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

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Running head: PD-L1 BLOCKADE IN MAP-INFECTED CATTLE

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 after administration. 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.

Keywords: cattle, immunotherapy, Johne's disease, programmed death-ligand 1, Th1 response

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-ligand 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: 1.36×10^8 CFU; #99: 2.50×10^8 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 IS*900* as described previously [10] and by Pourquier ELISA (Institut Pourquier, 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 cultures as described previously [16], the serum levels of Abs against MAP by Pourquier ELISA, and fecal shedding of MAP by qPCR. To examine the effects of anti-PD-L1 Ab in MAP-infected cattle, blood samples were collected from animal #80 on the day of administration (day 0), and on several points after administration (days 1, 3, 8, 15, 29, 43, 57, and 85). Blood samples on day 0 were obtained before administration. Blood samples were collected from animal #99 on days 0, 7, 14, 21, 28, 42, 56, 70, 84, 98, and 112. Blood samples on days 0, 14, and 28 were obtained before administration. Peripheral blood mononuclear cells (PBMCs) were purified from blood samples using density gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, UK), and cultured with 2 μg/ml of J-PPD or 20 μg/ml of concanavalin A (Con A; Sigma-Aldrich, St. Louis, MO, USA). Phosphate buffered saline (PBS) and PPD from *Mycobacterium bovis* BCG strain (B-PPD) were used as a negative control and a control antigen, respectively. After 6 days, collected culture supernatants were assayed for IFN-γ and TNF-α by Bovine IFN-γ ELISA Development Kit (Mabtech, Nacka Strand, Sweden) and Bovine TNF alpha Do-It Yourself ELISA (Kingfisher Biotech, St. Paul, MN, USA), respectively. Additionally, the isolation of T cells ($CD3^+$ cells) from PBMCs was performed according to a previous report [15], and the purity of $CD3^+$ cells (> 95%) was confirmed using FACS Verse (BD Biosciences, San Jose, CA, USA). To quantitate gene expression levels of *IFN-γ*, *TNF-α*, and *CD69*, an activation marker of

lymphocytes, qPCR was conducted as described previously [18]. Primers for *IFN-γ*, *TNF-α*, and *ACTB* were shown in a previous report [18]. Primers for *CD69* were 5'-ATA GCT CTC GTT GCT CTA TCA GTG-3' and 5'-CCT TGT GTC CAA TCC AAT CA-3'.

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 B). 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 administrated 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. Further clinical studies using a number of MAP-infected animals are required to examine the influence of these factors, such as the experimental conditions (administration time, interval, and dosage) and examined animals (disease stage, response to J-PPD, and age), on the clinical efficacy of anti-PD-L1 Ab.

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 E_2 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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FIGURE LEGENDS

Figure 1

Interferon (IFN)-γ responses in blood, antibody (Ab) levels in sera, and fecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). (A) Whole-blood cells were cultured with Johnin purified protein derivative for 24 hours. IFN-γ concentrations in culture supernatants were determined by ELISA. (B) The serum levels of Abs against MAP were examined by ELISA. Results were indicated as percentage S/P, and the dotted lines indicated cutoff as recommended by the manufacturer. (C) MAP DNA quantity in feces detected by qPCR. (A–C) Arrowheads show the points of anti-programmed death-ligand 1 Ab administration.

Figure 2

Activation of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) specific Th1 responses by anti- programmed death-ligand 1 chimeric antibody. (A and B) PBMCs were cultured with Phosphate buffered saline (PBS), purified protein derivative from *Mycobacterium bovis* BCG strain (B-PPD), Johnin-PPD (J-PPD), or concanavalin A (Con A) for 6 days. Interferon (IFN)-γ (A) and tumor necrosis factor (TNF)-α (B)

concentrations in culture supernatants were determined by ELISA. (A and B) The symbols represent the means of three independent culture wells, and white symbols show $p < 0.05$ compared with the value on day 0. (C) Gene expression levels of *IFN-γ*, *TNF-α*, and *CD69* in $CD3^+$ cells were quantitated by qPCR. The symbols represent the means of three independent experiments, and white symbols show $p < 0.05$ compared with the value on day 0. (A–C) Statistical significance was determined by the Dunnett's test.

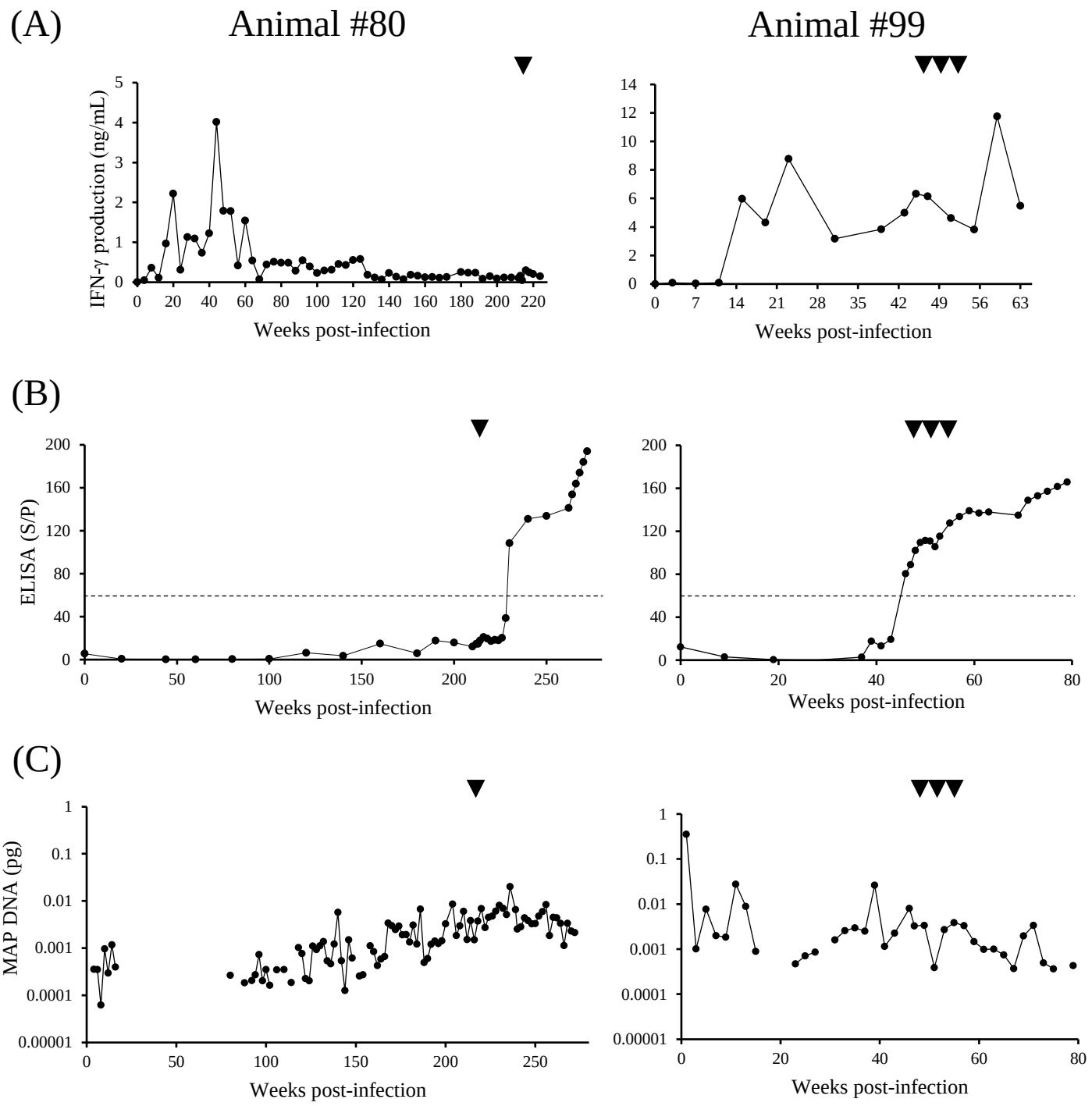
(A)
Animal #80
Animal #99
IFN-γ production (ng/mL)
Weeks post-infection
(B)
ELISA (S/P)
Weeks post-infection
(C)
MAP DNA (pg)
Weeks post-infection

Fig. 1

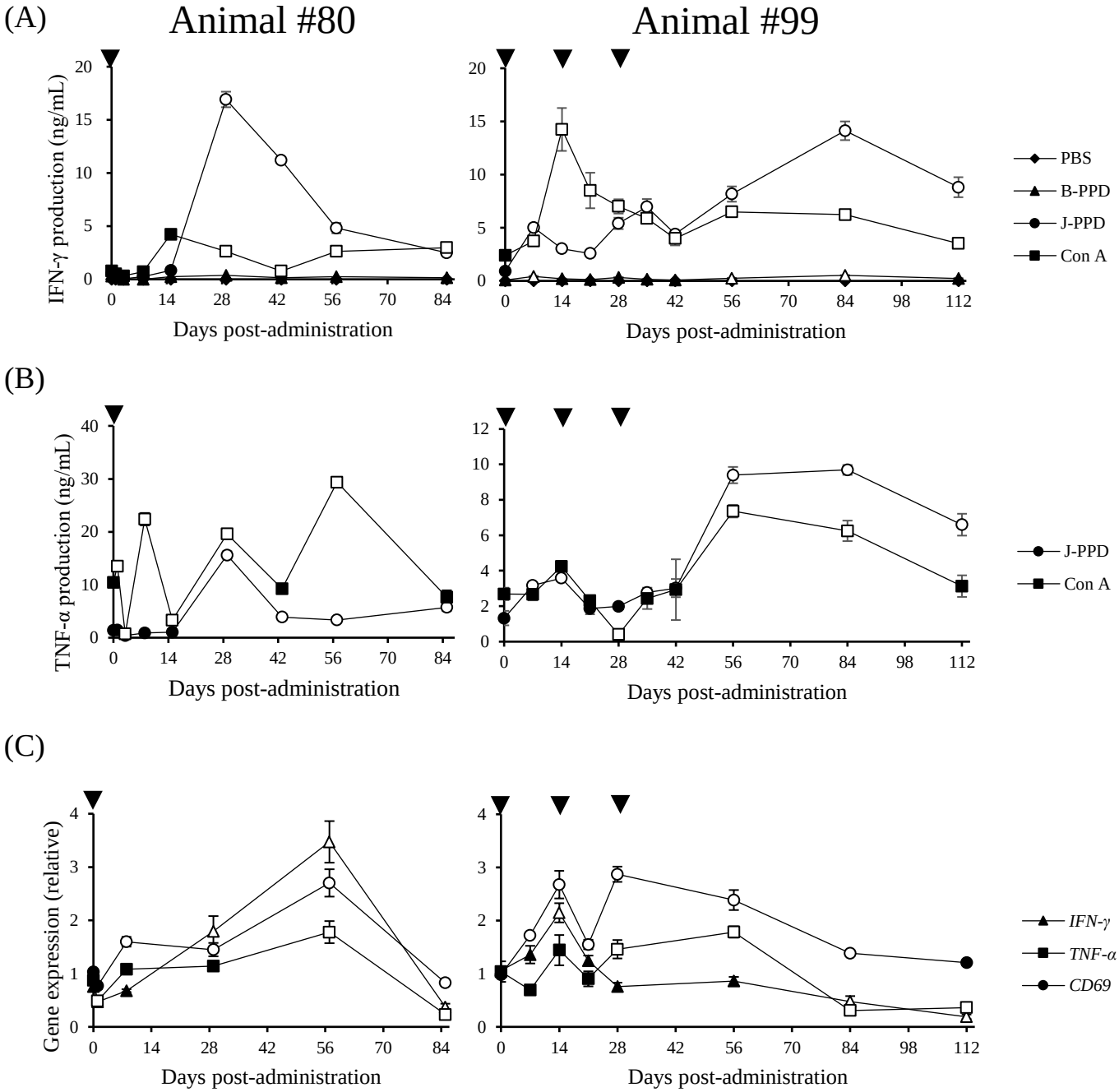
(A)
Animal #80
Animal #99
IFN-γ production (ng/mL)
Days post-administration
PBS
B-PPD
J-PPD
Con A
(B)
TNF-α production (ng/mL)
Days post-administration
J-PPD
Con A
(C)
Gene expression (relative)
Days post-administration
IFN-γ
TNF-α
CD69

Fig. 2