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Clinical efficacy of the combined treatment of anti-PD-L1 rat-bovine chimeric antibody with a COX-2 inhibitor in calves infected with *Mycoplasma bovis*

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Abstract

***Mycoplasma bovis* (*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, weight loss in surviving calves. In our previous studies, the programmed death-1 (PD-1)/ PD-ligand 1 (PD-L1) pathway and prostaglandin E_2 (PGE_2) were shown to be involved in the immunosuppression during chronic infectious diseases in cattle. In this study, the efficacy 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, Boch4G12, and cyclooxygenase 2 (COX-2) inhibitor, meloxicam. The calves treated with Boch4G12 and meloxicam significantly enhanced *M. bovis*-specific IFN-γ response after the administration. On the other hand, IFN-γ response was not activated in the controls and cattle treated with meloxicam alone throughout the experimental period. Interestingly, bacterial loads in nasal discharge and bronchoalveolar lavage fluid among calves treated with Boch4G12 with or without meloxicam were significantly decreased. These results suggest that the combination of anti-PD-L1 antibody with a COX-2 inhibitor is a candidate for therapeutic applications in calves infected with *M. bovis*.**

Key Words: *Mycoplasma bovis*, Immunotherapy, PD-L1, PGE_2, COX-2 inhibitor,

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Introduction

Mycoplasma bovis (*M. bovis*) was first isolated in the United States in 1961 from a severe case of mastitis in a dairy herd experiencing an outbreak, affecting more than 30% of the animals[17]. *M. bovis* has emerged as an important cause of not only mastitis but also other disease manifestations include pneumonia, arthritis and tenosynovitis, otitis media in dairy calves and beef calves[5,14,15]. *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, weight loss in surviving calves[5,14,15]. Unfortunately, no effective vaccines have been developed against *M. bovis* infection. The treatment with antibiotics is potentially effective against *M. bovis*, but strains which resistant to antibiotics have recently been spreading[1,24,37]. Therefore, a novel alternative strategy for the control of *M. bovis* infection is required.

Recently, several reports have demonstrated that immunoinhibitory molecules are one of the mechanisms of immunosuppression and contribute to the disease progression during various human chronic infections and cancers[9,22,38,43]. Cell surface immunoinhibitory receptors, such as programmed death-1 (PD-1), are negative regulators of T cell activation. After binding 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[42]. This dysfunction of T cells is called T cell exhaustion. Thus, the PD-1/PD-L1 pathway is recognized as a common mechanism of immunosuppression in chronic infections and cancers. Interestingly, the blockade of the PD-1/PD-L1 pathway by specific antibodies against either the receptor or ligand restores the function of exhausted T cells[42]. Indeed, anit-PD-1/PD-L1 antibodies are recognized as candidates for the treatment of several human cancers[9,22, 38,43]. As examples, fully humanized mAbs targeting PD-1, BMS-936558 (known as Nivolumab), and PD-L1, BMS-936559, have shown striking response against melanoma and other cancers[3].

In the veterinary field, during bovine chronic infection, 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 several cells in line with disease progression[19,20,21,31,32,34,35]. Furthermore, prostaglandin E_2 (PGE_2), a product of cyclooxygenase (COX) 2, induces immune check point molecules including PD-L1 and contributes to the progression of chronic infectious diseases in cattle[34,35]. Previously, we established anti-bovine PD-1 and PD-L1 rat-bovine chimeric Abs (chAbs) and conducted clinical studies of these chAbs in cattle infected with BLV[30,33,35]. 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 anti-BLV-specific $CD4^+$ T cells[30,33,35]. Moreover, the chAb treatment significantly reduced BLV provirus loads, clearly demonstrating that this treatment induced antiviral activities [30,33,35]. More interestingly, combined treatment of chAb with a COX-2 inhibitor substantially reduced the BLV provirus loads more effectively in cattle infected with BLV[35]. Therefore, the blockade of the PD-1/PD-L1 pathway and/or PGE_2 can be applied for the treatment of bovine chronic infection (Supplemental figure 1).

In *M. bovis* infection in cattle, immunosuppressive effects were observed, such as the inhibition of bovine peripheral blood mononuclear cell (PBMC) proliferation, induction of bovine lymphocyte apoptosis, and delay of bovine monocyte apoptosis, along with the suppression of IFN-γ and TNF-α productions[28,39,40]. However, the mechanisms involved in immunosuppression had not been fully elucidated. In a previous report, we had indicated that the immunosuppression was associated with the

Table I. The information of calves used in clinical studies.

Cattle	#1	#2	#3	#4	#5	#6	#7
Age	2 months old	7 months old	6 months old				
Species	Holstein	Holstein	Holstein	Holstein	Holstein	Holstein	Holstein
Sex	Male	Male	Male	Male	Male	Male	Male
Weight (kg)	85	252	213				
Respiratory symptom	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge	Cough and watery discharge
Serum PGE2 concentration at day 0 (pg/mL)	242	2,057	1,226	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.*

PD-1/PD-L1 pathway during *M. bovis* infection[16]. More specifically, it was elucidated that T cell exhaustion caused by the PD-1/PD-L1 pathway suppressed IFN-γ production against *M. bovis* and *in vitro* treatment with anti-bovine PD1/PD-L1 mAbs restored decreased *M. bovis*-specific IFN-γ response during *M. bovis* infection[16]. However, the clinical efficacy of blockade of the PD-1/PD-L1 pathway and/or PGE_2 on *M. bovis*-infected calves remains to be elucidated. Thus, in this study, the efficacy of combined treatment of anti-bovine PD-L1 chAb with a COX-2 inhibitor was examined using *M. bovis*-infected calves. The findings in this study indicated that novel biomedicines targeting the PD-1/PD-L1 pathway and PGE_2 are potentially applicable for immune therapies for the control of *M. bovis* infection.

Materials and methods

Animals

Calves, which clinical tests were performed in, were seven calves naturally infected by *M. bovis*, positive for respiratory mycoplasmas (nasal swab culture) obtained from farms at Hokkaido, Japan. *M. bovis* infection was confirmed by polymerase chain reaction (PCR) using clinical samples as previously described[18]. Animals were launched for this experiment and kept separately at the isolated rooms in a biosafety level I animal facility at the Animal Research Center, Agricultural Research Department, Hokkaido Research Organization (Shintoku, Hokkaido, Japan). The details of used cattle are shown in Table 1.

Administration of anti-bovine PD-L1 chimeric antibody (chAb) and a COX-2 inhibitor to M. bovis-infected calves

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)[30] was administered intravenously in four *M. bovis*-infected calves with pneumonia (animals #4-#7), and 0.5 mg/kg of meloxicam (Boehringer Ingelheim, Ingelheim, Germany) 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 with 100 mL of saline (Fuso Pharmaceutical Industries, Ltd. Osaka, Japan). After the treatments, peripheral blood samples, nasal discharge and bronchoalveolar lavage fluid (BALF) were then collected from these calves according to the schedule of sample collection as

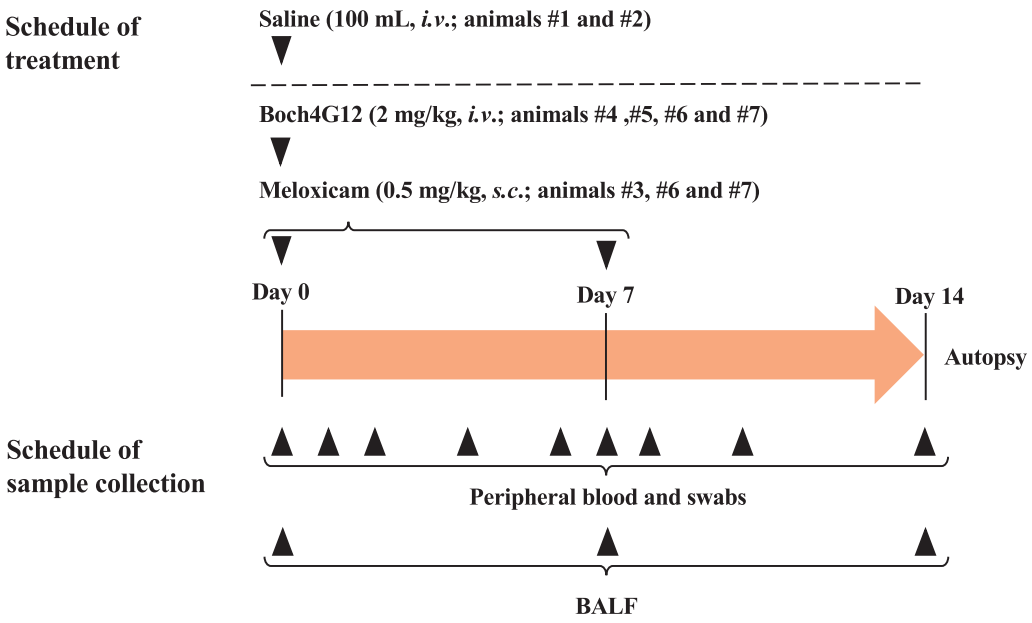

Fig.1
Schedule of treatment and sample collection in clinical study. 2 mg/kg of anti-PD-L1 chAb (Boch4G12) was administered intravenously in four *M. bovis*-infected calves with pneumonia (animal No #4-#7) and 0.5 mg/kg of meloxicam was co-administered by subcutaneous injection twice at a weekly interval in animals (animal No #3, #6 and #7). As controls, animals (animal No #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.

shown in Fig. 1. At 14 days after the treatments the inoculation of Boch4G12, animals #1, #4 and #6 were euthanized and subjected to autopsy.

Kinetic analysis of serum PGE_2 by ELISA

To confirm the kinetics of PGE_2 in treated calves, the serum PGE_2 levels were measured using the Prostaglandin E_2 Express ELISA kit (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The optical density was measured at 450 nm in a microplate reader (Corona Electronics, Tokyo, Japan).

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

To investigate the efficacy of the PD-1/PD-L1 blockade on *M. bovis*-specific immune response, peripheral blood samples were collected and PBMCs were purified by density gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, England, UK). The IFN-γ response against the heat-killed *M. bovis* antigen was investigated as previously described[16]. Briefly, to examine the IFN-γ production in treated calves, PBMCs were incubated with heat-killed *M. bovis* antigen (1.5 ng/mL) in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO, USA) containing 10% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA) 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 triplicate samples.

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

To investigate the efficacy of the PD-1/PD-

L1 blockade against *M. bovis* infection, bacterial load was measured in nasal discharge and bronchoalveolar lavage fluid (BALF) from treated calves using real-time PCR. Nasal discharges were collected by using sterilized P200 tips (Douo Rika Co., Sapporo, Japan) and 1.5-mL micro tubes (Fukaekasei, Tokyo, Japan). With regard to the collection of BALF from each animal, 100 mL of saline (Fuso Pharmaceutical Industries, Ltd.) 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, St. Louis, MO) 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).

Statistics

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.

Ethics statement

The animal experiments 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).

Results

Serum PGE_2 concentration in the treated calves

To investigate the efficacy of the treatment with Boch4G12 and/or meloxicam on the PGE_2 levels in peripheral blood from treated calves, serum PGE_2 concentration was measured by ELISA. The controls (animal No. #1 and #2) and one of two calves treated with Boch4G12 and meloxicam (animal No. #6) did not change serum PGE_2 concentration throughout the experimental period (Fig. 2). On the other hand, serum PGE_2 concentration in calves treated with meloxicam alone (animal No. #3), Boch4G12 alone (animal No. #4) and both Boch4G12 and meloxicam (animal No. #7) significantly decreased after the administration. Serum PGE_2 concentration in animal No. #5 calf treated with Boch4G12 alone significantly increased after the administration.

Clinical efficacy of M. bovis specific-IFN-γ response in the treated calves

We previously reported that meloxicam activates Th1 responses in the treated cattle[35]. To examine whether meloxicam activates the *M. bovis*-specific Th1 responses, PBMCs from meloxicam-treated calf (animal No. #3) was cultured in the presence of heat-killed *M. bovis*, and IFN-γ production was measured by ELISA (Fig. 3). However, unexpectedly, IFN-γ production was not enhanced in the meloxicam-treated calf (animal No. #3) as well as those of control cattle (animal No. #1 and #2). On the other hand, we also investigated the efficacy of the treatment with Boch4G12 alone/and meloxicam on the IFN-γ production because we previously reported that the blockade of the PD-1/PD-L1 pathway activates IFN-γ production *in vitro* from PBMCs of *M. bovis*-infected cattle[16]. Although the enhancement of IFN-γ production was not observed in one of two calves treated with Boch4G12 alone, IFN-γ production against *M. bovis* antigen was significantly higher than before administration in calves treatment with Boch4G12 alone (animal No. #4)/and meloxicam (animal No. #6 and #7) (Fig. 3). Interestingly, IFN-γ production against *M. bovis* antigen was strongly enhanced and maintained in calves treated with combinations of Boch4G12 and meloxicam (animal No. #6 and #7) (Fig. 3)

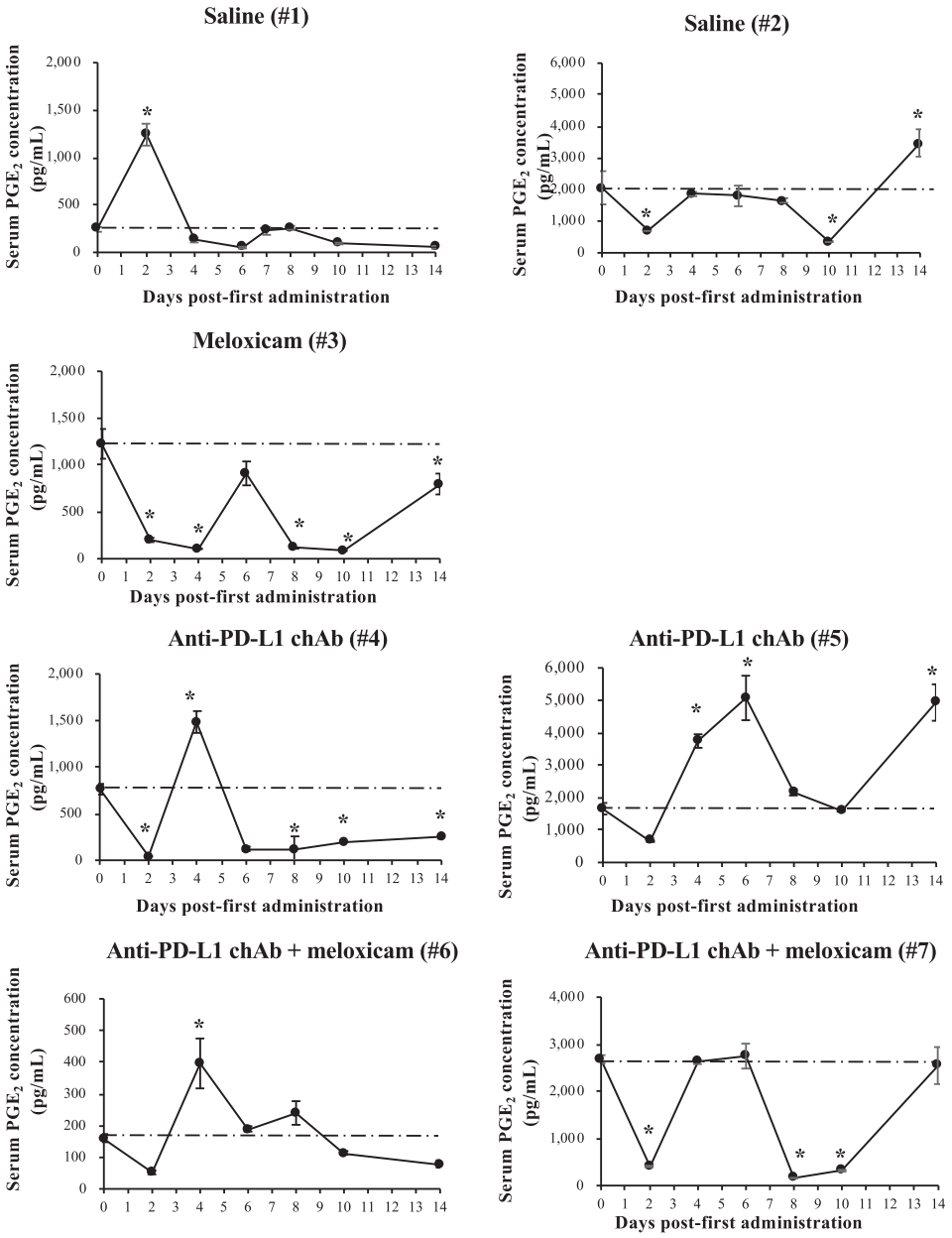

Fig.2
Kinetic analysis of serum PGE_2 in *M. bovis*-infected calves treated with anti-PD-L1 chAb (Boch4G12) and meloxicam. *M. bovis*-infected calves were intravenously administered 2 mg/kg of purified Boch4G12 (animal No #4-#7) or saline (control, animal No #1 and #2). In addition, animals (animal No #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).

Clinical efficacy of bacterial loads in the treated calves

To investigate the efficacy 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 No. #1) (Fig. 4), but those from treated cattle were significantly

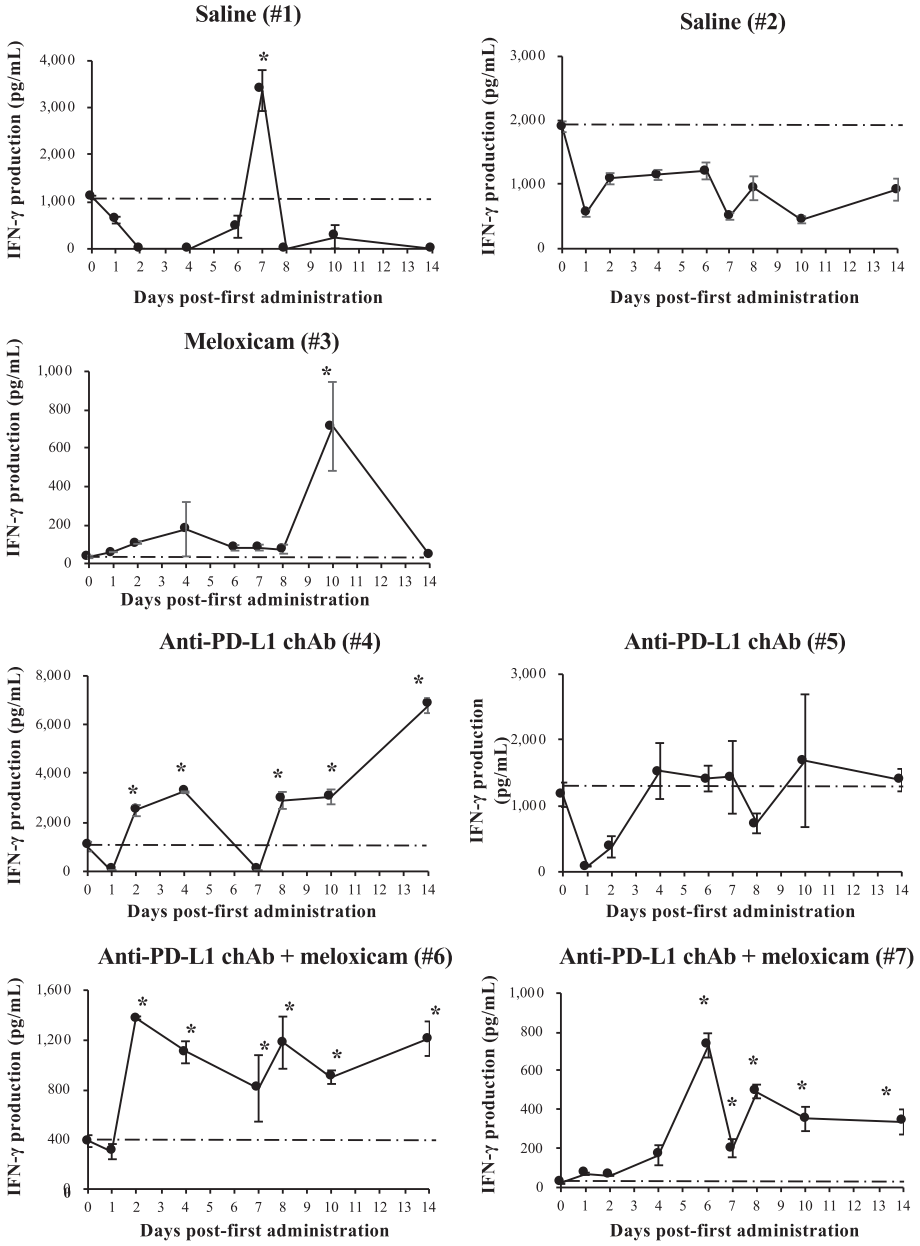

Fig.3
Activation of IFN-γ responses in *M. bovis*-infected calves treated with Boch4G12 and meloxicam. *M. bovis*-infected calves were intravenously administered 2 mg/kg of purified Boch4G12 (animal No. #4-#7) or saline (control, animal No. #1 and #2). In addition, animals (animal No.#3, #6 and #7) were 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).

decreased after the treatment of Boch4G12 with or without meloxicam (animal No #4 and #6) (Fig. 4). In other tested animals (animal No #2, #3, #5 and #7), the bacterial loads in nasal discharges and BALFs were not detectable by real-time PCR due to low bacterial loads (data not shown).

Gross lung lesions in the treated calves

To examines the pathologic findings of pneumonia in the treated calves, *M. bovis*-

detected calves (animal No #1, #4 and #6) were dissected at 14 days after administration. Saline-inoculated calf as control was observed sever findings of typical mycobacterial pneumonia such as pleural adhesion, multifocal white nodules and atelectasis in the cranioventral areas of the lungs[4] (Fig. 5). On the other hand, although mild atelectasis was partially observed, the sever findings including pleural adhesion and multifocal white nodules were not confirmed in the calves treatment with Boch4G12 alone (animal No. #4)/ and meloxicam (animal No. #6) (Fig. 5).

Discussion

Pneumonia is a typical symptom of *M. bovis* infection in calves, and tends to be chronic progression[4,5]. The chronicity and the persistence of *M. bovis* infection imply that the immune response is insufficient in eliminating the pathogen. In our previous studies, it was found that immunoinhibitory molecules, PD-1/PD-L1, and PGE_2 caused the immunosuppression during several bovine chronic infectious diseases including BLV infection, Johne's disease and bovine and these molecules had the potential as targets for control of these infection[34, 35]. During *M. bovis* infection, T cell exhaustion caused by upregulation of PD-1/PD-L1 expression on immune cells and blockade of PD-1/PD-L1 pathway using anti-bovine PD-1 or PD-L1 mAb restored *M. bovis*-specific immunoresponse *in vitro*[16]. In this study, to examine the efficacy of the treatment of anti-PD-L1 chAb with or without 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 efficacy 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 controls and cattle treated with meloxicam, cattle treated with Boch4G12 or both Boch4G12 and meloxicam showed the reactivation of the IFN-γ response against *M. bovis* antigen (Fig. 3). 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 (animal No. #5) was not reactivated by anti-PD-L1 Ab alone. The reason for this observation is unclear but this might be due to the high level of serum PGE_2 (Table 1). Several reports indicate that the treatment with anti-PD-1/PD-L1 Abs reactivates T cells, and that the production of Th1 cytokine such as TNF-α[13]. TNF-α is one of the inducers for PGE_2 production by the activation of COX-2 via NFκB[26,29]. Thus, the low IFN-γ response observed in animal No. #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 No. #5 was consistently higher compared to those of other tested calves (Fig. 2). Interestingly, *M. bovis*-specific IFN-γ response of animal No. #7, which decreased serum PGE_2 concentration after the meloxicam administration, was significantly enhanced throughout the experimental period. These findings might suggest an association between IFN-γ response correlated with PGE2 concentration. 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 Ab.

Bacterial loads in nasal discharge and BALF among calves treated with Boch4G12 with or without meloxicam were decreased (Fig. 3). The calves decreased bacterial loads clearly indicated IFN-γ production after the treatments as described above. In contagious bovine pleuropneumonia caused by *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC), a positive correlation was found between the number of MmmSC-specific IFN-γ-secreting $CD4^+$ T cells and disease recovery[10,11]. 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

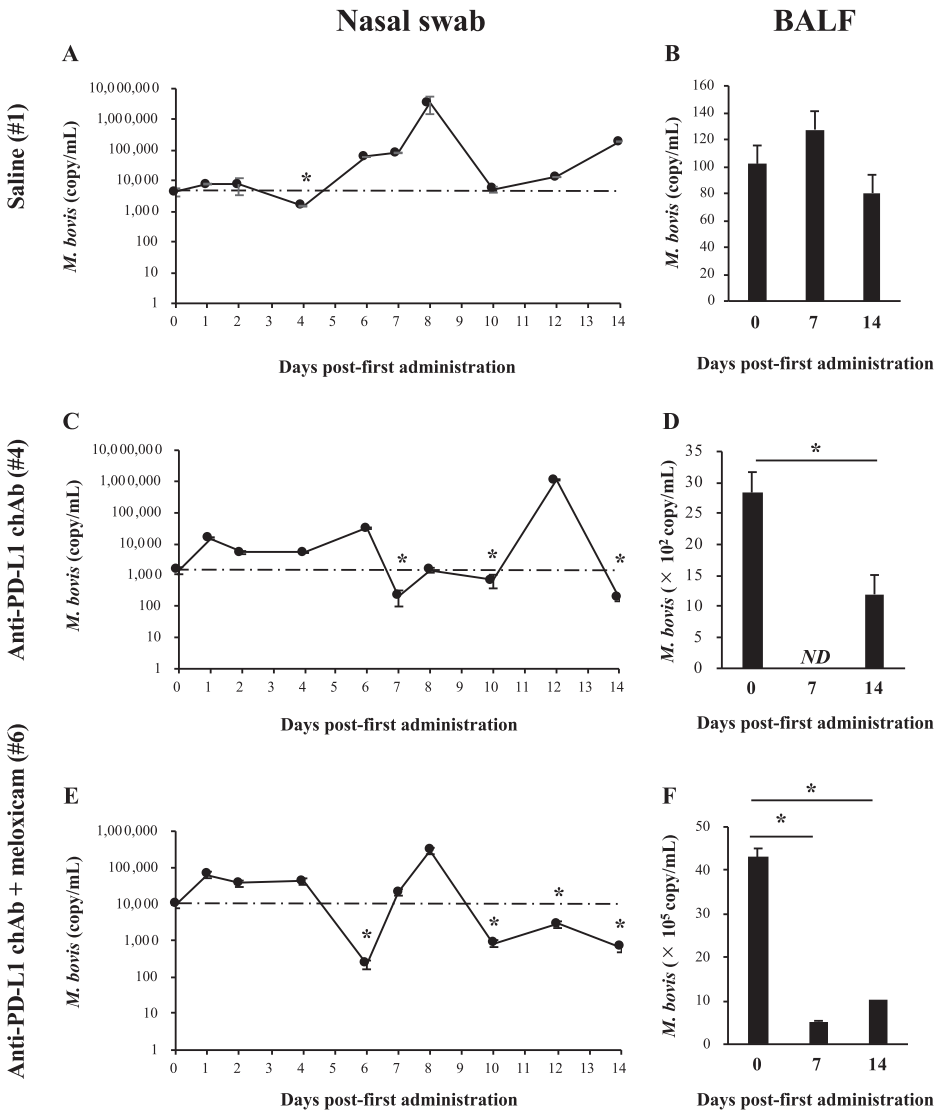

Fig.4
Bacterial load in nasal discharge and BALF from *M. bovis*-infected calves treated with Boch4G12 and meloxicam. *M. bovis*-infected calves were intravenously administered 2 mg/kg of purified Boch4G12 (animal No. #4 and #6) or saline (control, animal No. #1). In addition, animal (animal No. #6) 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 No. #1, C and D: data of animal No. #4, E and F: data of animal No. #6). Statistical significance was determined by Dunnett's test (A-F). *$P < 0.05$ (vs. day 0 for each result). ND; not detected.

by *Mycoplasma* spp., including *M. bovis*, and promotes persistent infection[12,23,36]. 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 animal susceptible to mycoplasma infection including *M. bovis*[7,13], *M. pneumoniae* which causes human pneumonia[41] and *M. pulmonis* which causes mice pneumonia[27]. IL-17A activates

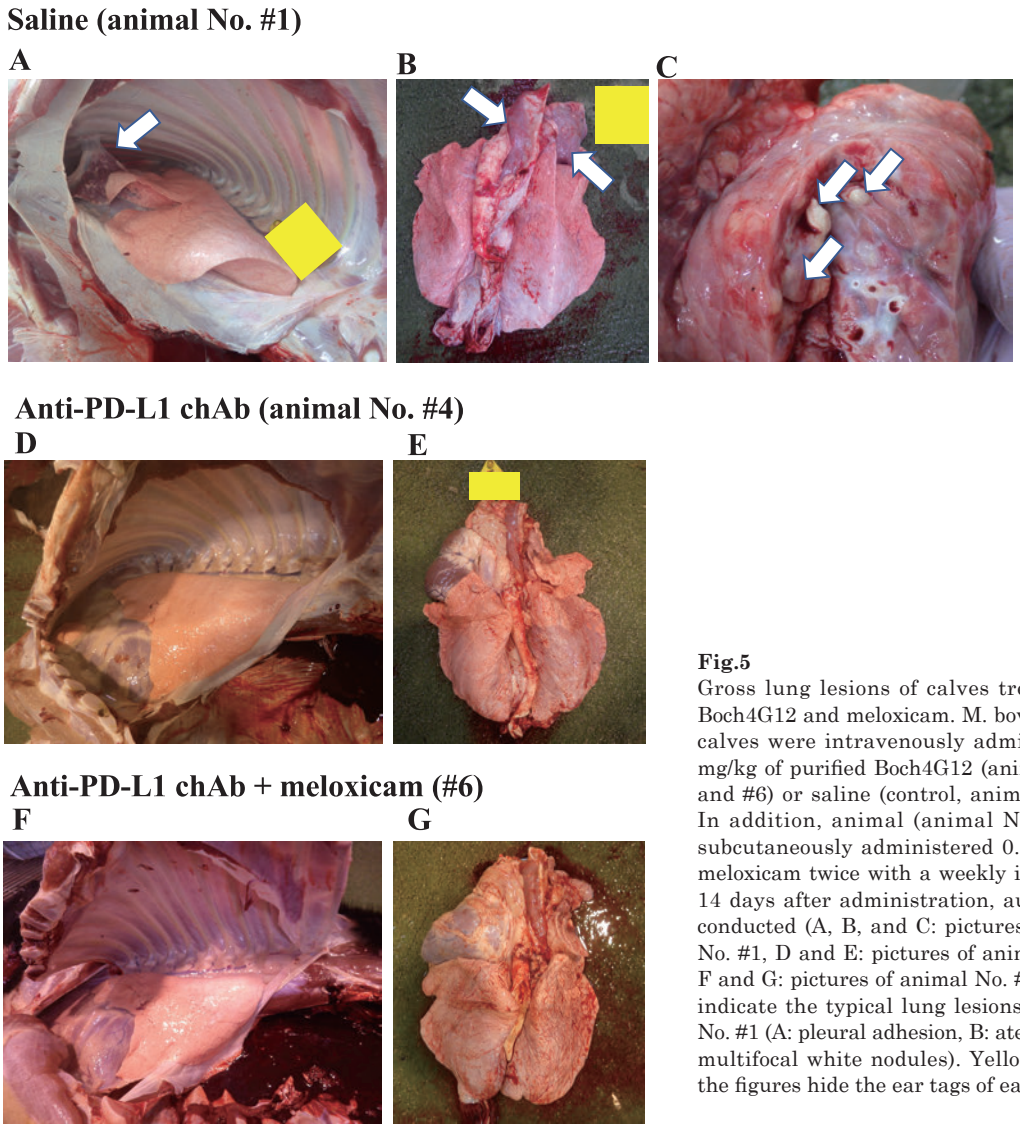

Fig.5
Gross lung lesions of calves treated with Boch4G12 and meloxicam. M. bovis-infected calves were intravenously administered 2 mg/kg of purified Boch4G12 (animal No. #4 and #6) or saline (control, animal No. #1). In addition, animal (animal No. #6) was subcutaneously administered 0.5 mg/kg of meloxicam twice with a weekly interval. At 14 days after administration, autopsy was conducted (A, B, and C: pictures of animal No. #1, D and E: pictures of animal No. #4, F and G: pictures of animal No. #6). Arrows indicate the typical lung lesions in animal No. #1 (A: pleural adhesion, B: atelectasis, C: multifocal white nodules). Yellow boxes in the figures hide the ear tags of each animal.

tissue-damaging inflammatory cascades[25]. Several studies demonstrated that PGE_2 promotes the differentiation and function of human and murine Th17 via EP2 and EP4 signaling[2,8,44]. In addition, a previous study reported a relationship between the PD-1/PD-L1 pathway and Th17. 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[6]. The present study found that the moderate lung lesion was observed in the treatment animals treated with Boch4G12 and meloxicam when compared to that of untreated cattle (Fig. 5). Taken together, it is hypothesized that the efficacy might be due to the suppression of Th17 by the treatment with Boch4G12 and meloxicam. Further investigation on Th17 is required to examin 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 calves were decreased. Thus, this study indicates that the combination therapy of anti-bovine PD-L1 chAb with a COX-2 inhibitor has a potential for the control of *M. bovis* infection. This pilot study could help developing potential novel methods for the control of *M. bovis* infection. Further experiments using a large number of infected cattle are required to confirm the efficacy of this novel treatment approach for clinical application.

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Supplemental data

Supplemental data associated with this article can be found, in the online version, at http://dx.doi.org/10.14943/jjvr.68.2.77

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