The enhancement of Th1 immune response by anti-PD-L1 antibody in cattle infected with *Mycobacterium avium* subsp. *paratuberculosis*

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**ABSTRACT.** Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic enteritis of ruminants. Previous studies have shown that programmed death-ligand 1 (PD-L1) is associated with the disease progression, and PD-L1 blockade activates MAP-specific Th1 responses *in vitro*. Here, we performed anti-PD-L1 antibody administration using 2 MAP-infected cattle at the late subclinical stage of infection. After administration, bacterial shedding was reduced or maintained at a low level. Additionally, MAP-specific Th1 cytokine production was upregulated, and CD69 expression was increased in T cells. Collectively, the treatment has a potential as a novel control method against Johne's disease.

**KEY WORDS:** cattle, immunotherapy, Johne's disease, programmed death-ligand 1, Th1 response

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Johne’s disease (paratuberculosis) is a chronic enteritis of ruminants, and is caused by the bacteria *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The clinical signs of Johne’s disease include chronic diarrhea, severe weight loss, reduced milk production, and mortality [17]. Johne’s disease is endemic in many countries, including Japan [13]. In an early stage of infection, MAP induces strong Th1 responses that cause the activation of macrophages to kill intracellular mycobacteria [4, 21, 22]. Th1 cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, are important for enhancing bactericidal activity of macrophages with the production of reactive oxygen and nitrogen species, T cell activation, and dendritic cell maturation [8, 12]. During the late subclinical stage, the Th1 response declines, which allows bacterial growth and progression to clinical disease [1, 2, 24]. Therefore, the Th1 response is essential for the prevention of the disease progression.

Programmed death (PD)-1 is one of the immunoinhibitory receptors expressed on T cells, and induces immunosuppression by binding to PD-lygand 1 (PD-L1) [11]. In chronic infections, the upregulation of PD-1 and PD-L1 expression is involved in the exhaustion of antigen-specific T cells which contributes to the disease progression [11, 25]. During human tuberculosis that is caused by *Mycobacterium tuberculosis*, the PD-1/PD-L1 pathway inhibits effector function of T cells [9], and PD-1 expression on *M. tuberculosis*-specific CD4+ T cells is associated with bacterial loads [5]. Previous studies have shown that the PD-1/PD-L1 pathway is involved in the suppression of Th1 responses in cattle infected with MAP [16]. The blockade of PD-L1 using a specific antibody (Ab) increases MAP-specific Th1 immune responses *in vitro* [18]. Thus, the PD-1/PD-L1 pathway is considered to have
a therapeutic potential for Johne’s disease. In addition, previous studies have demonstrated that anti-PD-L1 rat-bovine chimeric antibody (chAb) has therapeutic effects against other chronic bovine infections, such as bovine leukemia virus (BLV) infection and Mycoplasma bovis infection [7, 15, 19]. However, there is no report which evaluates the function of PD-L1 blockade in MAP-infected animals. Therefore, in this study, we performed the administration of anti-PD-L1 chAb using 2 MAP experimentally-infected cattle to examine the responses to the antibody administration by immunological and bacteriological analyses.

For the experimental infection of MAP, 2 male Holstein calves, animals #80 (3 weeks of age) and #99 (a week of age), were orally inoculated with intestinal tissue homogenate from an infected cow containing MAP (#80: 1.36 × 10⁸ CFU; #99: 2.50 × 10⁸ CFU) which was measured by using Middlebrook 7H10 agar-based slants as described in a previous paper [10]. Both animals were sourced from farms with no history of paratuberculosis and confirmed negative by fecal quantitative polymerase chain reaction (qPCR) targeting MAP-specific gene IS999 as described previously [10] and by Pouquier ELISA (Institut Pouquier, Montpellier, France) prior to inoculation with MAP. Animals #80 (770 kg, 212 weeks post-infection) and #99 (320 kg, 47 weeks post-infection) were intravenously administered with 2 mg/kg of the purified anti-PD-L1 chAb (Boch4G12) [15] a time and three times at 2 week-intervals, respectively. Both animals were kept in a biosafety level 2 animal facility at the National Institute of Animal Health, Tsukuba, Japan. All experiments using these animals were approved by the National Institute of Animal Health Ethics Committee (approval No. 17-077-2 and 18-077). After the experimental infection, we collected blood and fecal samples at intervals of 2–4 weeks, and monitored IFN-γ production responded to Johnin purified protein derivative (J-PPD) by whole-blood reaction (qPCR) targeting MAP-specific gene IS999 at intervals of 2–4 weeks, respectively.

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In animal #80, J-PPD-specific IFN-γ response peaked during the acute phase of infection (Fig. 1A). This response was gradually suppressed from approximately 60 weeks post-inoculation, and was maintained at a low level until the time of administration (Fig. 1A). In contrast, the serum levels of Abs against MAP were increased from 160 weeks post-infection (Fig. 1B). The tentative shedding of MAP was observed in feces of animal #80 from 4 to 16 weeks post-infection (Fig. 1C). Fecal shedding was not detected between 18–78 weeks, and then the MAP DNA quantity gradually increased from 80 weeks post-infection (Fig. 1C). In animal #99, J-PPD-specific IFN-γ response peaked at 23 weeks after the experimental infection, and was maintained at a high level (Fig. 1A). The serum levels of Abs against MAP were increased and then turned positive at 46 weeks post-infection, a week before the administration (Fig. 1B). We observed bacterial shedding intermittently until the time of administration (Fig. 1C). Although both animals #80 and #99 showed the bacterial shedding, these animals did not show clinical symptoms such as diarrhea. The serum levels of Abs against MAP was increasing before anti-PD-L1 Ab administration. A previous report has described that detectable levels of Abs against MAP return in mid- to late-stage subclinical infections [3]. From these data, we concluded that animals #80 and #99 were both in the late subclinical stage at the time of administration.

To evaluate anti-bacterial effects of Boch4G12 in MAP-infected cattle, fecal shedding of MAP was monitored by qPCR for 60 weeks for animal #80 and 34 weeks for #99 after administration. The bacterial loads in feces from #80 and #99 were maintained at a low level after administration (Fig. 1C). Remarkably, in animal #99, MAP DNA was not detected in feces at 30 and 34 weeks after Boch4G12 administration (correspondence to 77 and 81 weeks-post infection, respectively) (Fig. 1C). A previous report has shown that persistent shedding patterns related on ELISA-positive samples rarely reverse to negativity [14]. In addition, cattle detected more than 1.0 × 10⁻² pg MAP DNA in feces are associated with progressing to severe disease [23]. These data suggest that anti-PD-L1 Ab treatment has a possibility to regulate the bacterial shedding in MAP-infected cattle.

We then examined the effects of Boch4G12 on Th1 responses in vitro. IFN-γ and TNF-α production from PBMCs was significantly increased in the presence of J-PPD and Con A (Fig. 2A and 2B). TNF-α was not detected from culture supernatants of groups stimulated with PBS and B-PPD. In addition, the expression levels of IFN-γ, TNF-α, and CD69 in T cells of #80 were significantly upregulated after Boch4G12 administration (Fig. 2C). CD69 upregulation in T cells was also observed in animal #99 (Fig. 2C). Collectively, these results demonstrated that treatment with anti-PD-L1 chAb activated Th1 responses in both animals, suggesting a therapeutic potential for Johne’s disease.

In this study, the effects of anti-PD-L1 Ab treatment on bacterial shedding were different between #80 and #99. Animal #80 was administered with anti-PD-L1 Ab a time, whereas animal #99 was administrated three times. Before anti-PD-L1 Ab administration, IFN-γ production responded to J-PPD in animal #80 had been suppressed for more than 100 weeks, whereas IFN-γ production in animal #99 had not been suppressed. These differences might be factors to determine the effect of anti-PD-L1 Ab treatment on fecal shedding patterns related on ELISA-positive samples rarely reverse to negativity [14]. In addition, cattle detected more than 1.0 × 10⁻² pg MAP DNA in feces are associated with progressing to severe disease [23]. These data suggest that anti-PD-L1 Ab treatment has a possibility to regulate the bacterial shedding in MAP-infected cattle.

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Th1 responses, especially IFN-γ production, are considered to be essential for the prevention of the disease progression. Our previous and present studies have shown that treatment with anti-PD-L1 Ab enhanced MAP-specific Th1 responses both in vitro and in vivo [18]. Therefore, the observed effect of anti-PD-L1 Ab on the bacterial shedding is presumably due to the activation of Th1 responses. Additional experiments are required to elucidate the underlying mechanism of the anti-bacterial effect of anti-PD-L1 Ab on MAP-infected cattle. Further, recent studies have described that the combined treatment of anti-PD-L1 Ab with other medicines has a potential to enhance therapeutic effects of PD-1/PD-L1 blockade in several chronic bovine infections [6, 7, 18–20].

A previous study on Johne’s disease revealed that the dual blockade of the PD-1/PD-L1 pathway and prostaglandin E2 production enhanced MAP-specific Th1 responses in vitro [18]. Hence, the combination with other medicines could be a strategy to enhance the anti-bacterial effect of anti-PD-L1 Ab in cattle infected with MAP.
In conclusion, we showed a potential of anti-PD-L1 Ab treatment for the regulation of bacterial shedding. Additionally, anti-PD-L1 Ab treatment also activated MAP-specific Th1 cytokine production in MAP-infected cattle. To our best knowledge, this is the first study which shows immune activating effects of the PD-L1 blockade in cattle infected with MAP. Although the number of experimentally-infected cattle used in this study was limited, the observations in the present study could play an important role to establish a novel therapeutic strategy against Johne’s disease.

CONFLICT OF INTEREST. The authors declare that they have no competing interests.

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