade of PD-1/PD-L1 pathway and PGE_2

To investigate the effects of dual blockade by using a COX-2 inhibitor and anti-PD-L1 mAb, PBMCs (4×10^5 cells) from infected cattle were incubated with 10 μg/mL of anti-bovine PD-L1 mAb (4G12; Ikebuchi *et al*., 2014) and/or 10 μM of meloxicam (Sigma–Aldrich), a COX-2 inhibitor, in the presence of 1.5 μg/mL of heat-killed *M. bovis*, live *M. bovis* (MOI of 1) or 1.0 μg/mL of anti-bovine CD3 mAb (MM1A, Washington State University Monoclonal Antibody Center) and 1.0 μg/mL of anti-bovine CD28 mAb (CC220, Bio-Rad) in 96-well plates (Corning Inc.) at 37°C under 5% CO_2 for 5 days. DMSO (Nacalai Tesque) and rat IgG (Sigma–Aldrich) were used as negative controls. Culture supernatants were obtained from individual wells and were tested for IFN-γ production using the ELISA kit (Mabtech) as described in Chapter I.

Statistical analysis

Statistical differences were identified using Dunnett's test, Mann–Whitney U test and Steel-Dwass test. Correlation statistics were analyzed using Spearman correlation analysis. The statistical analysis program MEPHAS (*http://www.gen-info.osaka-u.ac.jp/MEPHAS/)* was used to perform statistical analysis. A p-value < 0.05 was considered statistically significant.

RESULTS

M. bovis infection upregulated PD-L1 expression via PGE_2

As described in Chapter I, the expression levels of PD-L1 on monocytes were increased in PBMCs of *M. bovis*-infected cattle. To confirm whether *M. bovis* directly upregulates PD-L1 expression, PBMCs from uninfected cattle were cultured with or without live *M. bovis* and PD-L1 expression was measured on monocytes by flow cytometry. PD-L1 expression was upregulated on $CD11b^+CD14^+$ monocytes when cultured with live *M. bovis* compared to that cultured without live *M. bovis* (Fig. II-1A). The previous study showed that PGE_2 is one of the inducers of PD-L1 expression in Johne's disease [Sajiki *et al*., 2018]. Thus, to evaluate whether PGE_2 induced by live *M. bovis* upregulated PD-L1 expression, PGE_2 production in culture supernatants was assessed using ELISA. Expectedly PGE_2 production was increased when PBMCs were cultured with live *M. bovis*. In addition, the increase in PGE_2 production was positively correlated with PD-L1 expression on $CD11b^+CD14^+$ monocytes (Figs. II-1B and C). To investigate whether recognition of *M. bovis* by pattern recognition receptors contribute to PGE_2 production and PD-L1 expression in the PBMC cultures, PBMCs from uninfected cattle were cultured with or without heat-killed *M. bovis* (Figs. II-1D–F). Heat-killed *M. bovis* also upregulated PD-L1 expression on $CD11b^+CD14^+$ monocytes in line with increased PGE_2 production in culture supernatants (Figs. II-1D–F). To test whether culture supernatants containing humoral factors, such as PGE_2, induced by *M. bovis* upregulate PD-L1 expression, PBMCs from uninfected cattle were cultured in supernatant from PBMCs that were co-cultured with live *M. bovis* for 24h. PD-L1 expression on $CD11b^+CD14^+$ monocytes cultured in 24-h culture supernatants of PBMCs with live *M. bovis* was upregulated compared to that without live *M. bovis* (Fig. II-1G). To confirm that PGE_2 induced PD-L1 expression, PD-L1 expression on monocytes in PBMCs that had been cultured with PGE_2 was analyzed by flow cytometry. PD-L1 expression on $CD11b^+CD14^+$ monocytes incubated with PGE_2 was increased compared to that treated with negative control (Fig. II-1H). These results show that *M. bovis* could upregulate PD-L1 expression on monocytes via PGE_2.

To identify the major cell type that produced PGE_2 in the PBMC culture, PGE_2

productions from isolated $CD14^+$ and $CD14^-$ cells cultured with live *M. bovis* were measured using ELISA. PGE_2 production from $CD14^+$ cells was significantly higher compared to that from $CD14^-$ cells (Fig. II-2A). In the $CD14^+$ fraction, PD-L1 expression on $CD11b^+CD14^+$ monocytes cultured with live *M. bovis* was increased (Fig. II-2B). These results indicate that $CD14^+$ cells could be a major cell type producing PGE_2 against *M. bovis*.

Induction of PGE_2 via TLR2

Because PGE_2 production was increased by heat-killed *M. bovis*, it was hypothesized that pattern-recognition receptors recognize *M. bovis* and their subsequent signaling induce PGE_2 production. Previous researches showed that other mycoplasma, *M. fermentans*, which was first isolated from the human urogenital tract [Ruiter *et al*., 1952], induced PGE_2 production via TLR2 in human monocytes [Kandasamy *et al*., 2011; Krausse-Opatz *et al*., 2004]. Thus, to test whether PGE_2 production and PD-L1 upregulation was induced in bovine PBMCs under TLR2 stimulation, PBMCs from uninfected cattle were incubated with a TLR2/6 agonist. FSL-1 is a synthetic lipoprotein of *M. salivarium* which has been one of the pathogens in eye and ear disorders, oral infection in human [Shibata *et al*., 2000]. FSL-1 upregulated PGE_2 production from PBMCs and increased PD-L1 expression on $CD11b^+CD14^+$ monocytes (Figs. II-3A and B). Consistent with the results by *M. bovis* stimulation, the level of PGE_2 production was positively correlated with PD-L1 expression on $CD11b^+CD14^+$ monocytes under FSL-1 stimulation (Fig. II-3C). Inhibition of TLR2/4 signaling by a selective antagonist, SsnB, decreased PGE_2 production induced by FSL-1 and *M. bovis* stimulation (Fig. II-3D). Since $CD14^+$ cells are considered as one of the major cell types expressing TLR2 [Flo *et al*., 2001], these results suggest that *M. bovis* could induce PGE_2 production from monocytes presumably via TLR2.

Upregulation of PGE_2 production in *M. bovis*-infected cattle

To confirm that PGE_2 is associated with immunosuppression in *M. bovis* infection, serum PGE_2 levels were analyzed in cattle naturally infected with *M. bovis*, which were diagnosed in the previous studies [Gondaira *et al*., 2015; Higuchi *et al*., 2011a]. Serum

PGE_2 levels were significantly increased in *M. bovis*-infected cattle compared to that in uninfected cattle (Fig. II-4A). No differences in serum PGE_2 levels were observed among the different clinical symptoms of mycoplasmosis in the *M. bovis*-infected cattle (Fig. II-4B). Because PBMC samples were not available for the previous clinical samples used in Figs. II-4A and B, blood samples were collected from *M. bovis*-infected cattle and the plasma levels of PGE_2 and other immunological factors were analyzed (Figs. II-4C–E). Plasma levels of PGE_2 were positively correlated with the proportions of PD-$L1^+CD11b^+CD14^+$ cells in infected cattle (Figs. II-4C). To investigate the relationship between PGE_2 and immune responses, plasma IFN-γ levels and IFN-γ production against heat-killed *M. bovis* from PBMCs of infected cattle were measured using ELISA and their correlation with plasma PGE_2 levels were analyzed. Plasma IFN-γ levels and IFN-γ production against *M. bovis* were negatively correlated with plasma PGE_2 levels in infected cattle (Figs. II-4D and E). These results suggest that PGE_2 is associated with PD-L1 expression and immune dysfunction in *M. bovis*-infected cattle.

Expressions of PD-L1 and PGE_2 associated with pneumonic lung lesions in *M. bovis*-infected cattle

To determine the expressions of PD-L1 and PGE_2 in lung lesions of infected cattle, immunohistochemical analysis was conducted on pneumonic lung tissues from cattle infected with *M. bovis*. PD-L1 was expressed on macrophages and fibroblasts infiltrating to the lesions (Fig. II-5A), and PGE_2 was produced by epithelial cells and macrophages infiltrating to the lesions (Fig. II-5C). On the other hand, the expressions of PD-L1 and PGE_2 in the healthy lung of *M. bovis*-uninfected cattle were very weak (Figs. II-5B and D).

Combined blockade of the PD-1/PD-L1 pathway and PGE_2 enhanced the IFN-γ response against *M. bovis* antigen

In Chapter I, the PD-1/PD-L1 blockade by anti-bovine PD-1/PD-L1 mAbs was shown to enhance the *M. bovis*-specific IFN-γ response *in vitro*. Recent researches showed that the dual blockade of the PD-1/PD-L1 pathway and PGE_2 by anti-bovine PD-L1 mAb and a COX-2 inhibitor enhance immune responses against several pathogens

[Chen *et al*., 2015; Sajiki *et al*., 2018; Sajiki *et al*., 2019]. Thus, to evaluate the enhancement of *M. bovis*-specific IFN-γ response, the dual blockade of the PD-1/PD-L1 pathway and PGE_2 using anti-bovine PD-L1 mAb and a COX-2 inhibitor, meloxicam, was performed. IFN-γ production from PBMCs stimulated with T cell stimulators (combination of anti-bovine CD3 mAb and anti-bovine CD28 mAb; Fig. II-6A) or heat-killed *M. bovis* antigen (Fig. II-6B) was significantly increased either by anti-bovine PD-L1 mAb or the combination of anti-bovine PD-L1 mAb with meloxicam compared to that treated with negative controls (combination of DMSO and control rat IgG). These results obtained by the combination of anti-bovine PD-L1 mAb with meloxicam support its potential as a novel treatment method for *M. bovis* infection.

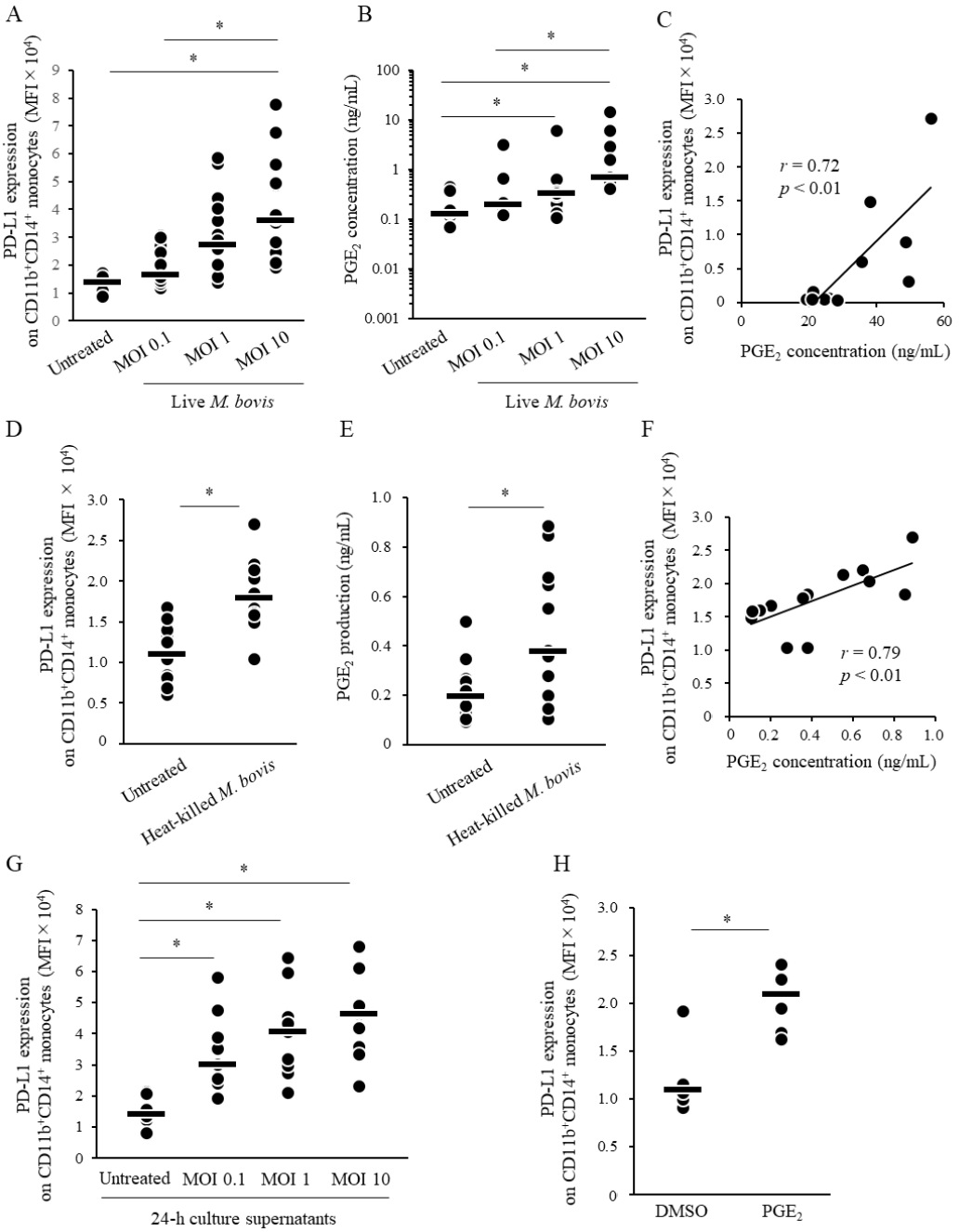

Figure II-1. *M. bovis* upregulates PD-L1 and PGE_2 expression. PBMCs from *M. bovis*-uninfected cattle were incubated with (A–C) live *M. bovis* (MOI of 0.1, 1 or 10) or (D–F) heat-killed *M. bovis* (1.5 ng/mL) for 24 h. (A) PD-L1 expression on $CD11b^+CD14^+$ monocytes (n = 14) was determined using flow cytometry. (B) PGE_2 levels in culture supernatants (n = 9) were determined using ELISA. (C) Positive correlation is noted between PD-L1 expression on $CD11b^+CD14^+$ monocytes and PGE_2 levels under live *M. bovis* stimulation (MOI of 10, n = 11). (D) PD-L1 expression on $CD11b^+CD14^+$ monocytes (n = 13) were determined using flow cytometry. (E) PGE_2 levels in culture supernatants (n = 13) were determined using ELISA. (F)

Positive correlation is noted between PD-L1 expression on $CD11b^{+}CD14^{+}$ monocytes and PGE_2 levels under heat-killed *M. bovis* stimulation ($n = 13$). (G) PD-L1 expression on $CD11b^{+}CD14^{+}$ monocytes cultured in 24-h culture supernatant of PBMCs with or without live *M. bovis* ($n = 9$) were analyzed by flow cytometry. (H) PBMCs from uninfected cattle were incubated with PGE_2, and PD-L1 expression on $CD11b^{+}CD14^{+}$ monocytes ($n = 6$) was determined using flow cytometry. The bars are the median of the values (A, B, D, E, G and H). Statistical significance was determined by the Steel-Dwass test (A, B and G) or Mann–Whitney U test (D, E and H). Correlation statistics were analyzed using Spearman's correlation analysis (C and F). $*p < 0.05$.

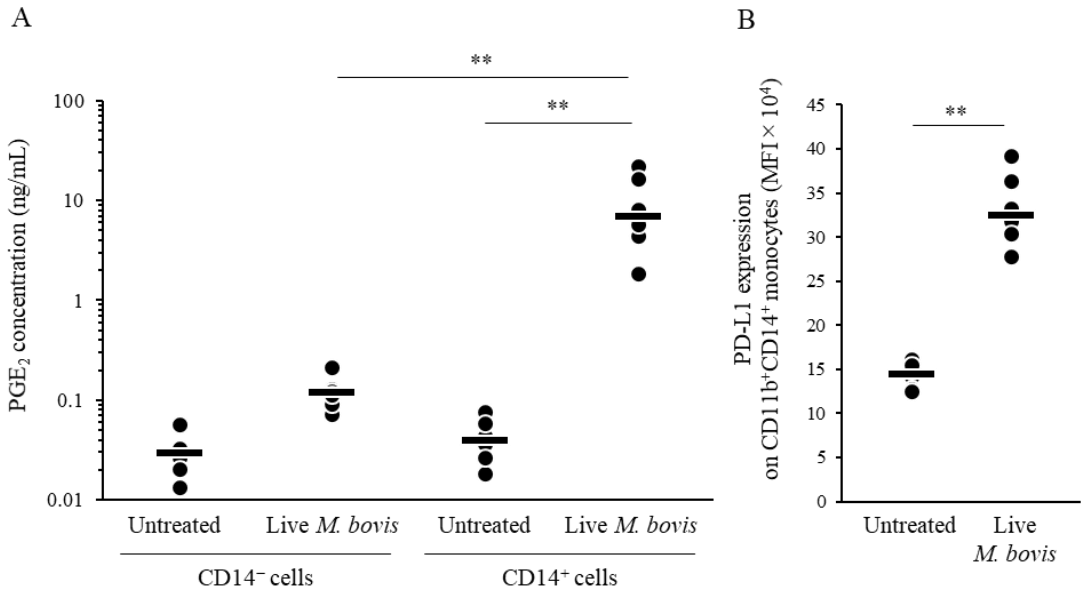

Figure II-2. PGE_2 production from $CD14^+$ cells induced by *M. bovis*. $CD14^+$ cells or $CD14^-$ cells from uninfected cattle were incubated with live *M. bovis* (MOI of 10) for 24 h. (A) PGE_2 production was determined by ELISA ($n = 6$). (B) PD-L1 expression on $CD11b^+CD14^+$ cells ($n = 6$) in the $CD14^+$ cell culture was determined by flow cytometry. The bars are the median of the values (A and B). Statistical significance was determined by the Steel-Dwass test (A) or Mann–Whitney U test (B). $^{**}p < 0.01$.

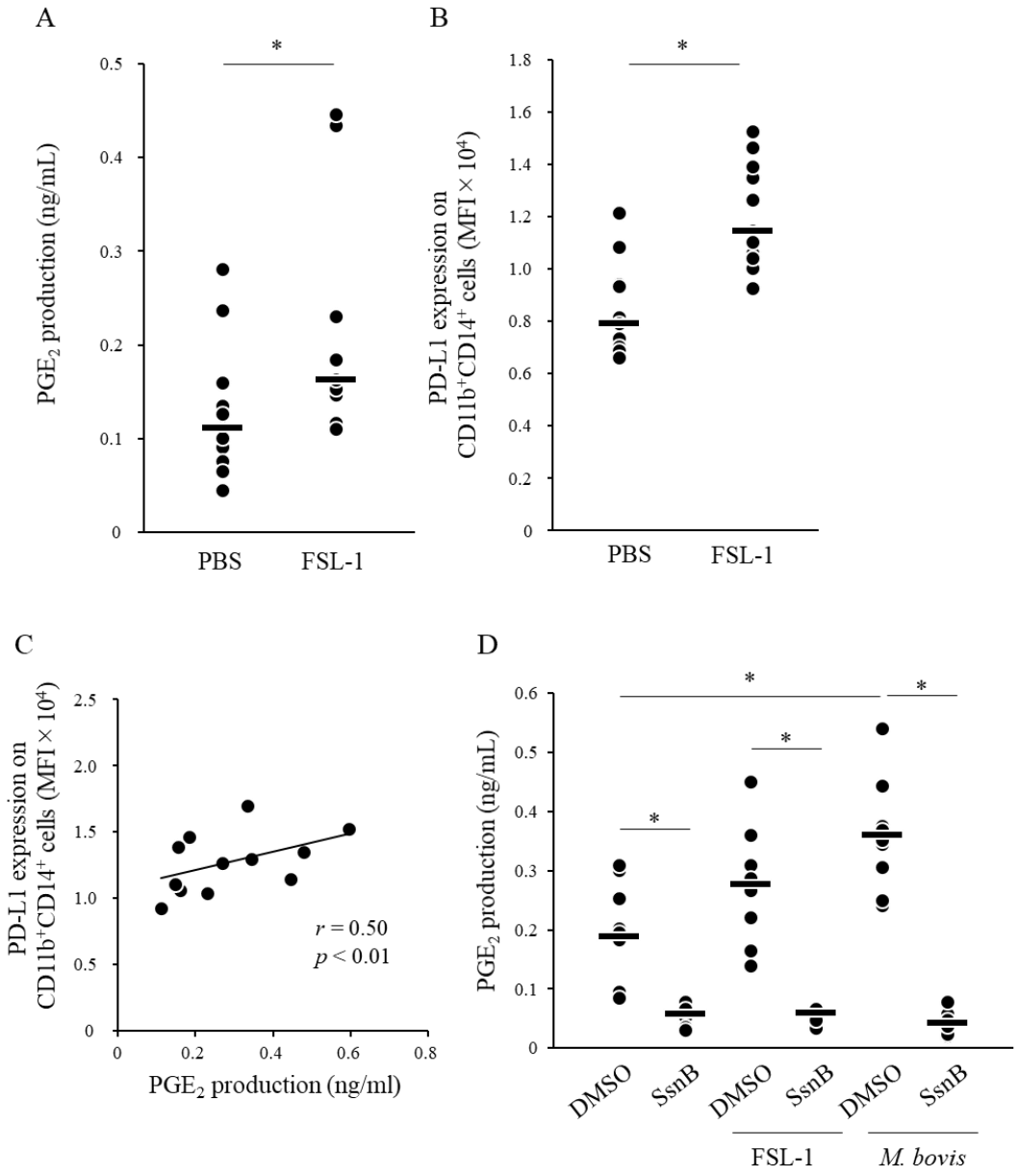

Figure II-3. TLR2 signaling upregulates PD-L1 and PGE_2 expression. PBMCs from uninfected cattle were incubated with FSL-1 (100 ng/mL). (A) PGE_2 production was determined by ELISA (n = 11). (B) PD-L1 expression on $CD11b^+CD14^+$ cells (n = 11) was determined by flow cytometry. (C) Positive correlation is noted between PD-L1 expression on $CD11b^+CD14^+$ monocytes and PGE_2 levels under FSL-1 stimulation (n = 13). (D) PBMCs from uninfected cattle were incubated with FSL-1 (100 ng/mL) or heat-killed *M. bovis* (1.5 ng/mL) under inhibition of TLR signaling by SsnB. PGE_2 production in the culture supernatant was determined by ELISA (n = 9). The bars are the median of the values (A, B and D). Statistical significance was determined by the Mann–Whitney U test (A and B) or the Steel-Dwass test (D). Correlation statistics were analyzed using Spearman's correlation analysis (C). *$p < 0.05$.

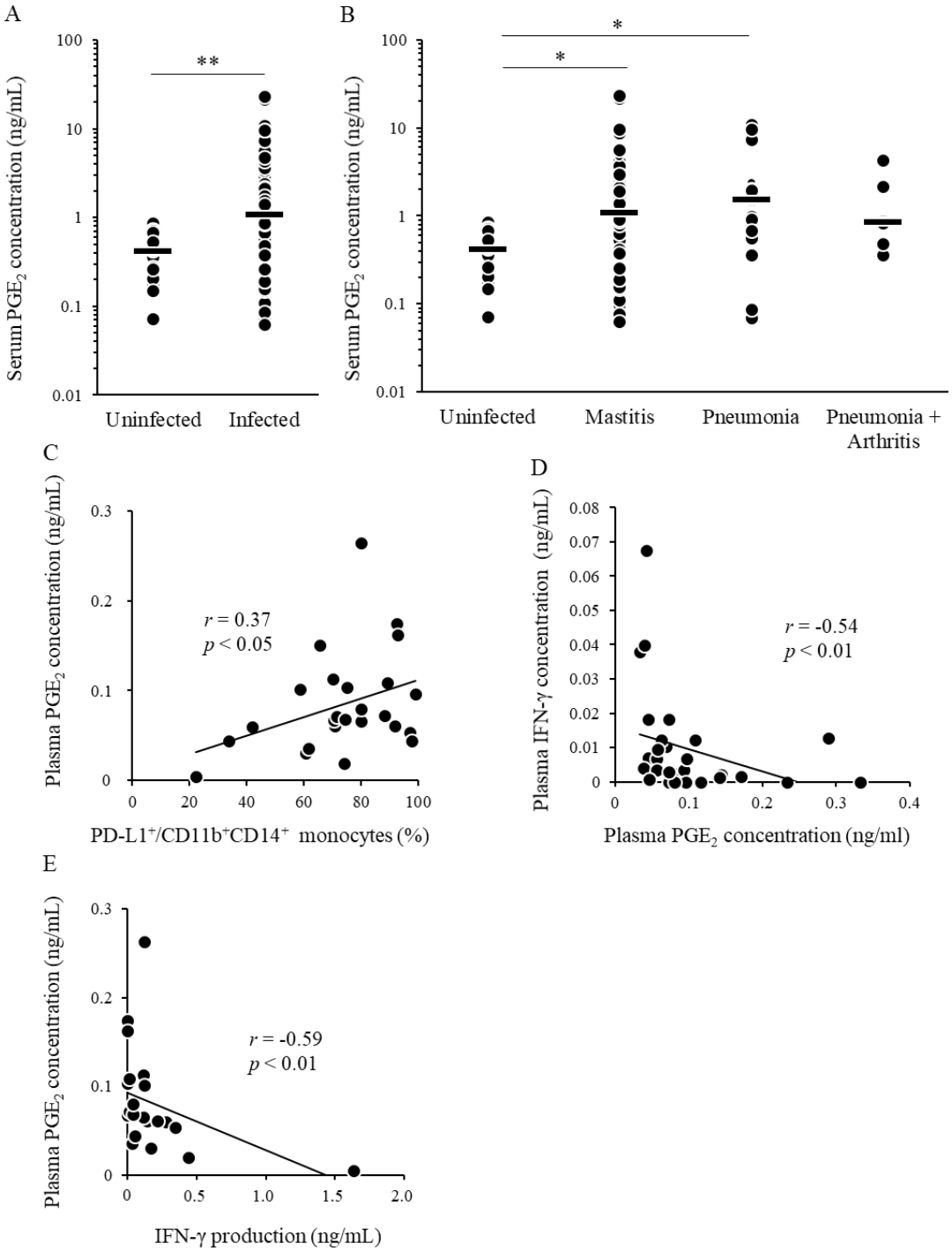

Figure II-4. Analysis of PGE_2 in cattle infected with *M. bovis*. (A and B) Serum PGE_2 levels in *M. bovis*-infected cattle (n = 89; mastitis: n = 62, pneumonia: n = 21, pneumonia with arthritis: n = 6) and uninfected cattle (n = 18) was determined by ELISA. (C–E) Correlation between plasma PGE_2 levels in *M. bovis*-infected cattle {arthritis (n = 13), otitis (n = 10), pneumonia (n = 5) and unknown (n =1)}. (C) Correlation between plasma PGE_2 levels and plasma IFN-γ levels (n = 29). (D) Correlation between the plasma levels of PGE_2 and the proportions of PD-$L1^+$ monocytes (n

= 25). (E) Correlation between plasma PGE_2 levels and IFN-γ production from PBMCs against heat-killed *M. bovis* (n = 25). The bars are the median of the values (A and B). Statistical significance was determined by the Mann–Whitney U test (A) or the Steel-Dwass test (B). Correlation statistics were analyzed using Spearman's correlation analysis (C–E). *$p < 0.05$, **$p < 0.01$.

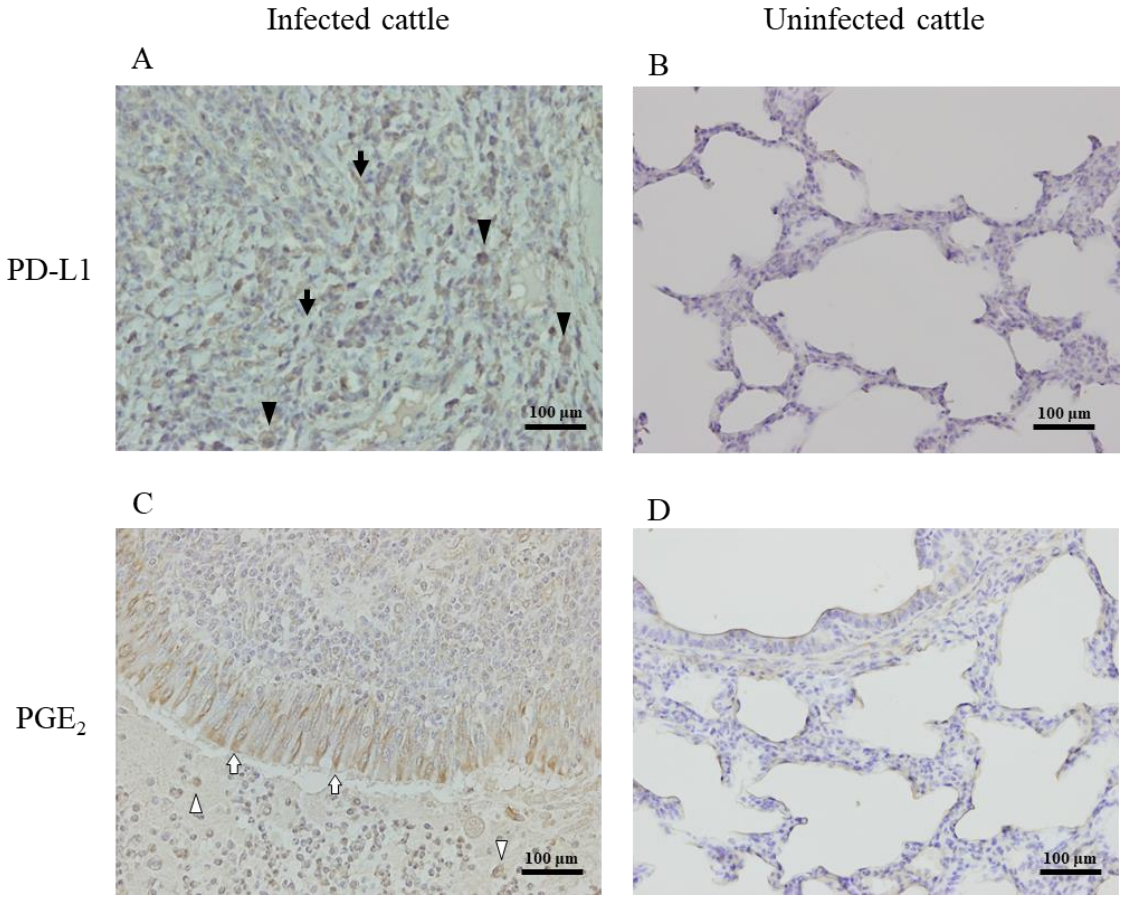

Figure II-5. PD-L1 and PGE_2 expression in the lung of *M. bovis*-infected cattle with pneumonia. Immunohistochemical staining of PD-L1 (A and B) and PGE_2 (C and D) in lung tissues of cattle with or without *M. bovis* infection was performed using anti-bovine PD-L1 mAb (6C11-3A11) and anti-human PGE_2 polyclonal Ab (rabbit polyclonal). Block arrowheads: PD-L1 positive macrophages, block arrows: PD-L1 positive fibroblasts, white arrowheads: PGE_2 positive macrophages, white arrows: PGE_2 positive epithelial cells.

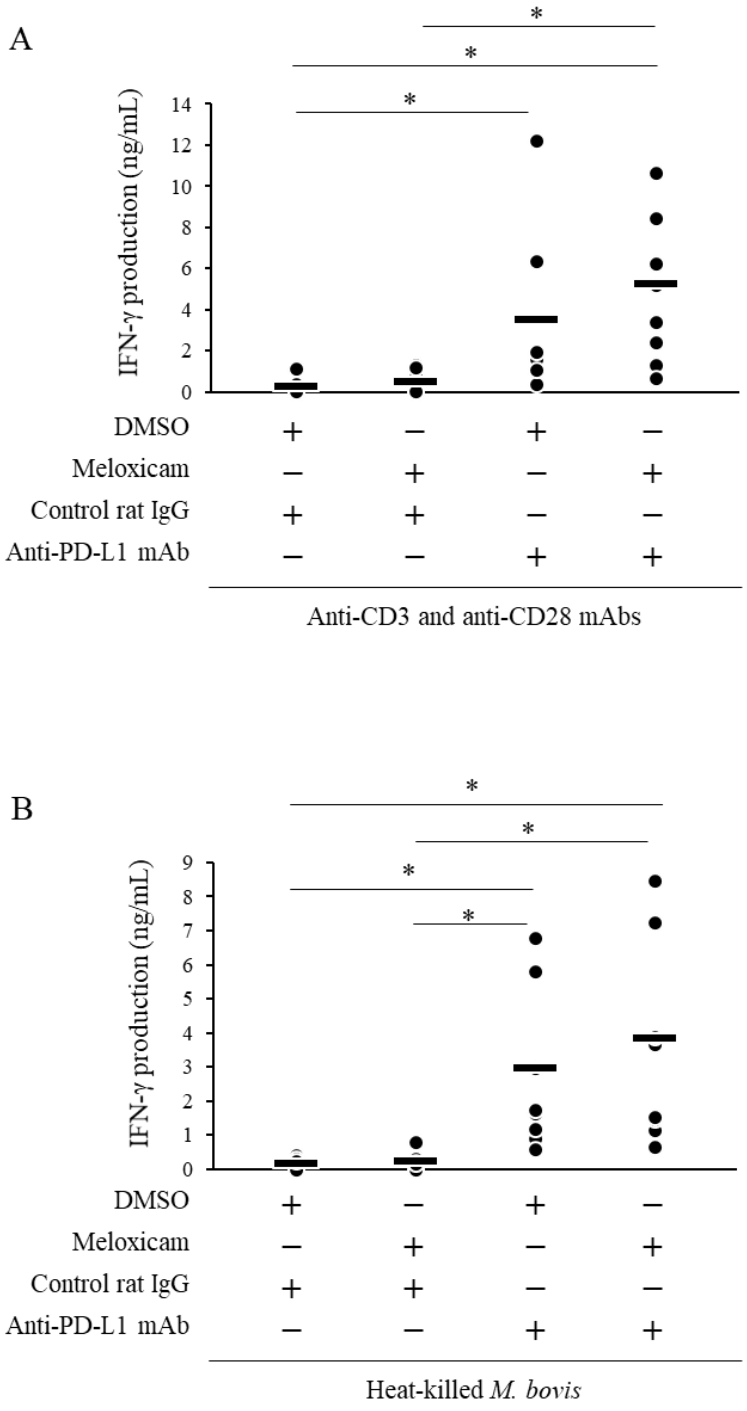

Figure II-6. Activation of IFN-γ responses by the combination of meloxicam and anti-PD-L1 mAb. PBMCs from cattle infected with *M. bovis* (n = 7) were incubated with meloxicam and anti-bovine PD-L1 mAb in the presence of anti-bovine CD3 and CD28 mAbs (A) or heat-killed *M. bovis* (B). IFN-γ production in culture supernatants was determined by ELISA. The bars are the median of the values (A and B). Statistical significance was determined by the Steel-Dwass test (A and B). *$p < 0.05$.

DISCUSSION

PGE_2 is typically known as pro- and anti-inflammatory mediator and its synthesis is induced by COX-2 and PGE synthases [Morita, 2002; Phipps *et al*., 1991]. Previous researches in cancer demonstrated the relationships between PGE_2 and the PD-1/PD-L1 pathway, indicating that PGE_2 was implicated in the induction of PD-L1 expression [Botti *et al*., 2017; Prima *et al*., 2017]. In addition, previous studies in cattle have demonstrated that PGE_2 upregulates PD-L1 expression on bovine PBMCs and is involved in the disease progression of BLV infection and Johne's disease, which are chronic infections in cattle [Sajiki *et al*., 2018; Sajiki *et al.*, 2019]. Those studies suggest that the immunosuppression via PGE_2–PD-L1 axis is commonly involved in bovine chronic infections. Indeed, in the present study, *M. bovis* induced PGE_2 production from monocytes in line with the upregulation of PD-L1 expression on monocytes (Figs. II-1A–F). Additionally, the levels of plasma PGE_2 were positively correlated with proportions of circulating PD-$L1^+$ monocytes (Fig. II-4C). These results show that PGE_2 could be associated with PD-L1 expression during *M. bovis* infection. Several reports showed that PGE_2 activates signal transducer and activator of transcription 3 (STAT3) signaling [Frias *et al*., 2006; Sajiki *et al*., 2018]. In tumor studies, STAT3 regulates PD-L1 expression transcriptionally by binding to its promoter [Marzec *et al*., 2008; Song *et al*., 2018]. Taken together, PGE_2 induced by *M. bovis* could upregulate PD-L1 expression on monocytes via STAT3 signaling. On the other hand, many researches have reported that PD-L1 expression can be induced by other factors, such as IFN-γ and TNF-α [Abiko *et al*., 2015; Lee *et al.*, 2006; Lim *et al*., 2016]. A previous report showed that *M. bovis* induced IFN-γ and TNF-α production from PBMCs of uninfected cattle [Gondaira *et al*., 2015]. Further studies on other inducer of PD-L1 expression such as cytokines are warranted to fully elcidate the mechanisms of PD-L1 expression during *M. bovis* infection.

TLRs are pattern-recognition receptors which play important roles in early innate recognition and are essential for host immune responses against several pathogens, including *Mycoplasma* [Brown *et al*., 2011; Kopp and Medzhitov, 1999, 3 Shimizu *et al*., 2007]. TLR2 recognizes lipoproteins from *Mycoplasma* spp. including *M. bovis* [Chu *et al*., 2005; He *et al*., 2009; Krausse-Opatz *et al*., 2004; Muneta *et al*., 2003; Ruiter *et al*.,

1952; Wang *et al*., 2015]. TLR2/MyD88 signaling induces PGE_2 production by COX-2 transcription via NF-κB [Echizen *et al*., 2016], during not only *Mycoplasma* infection [Kandasamy *et al*., 2011; Mitsunari *et al*., 2006; Müller *et al*., 2015] but also other bacterial infection such as Mycobacterial infection, including *Mycobacterium bovis* [Almeida *et al*., 2014] and *Mycobacterium leprae* [Mattos *et al*., 2010], and Staphylococcal infection [Falahee *et al*., 2013]. TLR2 expression has been demonstrated in several types of immune cells, such as lymphocytes and monocytes [Shibata *et al*., 2000] and some types of non-hematopoietic cells, such as epithelial cells [Muir *et al*., 2004]. A previous study in humans showed that $CD14^+$ monocytes expressed the highest level of TLR2 in blood [Flo *et al*., 2001]. Indeed, the present study suggested that $CD14^+$ monocytes were a major cell type producing PGE_2 in response to *M. bovis in vitro* (Fig. II-2A). In addition, immunohistochemical analysis demonstrated that macrophages infiltrating to lung lesions and epithelial cells in lung lesions from infected cattle expressed PGE_2 (Fig. II-5C). These findings suggest that *M. bovis* could induce PGE_2 from monocytes and epithelial cells via TLR2.

In the present study, SsnB, a selective antagonist for TLR2 and TLR4 [Liang *et al*., 2011], almost completely canceled the induction of PGE_2 by heat-killed *M. bovis*. This result suggested that *M. bovis* is recognized by monocytes and induces PGE_2 production via TLR2 and/or TLR4. TLR4 recognizes bacterial lipopolysaccharide and peptidoglycan which are the components of bacterial cell wall [Brown *et al*., 2011; Kopp and Medzhitov, 1999]. Although Mycoplasmas do not have cell wall [Trachtenberg, 2015], several researchers reported the relationships between TLR4 and the inflammation during *Mycoplasma* infection [Domingo-Gonzalez *et al*., 2016; Gondaira *et al*., 2017; Muir *et al*., 2004]. Shimizu *et al*. have demonstrated that *M. pneumoniae* induces TNF-α production from macrophages through autophagy and TLR4. Because the induction of TLR4 signaling was observed only when live *M. pneumoniae* was used as a stimulator, cytoadherence of the live bacterium to macrophages might be needed for the recognition. [Shimizu *et al.,* 2014]. In the present study, heat-killed *M. bovis* stimulation clearly induced PGE_2 production (Figs. II-1D and II-3A), suggesting that *M. bovis* induces PGE_2 production mainly via TLR2 signaling. However, further studies focusing on TLR4 using live *M. bovis* are needed to fully elucidate the molecular mechanisms of PGE_2 induction

in *M. bovis* infection *in vivo*.

PGE_2 suppresses Th1 immune responses, limiting the functions of NK cells, $CD4^+$ T cells and cytotoxic T cells via EP2 and EP4 receptors [Martínez-Colón and Moore, 2018]. In the present study, increased plasma PGE_2 levels were strongly correlated with lower *M. bovis*-specific IFN-γ production (Fig. II-4D). Additionally, plasma levels of PGE_2 were positively correlated with the proportions of circulating PD-$L1^+CD11b^+CD14^+$ monocytes (Fig. II-4C). These results showed that decreased IFN-γ levels during *M. bovis* infection might be associated with the upregulation of PGE_2 and PD-$L1^+$ cells. In addition. PGE_2 can inhibit not only adaptive immunity but also innate immunity. A previous report in humans demonstrated that PGE_2 inhibits neutrophil functions, such as neutrophil extracellular trap (NET) formation, via EP2 and EP4 [Domingo-Gonzalez *et al*., 2016]. Indeed, *M. bovis* was reported to inhibit NET formation and escape from neutrophil killing [Gondaira *et al*., 2017]. Therefore, PGE_2 in *M. bovis* infection might play a role not only as an inducer of PD-L1 expression but also as a direct suppressor of other immune responses, such as T cell and neutrophil responses. Further studies focusing on the inhibition induced by PGE_2 might help elucidate the detailed mechanisms of immunosuppression in *M. bovis* infection.

The previous studies demonstrated that the blockade of the PD-1/PD-L1 pathway and/or PGE_2 effectively improved antigen-specific immune reactions in bovine chronic diseases, such as bovine leukemia virus infection, Johne's disease and anaplasmosis [Ikebuchi *et al*., 2011; Ikebuchi *et al*., 2013; Okagawa *et al*., 2016a; Okagawa *et al*., 2016b; Sajiki *et al*., 2018; Sajiki *et al*., 2019]. Effective reactivation by dual blockade could be due to PGE_2 function that inhibits T cell activation and regulates PD-L1 expression. Indeed, single blockade of the PD-1/PD-L1 pathway reactivated the *M. bovis*-specific IFN-γ response *in vitro* (Fig. II-6B). Therefore, to evaluate the immune activation, the combined treatment of anti-bovine PD-L1 mAb with a COX-2 inhibitor, meloxicam, was performed *in vitro*. Indeed, the combined treatment of anti-bovine PD-L1 mAb with a COX-2 inhibitor significantly increased IFN-γ production in response to T cell stimulation and *M. bovis* antigen when compared to COX-2 inhibitor treatment alone. Although the difference was not significant between the antibody treatment alone and the combined treatment, IFN-γ production tended to be increased in the combined treatment

group. Although further investigation is needed to support the efficacy of the combined treatment, the combination of PD-1/PD-L1 blockade with COX-2 inhibition has therapeutic potential for the control of *M. bovis* infection.

In conclusion, the study in this chapter showed that *M. bovis* could induce PGE_2 and upregulate PD-L1 expression on monocytes via PGE_2, and that *M. bovis*-specific IFN-γ response could be upregulated by dual blockade of the PD-1/PD-L1 pathway and PGE_2 production. These results suggest that the combination of anti-PD-L1 antibody with a COX-2 inhibitor is a candidate for therapeutic application to treat *M. bovis* infection.

SUMMARY

PGE_2 suppresses immune responses and is associated with the upregulation of PD-L1 expression in several bovine chronic diseases, such as BLV infection and Johne's disease. In Chapter I, it was found that the PD-1/PD-L1 pathway was associated with immunosuppression during *M. bovis* infection. However, the role of PGE_2 in the immunosuppression and the relationship between PGE_2 and the PD-1/PD-L1 pathway during *M. bovis* infection is still unclear.

In Chapter II, *in vitro* stimulation with *M. bovis* upregulated the expressions of PGE_2 and PD-L1 presumably via TLR2 in bovine PBMCs. PGE_2 levels of peripheral blood in infected cattle were significantly increased compared to those in uninfected cattle. Remarkably, plasma PGE_2 levels were positively correlated with the proportions of PD-$L1^+$ monocytes in *M. bovis*-infected cattle. Additionally, plasma PGE_2 production in infected cattle was negatively correlated with *M. bovis*-specific IFN-γ production from PBMCs. These results suggest that PGE_2 is one of the inducers of PD-L1 expression and could be involved in the immunosuppression during *M. bovis* infection. On the other hand, *in vitro* dual blockade using anti-bovine PD-L1 mAb and a COX-2 inhibitor further enhanced the *M. bovis*-specific IFN-γ response. These results suggest that the combination of anti-PD-L1 antibody with a COX-2 inhibitor is a candidate for therapeutic application to treat *M. bovis* infection.

CHAPTER III

Analysis of efficacy of combined treatment of anti-PD-L1 rat-bovine chimeric antibody with a COX-2 inhibitor in *M. bovis*-infected cattle

INTRODUCTION

In Chapters I and II, the immunosuppression associated with the PD-1/PD-L1 pathway and PGE_2 during *M. bovis* infection was investigated. In Chapter I, it was elucidated that IFN-γ production against *M. bovis* was decreased due to T cell exhaustion caused by the PD-1/PD-L1 pathway, and *in vitro* treatment with anti-bovine PD1/PD-L1 mAbs restored decreased *M. bovis*-specific IFN-γ response during *M. bovis* infection. In Chapter II, it was found that PGE_2 was one of the inducers of PD-L1 expression during *M. bovis* infection and the combined treatment of anti-bovine PD-L1 mAb with a COX-2 inhibitor further enhanced the IFN-γ response against *M. bovis in vitro*. These results suggest that the PD-1/PD-L1 pathway and PGE_2 could be therapeutic targets for the control of *M. bovis* infection. However, the effect of combined treatment of anti-bovine PD-L1 Ab with a COX-2 inhibitor on *M. bovis*-infected cattle remains to be elucidated.

A previous study reported the establishment of anti-bovine PD-L1 chAb and clinical study which was conducted in BLV-infected cattle [Nishimori *et al*, 2017; Sajiki *et al*., 2019]. Interestingly, T cell function, such as IFN-γ response and the proliferation of BLV-specific $CD4^+$ T cells, was restored and BLV provirus loads were significantly reduced in cattle inoculated with anti-bovine PD-L1 chAb [Nishimori *et al*, 2017; Sajiki *et al*., 2019]. In addition, the combined treatment of anti-bovine PD-L1 chAb with a COX-2 inhibitor significantly decreased BLV provirus loads, clearly demonstrating that this combined treatment induces antivirus activities [Sajiki *et al*., 2019]. Therefore, the blockade of the PD-1/PD-L1 pathway and/or PGE_2 can be applied for the treatment of bovine chronic infection.

In this chapter, the efficacy of combined treatment of anti-bovine PD-L1 chAb with a COX-2 inhibitor was examined using *M. bovis*-infected cattle *in vivo*. The findings in this study indicated the therapeutic potential of targeting the PD-1/PD-L1 pathway and PGE_2 for the control of *M. bovis* infection.

MATERIALS AND METHODS

Animals

Seven calves naturally infected with *M. bovis*, that were positive for respiratory mycoplasmas (nasal swab culture) were obtained from farms at Hokkaido, Japan (Table III-1). Detection of *M. bovis* were conducted by PCR as described in Chapter I and II. All animal experiments in this chapter were approved by the Ethics Committee of the Animal Research Center, Agricultural Research Department, Hokkaido Research Organization (No. 1905) and the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University (No. 17-0024).

Administration of anti-bovine PD-L1 chAb and a COX-2 inhibitor to *M. bovis*-infected cattle

To investigate the effect of the PD-1/PD-L1 blockade and COX-2 inhibition against *M. bovis in vivo*, 2 mg/kg of anti-PD-L1 chAb (Boch4G12; Nishimori *et al*, 2017) was administered intravenously (*i. v.*) in four *M. bovis*-infected cattle with pneumonia (animals #4–7), and 0.5 mg/kg of meloxicam (Boehringer Ingelheim, Ingelheim, Germany) was co-administered by subcutaneous (*s. c.*) injection twice at a weekly interval in animals #3, #6 and #7. As negative controls, animals #1 and #2 were administered with 100 mL of saline. These animals were kept in a biosafety level I animal facility at the Animal Research Center, Agricultural Research Department, Hokkaido Research Organization (Shintoku, Hokkaido, Japan). After the inoculation of Boch4G12, peripheral blood samples, nasal discharge and bronchoalveolar lavage fluid (BALF) were collected from these cattle according to the schedule of sample collection as shown in Fig. III-1. Fourteen days after the inoculation of Boch4G12, animals #1, #3, #4 and #6 were euthanized and subjected to autopsy.

Kinetic analysis of serum PGE_2 by ELISA

To investigate the effect of treatments on *M. bovis*-specific immune response, the serum PGE_2 levels were measured as described in Chapter II.

In vivo effect of anti-PD-L1 chAb and a COX-2 inhibitor on IFN-γ response

To investigate the effect of treatments on *M. bovis*-specific immune response, peripheral blood samples were collected and PBMCs were purified, and the IFN-γ response against the heat-killed *M. bovis* antigen was investigated as described in Chapter I.

The effect of anti-PD-L1 chAb and a COX-2 inhibitor on bacterial load in nasal discharge and bronchoalveolar lavage fluid from infected cattle

To investigate the effect of the PD-1/PD-L1 blockade against *M. bovis* infection, bacterial load was measured in nasal discharge and BALF from infected cattle using real-time PCR. Nasal discharges were collected by using sterilized P200 tips (Dooh Rika, Sapporo, Japan) and 1.5-mL microtubes (Fukaekasei, Tokyo, Japan) (Fig. III-2). With regard to the collection of BALF from each animal, 100 mL of saline were injected into the trachea using a cannula tube (Fukaekasei) and collected the injected saline as BALF by aspiration. DNA was extracted from total nasal discharge or 2 mL of BALF using GenElute Bacterial Genomic DNA Kits (Sigma–Aldrich) according to the manufacturer's instructions. Bacterial load was evaluated by using real-time PCR with Thunderbird SYBR qPCR Mix (Toyobo, Tokyo, Japan) and Cica genus *Mycoplasma bovis* detection kit (Kanto Chemical, Tokyo, Japan). Real-time PCR was performed in triplicate using a thermal cycler (LightCycler 480 system II; Roche Diagnostic, Mannheim, Germany). Using the values obtained from the experiments, significant difference between the bacterial load at day 0 and each time point was tested by statistical analysis as described below.

Statistical analysis

Statistical significance was determined by Dunnett's test (vs. day 0 for each result) using the statistical analysis program MEPHAS (*http://www.gen-info.osaka-u.ac.jp/MEPHAS/*). A p-value < 0.05 was considered statistically significant.

Table III-1. The information of cattle used in the clinical study.

Cattle	**#1**	**#2**	**#3**	**#4**	**#5**	**#6**	**#7**
Age	2 months old	7 months old	6 months old	2 months old	7 months old	2 months old	4 months old
Species	Holstein	Holstein	Holstein	Holstein	Holstein	Holstein	Holstein
Sex	Male	Male	Male	Male	Male	Male	Male
Weight (kg)	85	252	213	83	239	87	164
Respiratory symptom	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge	Cough and mucus discharge	Cough and discharge	Cough and mucus discharge
Serum PGE_2 concentration at day 0 (pg/mL)	242	2,057	1226	762	1,657	160	2,681
Inoculation dose (Boch4G12)	Saline 100mL, *i. v.*	Saline 100mL, *i. v.*	-	2 mg/kg, *i. v.*	2 mg/kg, *i. v.*	2 mg/kg, *i. v.*	2 mg/kg, *i. v.*
Administration dose (Meloxicam)	-	-	0.5 mg/kg, once a week., totally 2 times, *s. c.*	-	-	0.5 mg/kg, once a week., totally 2 times, *s. c.*	0.5 mg/kg, once a week., totally 2 times, *s. c.*

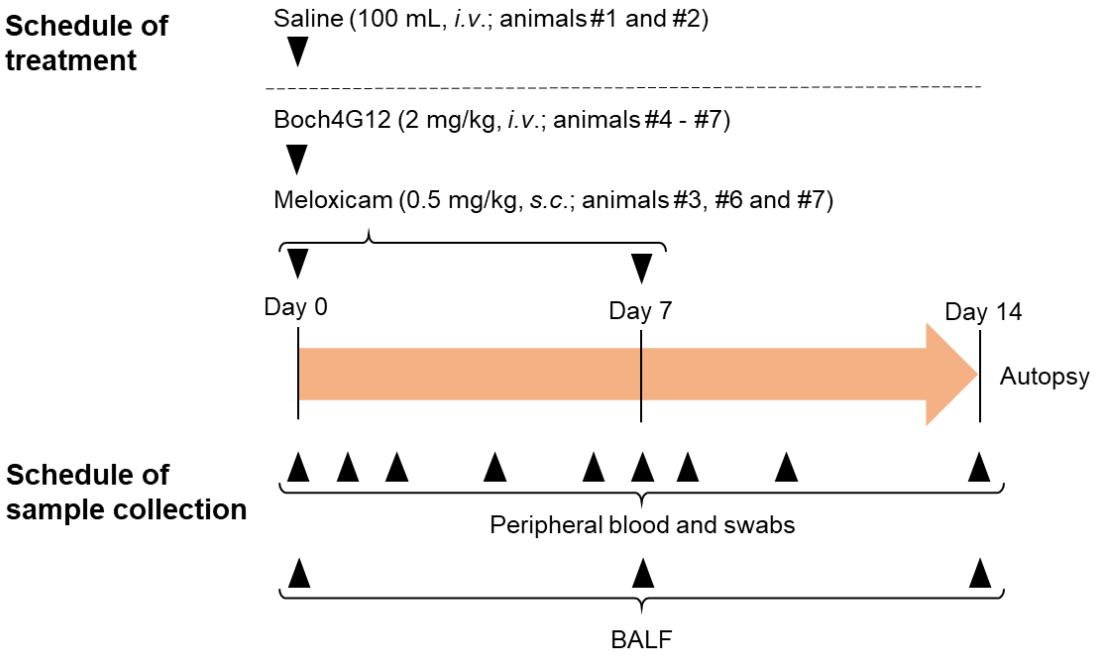

Figure III-1. Schedule of treatment and sample collection in clinical study. 2 mg/kg of anti-PD-L1 chAb (Boch4G12) was administered intravenously in four *M. bovis*-infected cattle with pneumonia (animals #4–#7) and 0.5 mg/kg of meloxicam was co-administered by subcutaneous injection twice at a weekly interval in animals #3, #6 and #7. As negative controls, animals #1 and #2 were administered 100 mL of saline. Peripheral blood and nasal swabs were collected at days 0, 1, 2, 4, 6, 7, 8, 10 and 14. BALF was collected at days 0, 7 and 14.

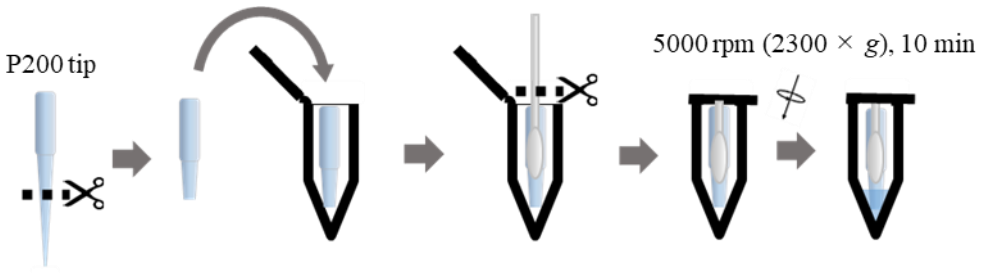

Figure III-2. Preparation of nasal discharge specimens. (1) Cut a P200 tip; (2) Place a cut tip into a 1.5-mL collection tube; (3) Place a nasal swab; (4) Centrifuge at 2,300 × *g* (10 min, 4°C).

RESULTS

The effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 on serum PGE_2 concentration in tested cattle

To investigate the effect of the treatment with Boch4G12 and/or meloxicam on the PGE_2 levels in peripheral blood from treated cattle, serum PGE_2 concentration was measured by ELISA. Serum PGE_2 concentration was not changed throughout the experimental period in the negative controls (animals #1 and #2) and one of the cattle treated with Boch4G12 and meloxicam (animal #6) (Fig. III-3). Decreased serum PGE_2 concentration was observed in cattle treated with meloxicam alone (animal #3), Boch4G12 alone (animal #4) and both Boch4G12 and meloxicam (animal #7), (Fig. III-3). Serum PGE_2 concentration in one of the cattle treated with Boch4G12 alone (animal #5) was significantly higher than that before administration (Fig. III-3).

***In vivo* effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 on *M. bovis* specific-IFN-γ response**

Because the combined blockade of the PD-1/PD-L1 pathway and PGE_2 enhanced the *M. bovis*-specific immune response *in vitro* as described in Chapter II, IFN-γ responses of PBMCs against *M. bovis* antigen were evaluated in the test animals. IFN-γ response was not increased in the negative controls (animals #1 and #2) and cattle treated with meloxicam alone (animal #3) (Fig. III-4). Although the enhancement of IFN-γ production was not observed in one of the cattle treated with anti-PD-L1 antibody alone (animal #5; Fig. III-4), the responses were significantly higher than those before administration in the other cattle treated with anti-PD-L1 antibody alone (animal #4) and in both two cattle treated with the combined treatment (animals #6 and #7) (Fig. III-4).

In vivo* effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 on bacterial loads of *M. bovis

To investigate the effect of the combined treatment of Boch4G12 and meloxicam, bacterial load of *M. bovis* in nasal discharges and BALFs was measured by real-time PCR. Bacterial load in nasal discharges and BALFs from negative control was not changed throughout the experimental period (animal #1; Figs. III-5A and B), but those from treated cattle were significantly decreased after the treatment of Boch4G12 with or without meloxicam (animals #4 and #6; Figs. III-5C–F). In the other tested animals, namely #2,

#3, #5 and #7, bacterial loads in nasal discharges and BALFs were not detectable by real-time PCR due to low bacterial loads.

Gross lung lesions of cattle treated with Boch4G12 and meloxicam

At 14 days after administration, autopsy was conducted (Figs. III-6A, B and C: pictures of animal #1; Figs. III-6D and E: pictures of animal #3; Figs. III-6F and G: pictures of animal #4; Figs. III-6H and I: pictures of animal #6). Pleural adhesion is observed in animals #1 and #3 (Fig. III-6A), but not in animals #4 and #6 (Figs. III-6D and F). Multifocal white nodules and atelectasis were observed in the cranial lung lobes from animal #1 (Figs. III-6B and C). Mild atelectasis is found in the cranioventral areas of the lungs from animals #3, #4 and #6 (Figs. III-6E, G and I).

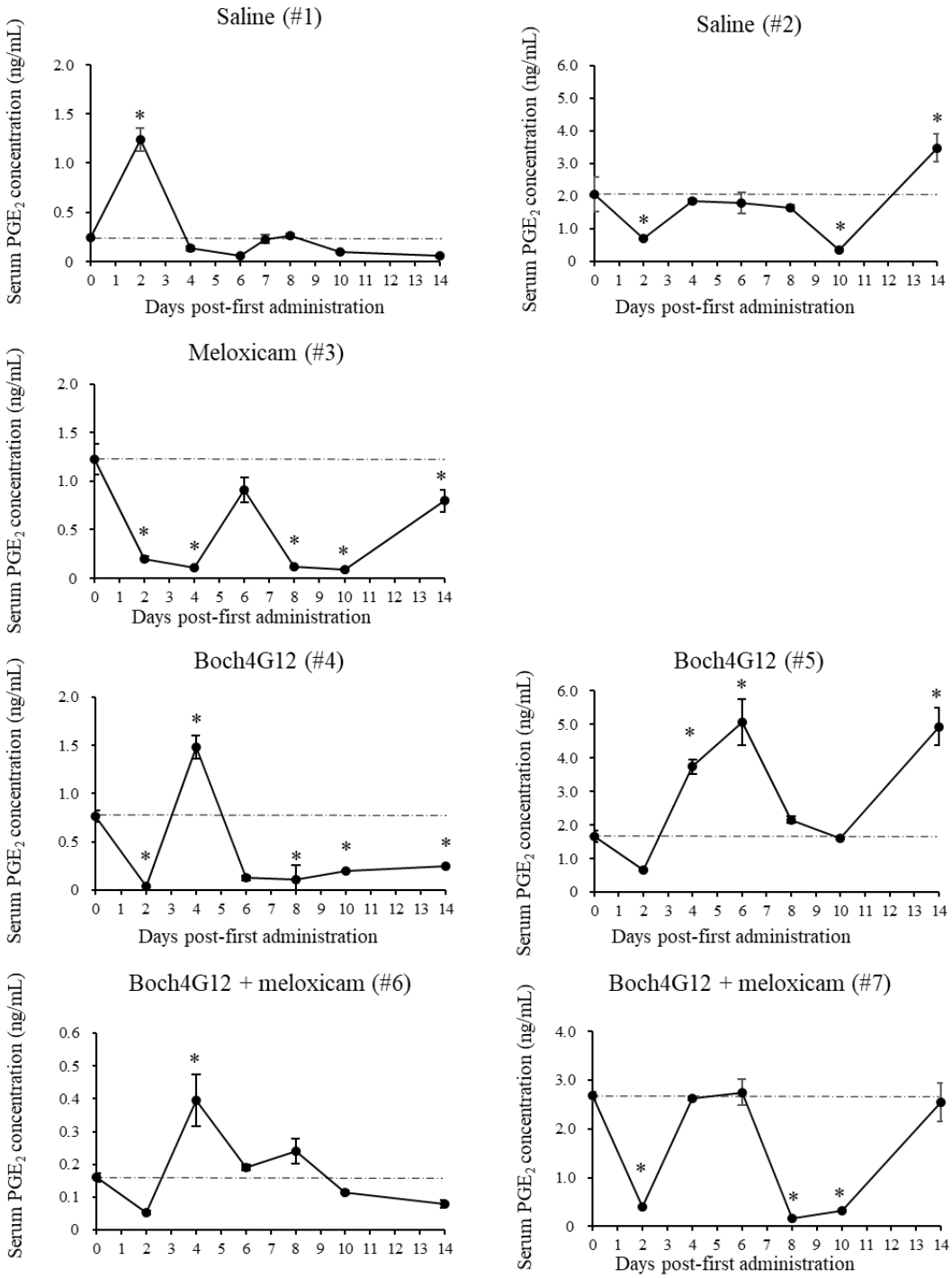

Figure III-3. Kinetic analysis of serum PGE_2 in *M. bovis*-infected cattle treated with anti-PD-L1 chAb (Boch4G12) and meloxicam. *M. bovis*-infected cattle were intravenously administered 2 mg/kg of Boch4G12 (animals #4–#7) or saline (negative control, animals #1 and #2). In addition, animals #3, #6 and #7 were subcutaneously administered 0.5 mg/kg of meloxicam twice with a weekly interval. Serum PGE_2 was measured by ELISA. Statistical significance was determined by Dunnett's test. *$p < 0.05$ (vs. day 0 for each result).

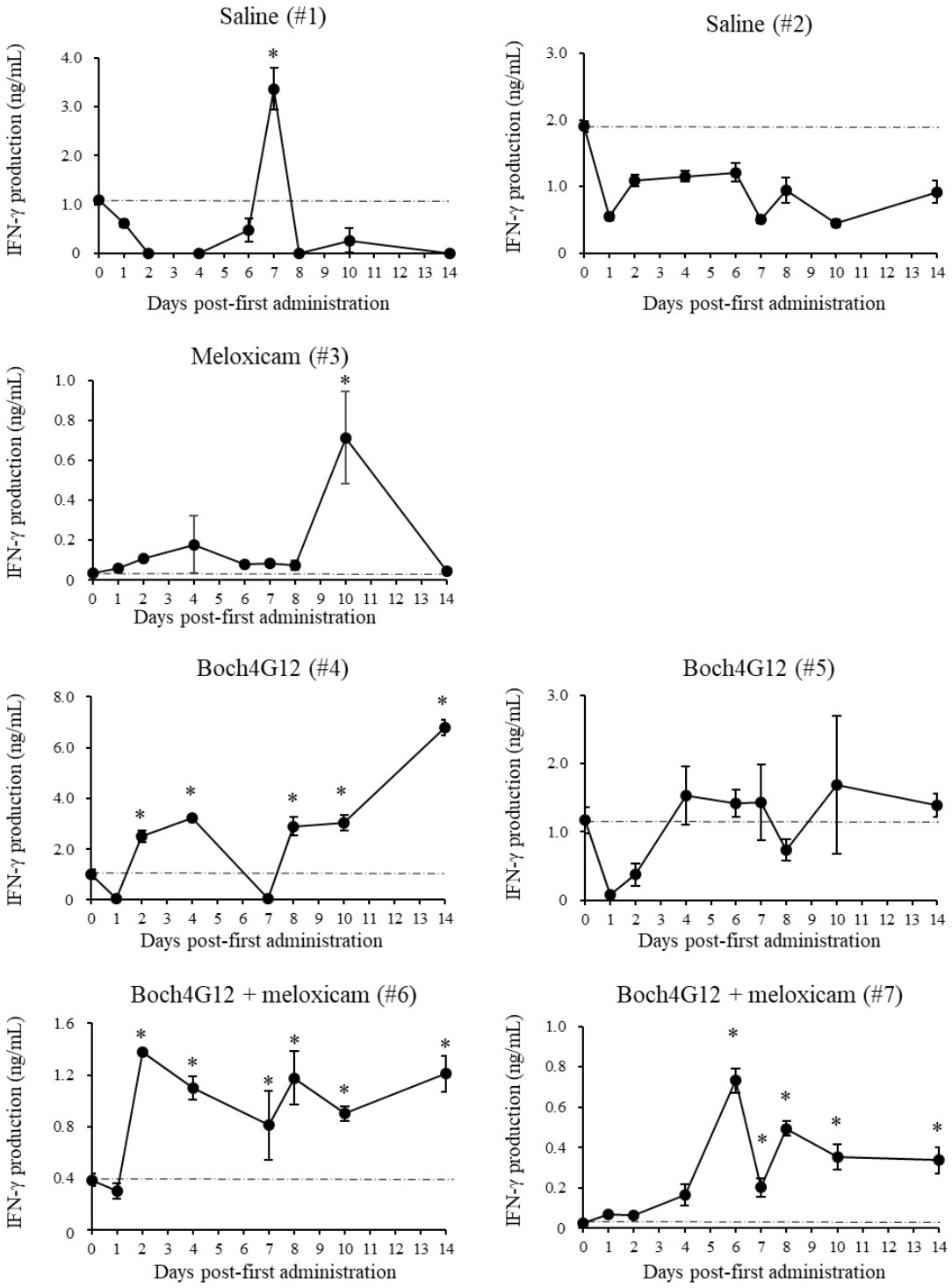

Figure III-4. Activation of IFN-γ responses in *M. bovis*-infected cattle treated with anti-PD-L1 chAb (Boch4G12) and meloxicam. *M. bovis*-infected cattle were intravenously administered 2 mg/kg of Boch4G12 (animals #4–#7) or saline (negative control, animals #1 and #2). In addition, animals #3, #6 and #7 were subcutaneously administered 0.5 mg/kg of meloxicam twice with a weekly interval. PBMCs from treated cattle were incubated in the presence of heat-killed *M. bovis* (1.5 ng/mL). IFN-γ production in culture supernatants was measured by ELISA. Statistical significance was determined by Dunnett's test. *$p < 0.05$ (vs. day 0 for each result).

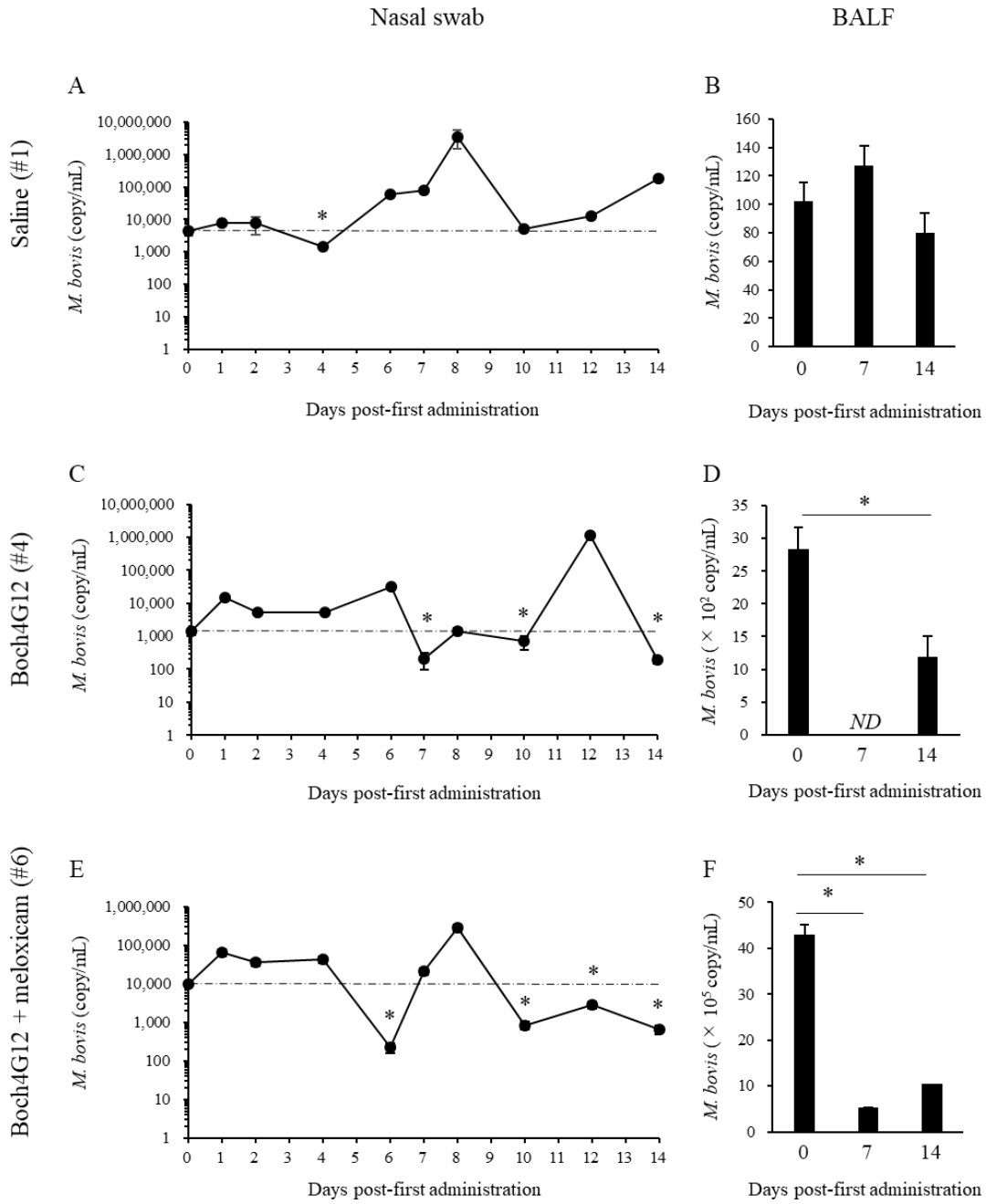

Figure III-5. Bacterial load in nasal discharge and BALF from *M. bovis*-infected cattle treated with anti-PD-L1 chAb (Boch4G12) and meloxicam. *M. bovis*-infected cattle were intravenously administered 2 mg/kg of purified Boch4G12 (animals #4 and #6) or saline (negative control, animal #1). In addition, animal #6 was subcutaneously administered 0.5 mg/kg of meloxicam twice with a weekly interval. Bacterial load in nasal discharge and BALF was measured by real-time PCR (A and B: data of animal #1, C and D: data of animal #4, E and F: data of animal #6). Statistical significance was determined by Dunnett's test. *$p < 0.05$ (vs. day 0 for each result). *ND*; not detected.

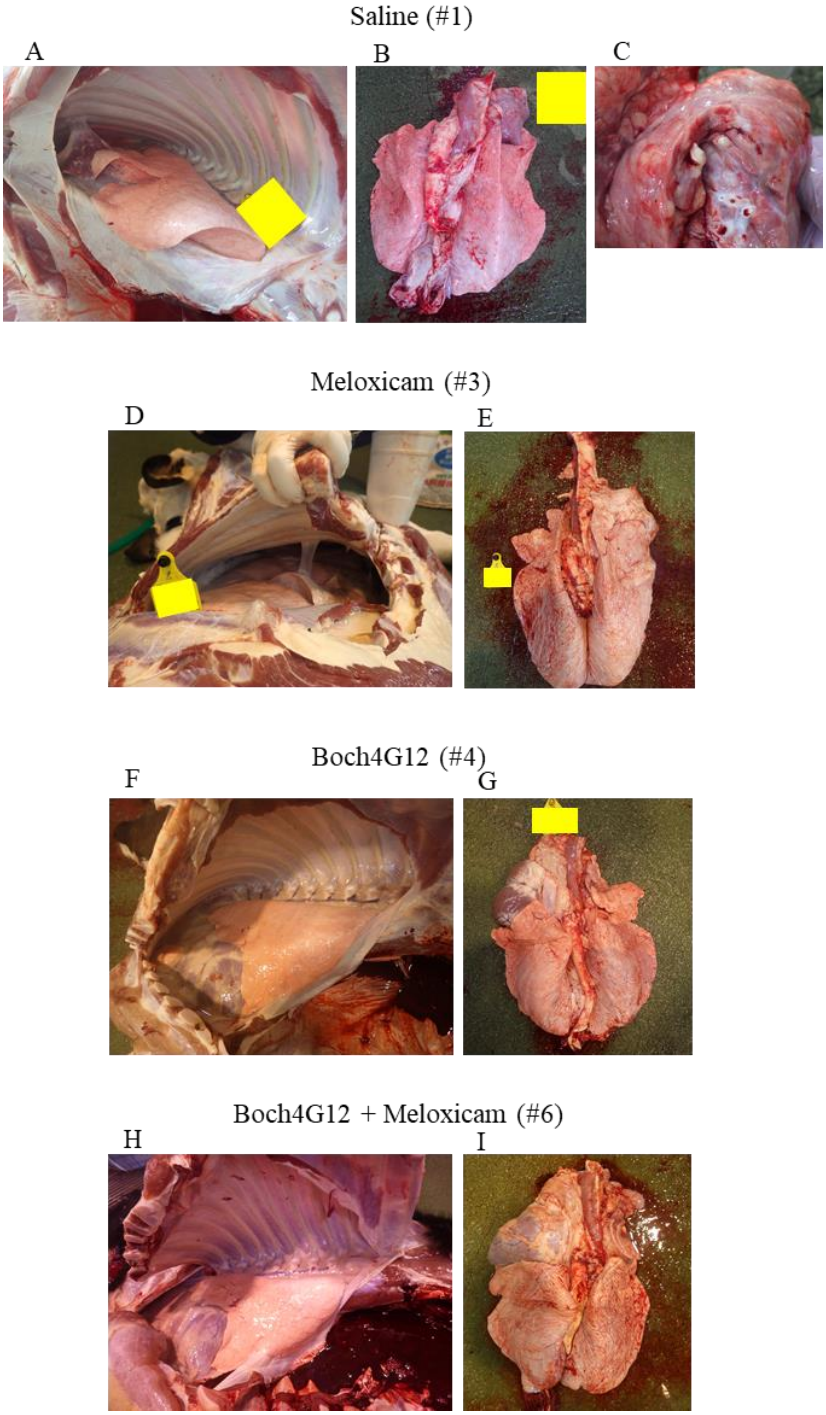

Figure III-6. Gross lung lesions of cattle treated with anti-PD-L1 chAb (Boch4G12) and meloxicam. *M. bovis*-infected cattle were intravenously administered 2 mg/kg of purified Boch4G12 (animals #4 and #6) or saline (negative control, animal #1). In addition, animal #3 and #6 was subcutaneously administered 0.5 mg/kg of meloxicam twice with a weekly interval. At 14 days after administration, autopsy was conducted (A, B and C: pictures of animal #1, D and E: pictures of animal #3, F and G: pictures of animal #4, H and I: pictures of animal #6). Yellow boxes in A, B, D, E and G hide the ear tags of each animal.

DISCUSSION

M. bovis infection, especially pneumonia, tends to progress chronically [Caswell *et al*., 2010]. The chronicity and the persistence of *M. bovis* infection imply that the immune response against the pathogen is insufficient. In Chapters I and II, it was found that immunoinhibitory molecules, PD-1/PD-L1, and PGE_2 caused the immunosuppression during *M. bovis* infection and these molecules had the potential as targets for control of *M. bovis* infection by *in vitro* blockade of the PD-1/PD-L1 pathway and PGE_2. In this chapter, to confirm the effect of the combined treatment of anti-PD-L1 chAb with a COX-2 inhibitor on *M. bovis* infection *in vivo*, the clinical study was performed using *M. bovis*-infected cattle with pneumonia.

Firstly, to evaluate the effect of the blockade of the PD-1/PD-L1 pathway and PGE_2 *in vivo,* IFN-γ response against *M. bovis* was analyzed. Although IFN-γ response was not activated in negative controls and cattle treated with meloxicam (animals #1–#3; Fig. III-4), cattle treated with Boch4G12 or both Boch4G12 and meloxicam (animals #4, #6 and #7) showed the reactivation of the IFN-γ response against *M. bovis* antigen (Fig. III-4). This result suggests that the treatment of Boch4G12 and meloxicam enhances *M. bovis*-specific immune response *in vivo*. However, IFN-γ response against *M. bovis* in animal #5 was not reactivated by Boch4G12 alone. The reason for this observation is unclear but this might be due to the high level of serum PGE_2 (Table III-1). Several reports indicate that the treatment with anti-PD-1/PD-L1 Abs reactivates T cell response and increases the production of Th1 cytokine, such as TNF-α [Dulos *et al.*, 2012]. TNF-α is one of the inducers for PGE_2 production by the activation of COX-2 via NF-κB [Lin *et al*., 2004; Nakao *et al*., 2002]. Thus, the low IFN-γ response observed in animal #5 might be due to the increased-PGE_2 production resulted from the enhancement of immune activity by Boch4G12. Indeed, serum PGE_2 concentration of animal #5 was consistently higher compared to those of other tested cattle (Fig. III-3). Further studies on the relationship between Th1 cytokine and PGE_2 production might help to understand the unresponsiveness of immune reaction by the treatment with anti-PD-L1 antibody.

Bacterial loads in nasal discharge and BALF among cattle treated with Boch4G12 with or without meloxicam were decreased (animals #4 and #6; Figs. III-5C–F). In contagious bovine pleuropneumonia caused by MmmSC, a positive correlation was found between the number of MmmSC-specific IFN-γ-secreting $CD4^+$ T cells and disease recovery [Dedieu *et al*., 2005; Dedieu *et al*., 2006]. IFN-γ promotes the activation and

proliferation of T cells and macrophages, which play vital roles in host defenses in the lungs. Previous studies showed that macrophage dysfunction is induced by *Mycoplasma* spp., including *M. bovis*, and promotes persistent infection [Caswell and Archambault, 2007; Deeney *et al*., 2019; Lai *et al*., 2010; Shaw *et al*., 2012;]. Therefore, macrophages activated by IFN-γ might have been involved in the decreased bacterial loads in nasal discharge and BALF.

The pathogenesis of severe pneumonia during *M. bovis* infection is still unclear. Recently, several studies reported that type 17 helper T cell (Th17), which mainly produced proinflammatory cytokine IL-17A, worsens the disease by making anmal susceptible to mycoplasma infection including *M. bovis* [Chao *et al*., 2019; Dulos *et al*., 2012], *M. pneumoniae* which causes human pneumonia [Wang *et al*., 2016] and *M. pulmonis* which causes mice pneumonia [Mize *et al*., 2018]. IL-17A activates tissue-damaging inflammatory cascades [Li *et al*., 2009]. Several studies demonstrated that PGE_2 promoted the differentiation and function of human and murine Th17 via EP2 and EP4 signaling [Boniface *et al*., 2009; Chizzolini *et al*., 2008; Yao *et al*., 2009]. In addition, a previous study reported a relationship between the PD-1/PD-L1 pathway and Th17 cell kinetics and function. In patients of pulmonary fibrosis, the number of PD-1^+ Th17 was increased in peripheral blood. Furthermore, the blockade of the PD-1/PD-L1 pathway in mouse model of fibrosis resulted in reductions of IL-17A expression from $CD4^+$ T cells and in improved fibrosis symptoms [Celada *et al*., 2018]. The present study found that the lung lesions of cattle treated with Boch4G12 and meloxicam appeared to improve when compared to that of untreated cattle although the clinical symptoms of the tested animals, such as cough and nasal discharges, were not improved (Fig. III-6). Taken together, it is hypothesized that the improvement of lung lesion as shown in this study might be due to the suppression of Th17 by the treatment with Boch4G12 and meloxicam. Further investigation on Th17 is required to examine this hypothesis and it might support to understand the mechanisms of action of the combination of PD-1/PD-L1 blockade with COX-2 inhibition.

In conclusion, dual blockade of the PD-1/PD-L1 pathway and PGE_2 *in vivo* significantly restored *M. bovis*-specific IFN-γ response. In addition, bacterial loads in nasal discharge and BALF among treated cattle were decreased. Thus, this study indicates that the combination therapy of anti-bovine PD-L1 chAb with a COX-2 inhibitor has a potential to become a novel immunotherapy for the control of *M. bovis* infection. To confirm the efficacy of this novel treatment for clinical application, additional

experiments using a large number of infected cattle are required.

SUMMARY

In Chapters I and II, the PD-1/PD-L1 pathway and PGE_2 were shown to be involved in the immunosuppression during *M. bovis* infection. In this chapter, the effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 in *M. bovis* infection *in vivo* was investigated using seven calves naturally infected by *M. bovis*. Calves were treated with anti-bovine PD-L1 rat-bovine chimeric antibody alone, a COX-2 inhibitor alone, or both. *M. bovis*-specific IFN-γ response in cattle treated with anti-bovine PD-L1 antibody and a COX-2 inhibitor was significantly increased. On the other hand, IFN-γ response was not activated in the negative controls and cattle treated with a COX-2 inhibitor alone throughout the experimental period. These results suggest that the combination of anti-PD-L1 antibody with a COX-2 inhibitor is a candidate for therapeutic applications to treat *M. bovis* infection.

CONCLUSION

Bovine mycoplasmosis is a disease of cattle characterized by chronic pneumonia, therapy-resistant mastitis, otitis and arthritis. Many mycoplasmas such as *Mycoplasma bovis*, *M. mycoides* subsp. *mycoides* biotype Small Colony, *M. bovigenitalium*, *M. californicum*, are known to be pathogenic to cattle. Recently, *M. bovis* is recognized as one of the most important *Mycoplasma* species associated with disease in cattle. So far, *M. bovis* is widely spread in the world and gets negative impacts on farm management. However, there is no effective vaccine to control *M. bovis* and antibiotics for the treatment are limited due to the emergence and spread of resistant strains. Thus, the elucidation of the mechanisms underlying the disease progression and the development of the novel strategy to control *M. bovis* infection are required. Thus, in the present study, to elucidate the mechanisms of immunosuppression during *M. bovis* infection, the immunoinhibitory molecules, programmed death 1 (PD-1) and PD-ligand 1 (PD-L1) which induce T cell exhaustion and are associated with disease progression of several bovine chronic infections, and prostaglandin E_2 (PGE_2), which is also an immunosuppressive factor in bovine chronic infections, were investigated. In addition, clinical tests targeting both the PD-1/PD-L1 pathway and PGE_2 were performed in *M. bovis*-infected cattle.

CHAPTER I: The role of the PD-1/PD-L1 pathway in *M. bovis* infection were investigated. The proportions of PD-1^+CD4^+ and PD-1^+CD8^+ T cells as well as PD-$L1^+$CD14^+ cells were increased in peripheral blood of *M. bovis*-infected cattle. The increases in the proportions of PD-1^+CD4^+ and PD-1^+CD8^+ T cells were negatively correlated with IFN-γ production from peripheral blood mononuclear cells (PBMCs) during *M. bovis* infection. On the other hand, the blockade of the PD-1/PD-L1 pathway *in vitro* by anti-bovine PD-1 or anti-bovine PD-L1 antibody (Ab) significantly upregulated the IFN-γ production from *M. bovis*-specific cells. These results suggest that the PD-1/PD-L1 pathway could be involved in immune exhaustion of *M. bovis*-specific T cells.

CHAPTER II: The role of PGE_2 in the immunosuppression and the relationship between PGE_2 and the PD-1/PD-L1 pathway during *M. bovis* infection were investigated. The stimulation with *M. bovis in vitro* upregulated the expressions of PGE_2 and PD-L1 presumably via Toll-like receptor 2 in bovine PBMCs obtained from healthy cattle. PGE_2 levels of peripheral blood from *M. bovis*-infected cattle were significantly increased in line with increased proportions of PD-$L1^+$ monocytes. On the other hand, the dual

blockade, using anti-bovine PD-L1 Ab and a cyclooxygenase (COX)-2 inhibitor *in vitro*, significantly enhanced the *M. bovis*-specific IFN-γ response. These observations show that PGE_2 could be one of the inducers of PD-L1 expression and could be involved in the immunosuppression during *M. bovis* infection.

CHAPTER III: The effect of dual blockade of the PD-1/PD-L1 pathway and PGE_2 in *M. bovis* infection *in vivo* was investigated using anti-bovine PD-L1 rat-bovine chimeric antibody (chAb) and a COX-2 inhibitor in seven calves naturally infected with *M. bovis*. *M. bovis*-specific IFN-γ response in cattle treated with anti-PD-L1 chAb and a COX-2 inhibitor was significantly increased. On the other hand, IFN-γ response was not activated in the negative controls and cattle treated with a COX-2 inhibitor alone throughout the experimental period. In addition, bacterial loads in nasal discharge and BALF were decreased in cattle treated with alone and combination of anti-PD-L1 chAb with a COX-2 inhibitor. These results suggest that the combination of anti-PD-L1 antibody with a COX-2 inhibitor is a candidate for therapeutic applications to prevent *M. bovis* infection.

In conclusion, this study showed that the immunosuppression during *M. bovis* infection is caused by T cell exhaustion induced by the upregulation of PD-1/PD-L1. In addition, PGE_2 was shown to be one of the inducers of PD-L1 expression and involved in the immunosuppression observed in *M. bovis*-infected cattle. Furthermore, the combination of anti-PD-L1 Ab with a COX-2 inhibitor reactivates *M. bovis*-specific immune reaction effectively both *in vitro* and *in vivo*. Taken together, the combined treatment of therapeutic antibody specific for PD-1/PD-L1 with a COX-2 inhibitor could be an effective strategy for the control of *M. bovis* infection. To confirm the efficacy of these treatments, additional experiments using a large number of infected cattle are required.

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SUMMARY IN JAPANESE

和文要旨

牛マイコプラズマ感染症は、ウシの伝染性疾患であり、慢性肺炎や難治性乳房炎、中耳炎、関節炎が認められる。現在までに、ウシに病原性を示すマイコプラズマとして、*Mycoplasma bovis* や *M. mycoides* subsp. *mycoides* biotype Small Colony, *M. bovigenitalium*, *M. californicum* など多くが報告されている。その中でも特に *M. bovis* は近年、世界各地で最も高率に分離され、畜産業に甚大な経済的損失を与えている。*M. bovis* 感染牛は慢性に病態が進行し、難治性に至る。現在 *M. bovis* には対する有効なワクチンはなく、本疾患の病態解明と新たな治療・制御法の開発が望まれている。そこで本研究では、様々な牛の慢性感染症の病態形成への関与が疑われている免疫抑制因子、programmed death 1 (PD-1) およびそのリガンド PD-ligand 1 (PD-L1) に着目し、*M. bovis* 感染症の免疫抑制機序の解明と新規治療法の開発を試みた。

第 1 章：免疫抑制が認められる *M. bovis* 感染症において、PD-1/PD-L1 の発現解析およびそれらの分子の機能解析を行った。*M. bovis* 感染牛の末梢血中において、PD-1 発現 $CD4^+$ および $CD8^+$ T 細胞、ならびに PD-1 のリガンドである PD-L1 発現単球の割合が、非感染牛と比較して有意に増加していた。さらに PD-1 発現 T 細胞の増加とインターフェロン-γ (IFN-γ) 応答能の低下との間には負の相関が認められた。PD-1 あるいは PD-L1 に特異的な抗体を用いた PD-1/PD-L1 経路阻害試験では、感染牛由来末梢血単核球における *M. bovis* 特異的な IFN-γ 応答が増強された。これらの結果より、PD-1/PD-L1 経路が *M. bovis* 特異的 T 細胞の疲弊化に関与していることが示唆された。

第 2 章：近年、PD-1/PD-L1 に関連した免疫抑制因子としてプロスタグランジン E_2 (PGE_2) 注目されている。そこで、本症における PGE_2 による免疫抑制機序と PD-1/PD-L1 経路との関連性の解明を試みた。健康牛から分離したウシ末梢血単核球 (PBMC) を用いた *in vitro* における *M. bovis* 感染試験では、PGE_2 産生量の増加に伴って、単球における PD-L1 発現量の上昇が認められた。さらに、*M. bovis* 感染牛における末梢血中 PGE_2 濃度の上昇は PD-L1 発現単球の割合の増加と正の相関を示した。一方で、PGE_2 合成の律速酵素である cyclooxygenase (COX) -2 に対する阻害剤と抗 PD-L1 抗体を併用して、感染牛由来 PBMC における PD-1/PD-L1 経路と PGE_2 の阻害試験を行うと、*M. bovis* 特異的 IFN-γ 応答がより強力に再活性化された。以上より、PGE_2 は *M. bovis* 感染牛における PD-L1 発現誘導因子のひとつであり、本疾患における免疫抑制に関与することが示唆された。

第 3 章：肺炎症状を呈した *M. bovis* 野外感染牛を用いて、抗 PD-L1 ラット-ウシキメラ抗体 (Boch4G12) と COX-2 阻害剤 (Meloxicam) の生体投与試験を行い、*in vivo* における PD-1/PD-L1 経路と PGE_2 の阻害効果を検討した。Boch4G12 と Meloxicam 併用投与牛では、投

与前と比較して *M. bovis* 抗原に対する IFN-γ 応答が有意に上昇し、試験最終日まで高い値を維持していた。一方で、無治療牛あるいは Meloxicam 単剤投与牛では、IFN-γ 応答の継続的な上昇は認められなかった。以上の結果より、抗 PD-L1 抗体と COX-2 阻害剤の併用による治療が本疾患に応用できることが示唆された。

本研究により、*M. bovis* 感染症における免疫抑制には、PD-1/PD-L1 の発現上昇により誘導された T 細胞の疲弊化が関与していることが明らかになった。また、*M. bovis* によって誘導される PGE_2 が本疾患における PD-L1 の発現誘導因子のひとつであることを示した。さらに、抗 PD-L1 抗体と COX-2 阻害剤を併用することにより、*M. bovis* 特異的免疫応答が強く再活性化されることが明らかとなった。本研究は、免疫抑制因子に着目した分子標的薬が根本的な制御法のない *M. bovis* 感染症に対する新規制御法となる可能性を示している。今後は、本研究をもとにした新規制御法が *M. bovis* 感染症を含む牛マイコプラズマ感染症の制圧の一助となることが期待される。