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HOST RESPONSE AND INFLAMMATION

# Prostaglandin $E_2$ Induction Suppresses the Th1 Immune Responses in Cattle with Johne's Disease

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**ABSTRACT** Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis*, is a bovine chronic infection that is endemic in Japan and many other countries. The expression of immunoinhibitory molecules is upregulated in cattle with Johne's disease, but the mechanism of immunosuppression is poorly understood. Prostaglandin $E_2$ ($PGE_2$) is immunosuppressive in humans, but few veterinary data are available. In this study, functional and kinetic analyses of $PGE_2$ were performed to investigate the immunosuppressive effect of $PGE_2$ during Johne's disease. *In vitro* $PGE_2$ treatment decreased T-cell proliferation and Th1 cytokine production and upregulated the expression of immunoinhibitory molecules such as interleukin-10 and programmed death ligand 1 (PD-L1) in peripheral blood mononuclear cells (PBMCs) from healthy cattle. $PGE_2$ was upregulated in sera and intestinal lesions of cattle with Johne's disease. *In vitro* stimulation with Johnin purified protein derivative (J-PPD) induced cyclooxygenase-2 (COX-2) transcription, $PGE_2$ production, and upregulation of PD-L1 and immunoinhibitory receptors in PBMCs from cattle infected with *M. avium* subsp. *paratuberculosis*. Therefore, Johnin-specific Th1 responses could be limited by the $PGE_2$ pathway in cattle. In contrast, downregulation of $PGE_2$ with a COX-2 inhibitor promoted J-PPD-stimulated $CD8^+$ T-cell proliferation and Th1 cytokine production in PBMCs from the experimentally infected cattle. PD-L1 blockade induced J-PPD-stimulated $CD8^+$ T-cell proliferation and interferon gamma production *in vitro*. Combined treatment with a COX-2 inhibitor and anti-PD-L1 antibodies enhanced J-PPD-stimulated $CD8^+$ T-cell proliferation *in vitro*, suggesting that the blockade of both pathways is a potential therapeutic strategy to control Johne's disease. The effects of COX-2 inhibition warrant further study as a novel treatment of Johne's disease.

**KEYWORDS** $PGE_2$, immunoinhibitory molecules, PD-1, PD-L1, T-cell exhaustion, immunotherapy, COX-2 inhibitor, Johne's disease, cattle

Prostaglandin $E_2$ ($PGE_2$) is an inflammatory mediator (1) derived from arachidonic acid by cyclooxygenase isoenzymes (COX-1 and -2) and PGE synthases (2). COX-1 is a widely expressed constitutive enzyme in many tissues, including the kidneys, stomach, and vascular endothelium (3). COX-2 is an inducible enzyme, and its expression is regulated by inflammatory cytokines and growth factors via activation of nuclear factor-kappa B (NF-κB) (4). $PGE_2$ has suppressive activity in T cells, natural killer (NK)

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cells, dendritic cells (DCs), and macrophages via specific EP2 and EP4 receptors. The interaction of $PGE_2$ and EP2 or EP4 activates the cyclic AMP (cAMP)/protein kinase A (PKA)/cAMP response element binding protein (CREB) pathway to induce the expression of anti-inflammatory and immunosuppressive genes (5, 6). Previous studies in humans have demonstrated that $PGE_2$ inhibits Th1 responses and the differentiation of DCs and induces several subsets of immunosuppressive cells, including M2 macrophages, myeloid-derived suppressor cells (MDSCs), and regulatory T (Treg) cells (7–9). Other studies have found an association of $PGE_2$ with progression of chronic infections. $PGE_2$ upregulates the human immunodeficiency virus type 1 (HIV-1) long terminal repeat-driven gene in T cells (10, 11). COX-2 expression is upregulated in hepatitis C virus (HCV) infection, and $PGE_2$ induces RNA replication of HCV (12). Because of the involvement of $PGE_2$ in the immunopathogenesis of chronic infections, COX-2 inhibitors have been considered candidate immunotherapy drugs. COX-2 inhibitors activate immune responses *in vitro* and *in vivo* (13, 14). COX-2 inhibition results in the activation of interleukin-12 (IL-12) production from antigen-presenting cells and the suppression of IL-10 production in splenocytes of tumor-bearing mice (13). In patients with chronic HIV-1 infection, COX-2 inhibitors were found to improve T-cell-dependent functions and vaccine responses (14). Thus, the COX-2–$PGE_2$ pathway limits Th1 responses during chronic diseases.

*Mycobacterium avium* subsp. *paratuberculosis* is a facultative intracellular pathogen causing Johne's disease (paratuberculosis) in ruminants (15). The clinical signs of Johne's disease include chronic diarrhea, severe weight loss, reduction in milk production, and mortality (15). Johne's disease is endemic worldwide; no country or region has been found to be free of this disease (16). In early infections, *M. avium* subsp. *paratuberculosis* induces strong Th1 responses characterized by interferon gamma (IFN-$\gamma$), and macrophages activated by IFN-$\gamma$ kill intracellular mycobacteria (17–19). The Th1 response declines during the late subclinical stage, which allows bacterial growth and progression to clinical disease (20–22). The Th1 response is the key in the control of progression of Johne's disease.

Programmed death 1 (PD-1) and lymphocyte activation gene 3 (LAG-3) are immunoinhibitory receptors that act in a negative-feedback system to inhibit excessive immune responses via interactions with their ligands, programmed death ligand 1 (PD-L1) and major histocompatibility complex class II (MHC II) (23, 24). In chronic infections, these immunoinhibitory molecules are involved in the exhaustion of antigen-specific T cells (25, 26). PD-1 and LAG-3 are upregulated on $CD4^+$ and/or $CD8^+$ T cells during subclinical Johne's disease in cattle, and an immunoinhibitory ligand, PD-L1, is expressed on *M. avium* subsp. *paratuberculosis*-infected macrophages of the ileum (27). In addition, antibody blockade of the PD-1 and LAG-3 pathways reactivates *M. avium* subsp. *paratuberculosis*-specific Th1 cytokine production *in vitro* (27). The dysfunction of the Th1 response during Johne's disease is mediated by immunoinhibitory molecules on T cells, but it is not known how these immunoinhibitory molecules are upregulated during the course of the disease.

The association of $PGE_2$ and immunoinhibitory molecules has been investigated in mouse models and in human patients (28–30). In a murine tumor model, $PGE_2$ regulated PD-L1 expression in tumor-associated macrophages and MDSCs (28). Another study reported a positive correlation between COX-2 and PD-L1 expression in human melanoma cells (29). Additionally, in a mouse model of chronic infection, EP2 and EP4 were upregulated on $CD8^+$ cytotoxic T cells (CTLs) and impaired CTL function and survival via $PGE_2$ signaling (30). Concurrent blockade of the $PGE_2$ and PD-1/PD-L1 pathways was shown to restore CTL function and improve viral control (30).

Few veterinary studies are available on the immunosuppressive effect of $PGE_2$, the association of $PGE_2$ and immunoinhibitory pathways, and the contribution to T-cell dysfunction or chronic disease progression. This study investigated the immunosuppressive function and kinetics of $PGE_2$ to investigate immunopathogenesis in Johne's disease in cattle.

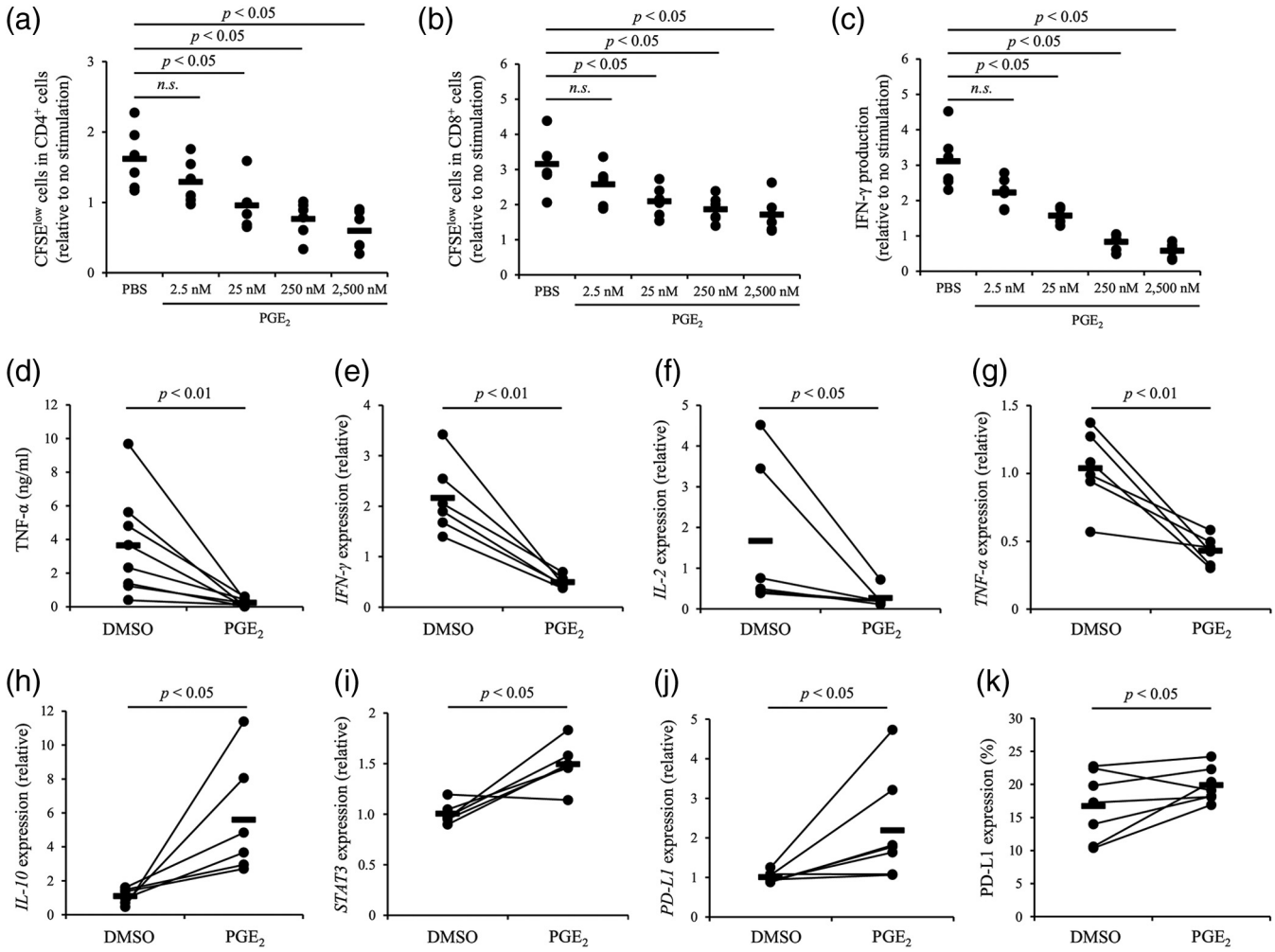


**FIG 1** Immunosuppressive effects of $PGE_2$. (a to d) PBMCs from uninfected cattle ($n = 6$ [a to c] or 8 [d]) were cultured with $PGE_2$ in the presence of anti-CD3 and anti-CD28 MAbs for 72 h. The proliferation of $CD4^+$ cells (a) and $CD8^+$ cells (b) was assayed by flow cytometry. IFN-γ (c) and TNF-α (d) production was determined by ELISA. (e to k) PBMCs from uninfected cattle ($n = 6$ [e to i] or 7 [j and k]) were cultured with $PGE_2$ for 24 h. Real-time PCR was performed in duplicate to quantitate the mRNA expression of IFN-γ (e), IL-2 (f), TNF-α (g), IL-10 (h), STAT3 (i), and PD-L1 (j). The expression of PD-L1 protein was measured by flow cytometry (k). Statistical significance was determined by the Steel-Dwass test (a to c) or the Wilcoxon signed-rank test (d to k).

## RESULTS

**Immunosuppressive effects of $PGE_2$.** To evaluate immunosuppression induced by $PGE_2$, T-cell proliferation, cytokine secretion, and gene expression (cytokine and STAT3 genes) were analyzed by cultivation assay of peripheral blood mononuclear cells (PBMCs) from uninfected cattle under $PGE_2$ treatment. $PGE_2$ inhibited proliferation of $CD4^+$ and $CD8^+$ T cells (Fig. 1a and b) and IFN-γ and TNF-α production from PBMCs (Fig. 1c and d). $PGE_2$ downregulated the mRNA expression of IFN-γ, IL-2, and tumor necrosis factor alpha (TNF-α) (Fig. 1e to g) and upregulated IL-10 and STAT3 mRNA expression (Fig. 1h and i). The results indicate that $PGE_2$ promotes IL-10 signaling and inhibits Th1 responses in cattle. Since $PGE_2$ is known to regulate PD-L1 expression in humans (28), $PGE_2$ regulation of PD-L1 expression was investigated in PBMCs of the healthy cattle. As shown in Fig. 1j and k, $PGE_2$ upregulated PD-L1 expression in PBMCs. Overall, these results indicate that $PGE_2$ has immunosuppressive activity against bovine PBMCs.

**Activation of immune responses by COX-2 inhibition.** To demonstrate the effects of COX-2 inhibition on T-cell function, production of IFN-γ and TNF-α and T-cell proliferation were evaluated by the 3-day culture assay using PBMCs from uninfected animals in the presence of meloxicam. Meloxicam treatment significantly increased

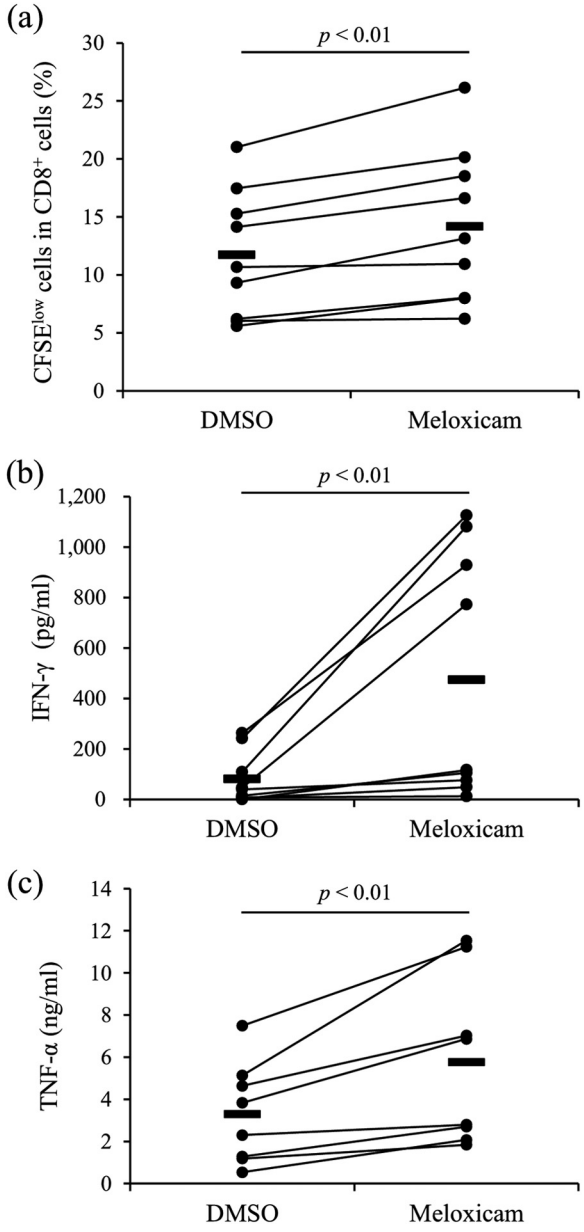


**FIG 2** Functional analysis of the COX-2 inhibitor. PBMCs from uninfected cattle ($n = 9$ [a and b] or 8 [c]) were cultured with meloxicam in the presence of anti-CD3 and anti-CD28 MAbs. $CD8^+$ cell proliferation was assayed by flow cytometry (a). IFN-$\gamma$ (b) and TNF-$\alpha$ (c) production was determined by ELISA in duplicate. Statistical significance was determined by the Wilcoxon signed-rank test.

both IFN-$\gamma$ and TNF-$\alpha$ production in PBMCs and the proliferation of $CD8^+$ T cells (Fig. 2a to c). This result indicates that meloxicam activates the T-cell response in cattle.

**Kinetic analysis of $PGE_2$ in cattle infected with *M. avium* subsp. *paratuberculosis*.** In humans, $PGE_2$ is associated with the progression of some chronic infections (10–12). Compared with that in uninfected cattle, the serum $PGE_2$ concentration was significantly increased in cattle with natural infection of *M. avium* subsp. *paratuberculosis* (Fig. 3a). Johnin purified protein derivative (J-PPD) antigen stimulation increased $PGE_2$ production in PBMCs from the infected animals but not in PBMCs from the uninfected animals (Fig. 3c), and meloxicam inhibited $PGE_2$ production under the J-PPD stimulation (Fig. 3c). COX-2 expression in PBMCs from the infected animals was induced by the stimulation of J-PPD (Fig. 3b). The results indicate that the COX-2–$PGE_2$ pathway

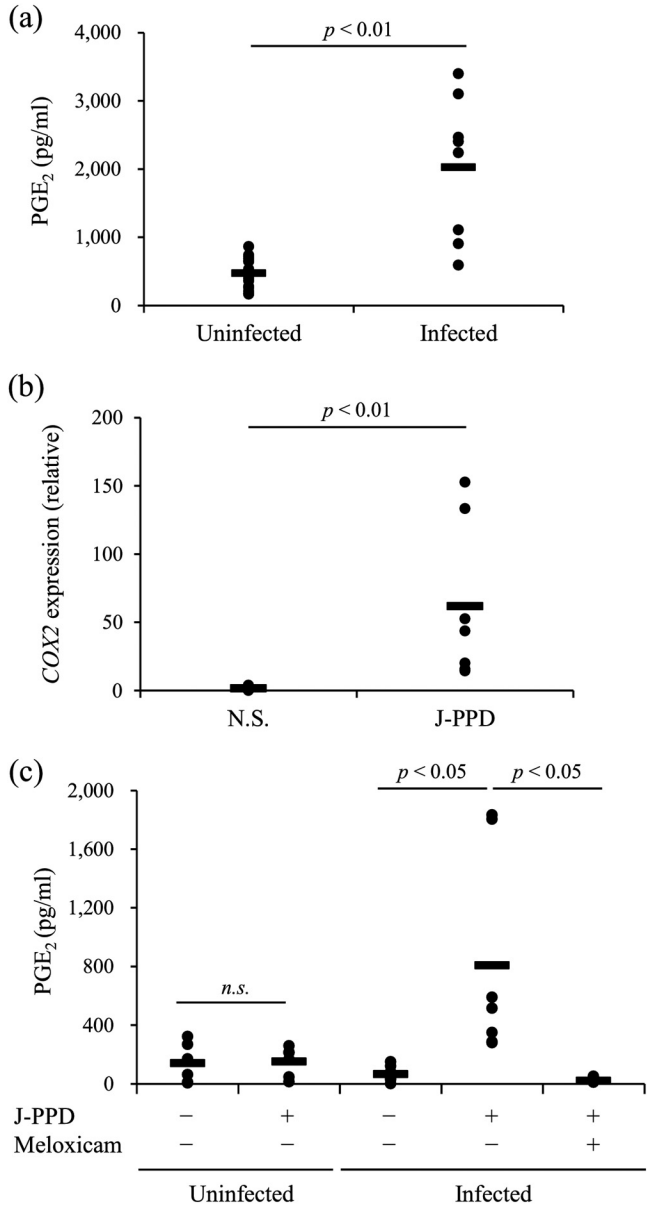


**FIG 3** Kinetic analysis of $PGE_2$ in cattle infected with *M. avium* subsp. *paratuberculosis*. (a) Serum $PGE_2$ concentrations in cattle uninfected ($n = 16$) or naturally infected with *M. avium* subsp. *paratuberculosis* ($n = 8$) were determined by ELISA. (b) The expression of COX-2 mRNA in PBMCs from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ($n = 7$) cultured with J-PPD was assayed by real-time PCR. (c) PBMCs of the uninfected ($n = 6$) and experimentally infected ($n = 7$) cattle were incubated with J-PPD. The concentration of $PGE_2$ in culture supernatants was determined by ELISA in duplicate. Statistical significance was determined by the Wilcoxon signed-rank test (a and b) or the Steel-Dwass test (c). N.S., no stimulation.

was activated by Johnin antigen stimulation in PBMCs of cattle infected with *M. avium* subsp. *paratuberculosis*.

**Upregulation of PD-L1, PD-1, and LAG-3 expression by J-PPD stimulation.** The expression of the immunoinhibitory molecules PD-L1, PD-1, and LAG-3 in PBMCs from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* was analyzed following stimulation by J-PPD for 24 h. Compared with that in the uninfected cattle, J-PPD significantly increased PD-L1 expression at the protein and mRNA levels in PBMCs of the experimentally infected animals (Fig. 4a and b). Flow cytometric analysis indicated that the expression of PD-L1 was significantly upregulated in $CD4^+$ T cells, $CD8^+$

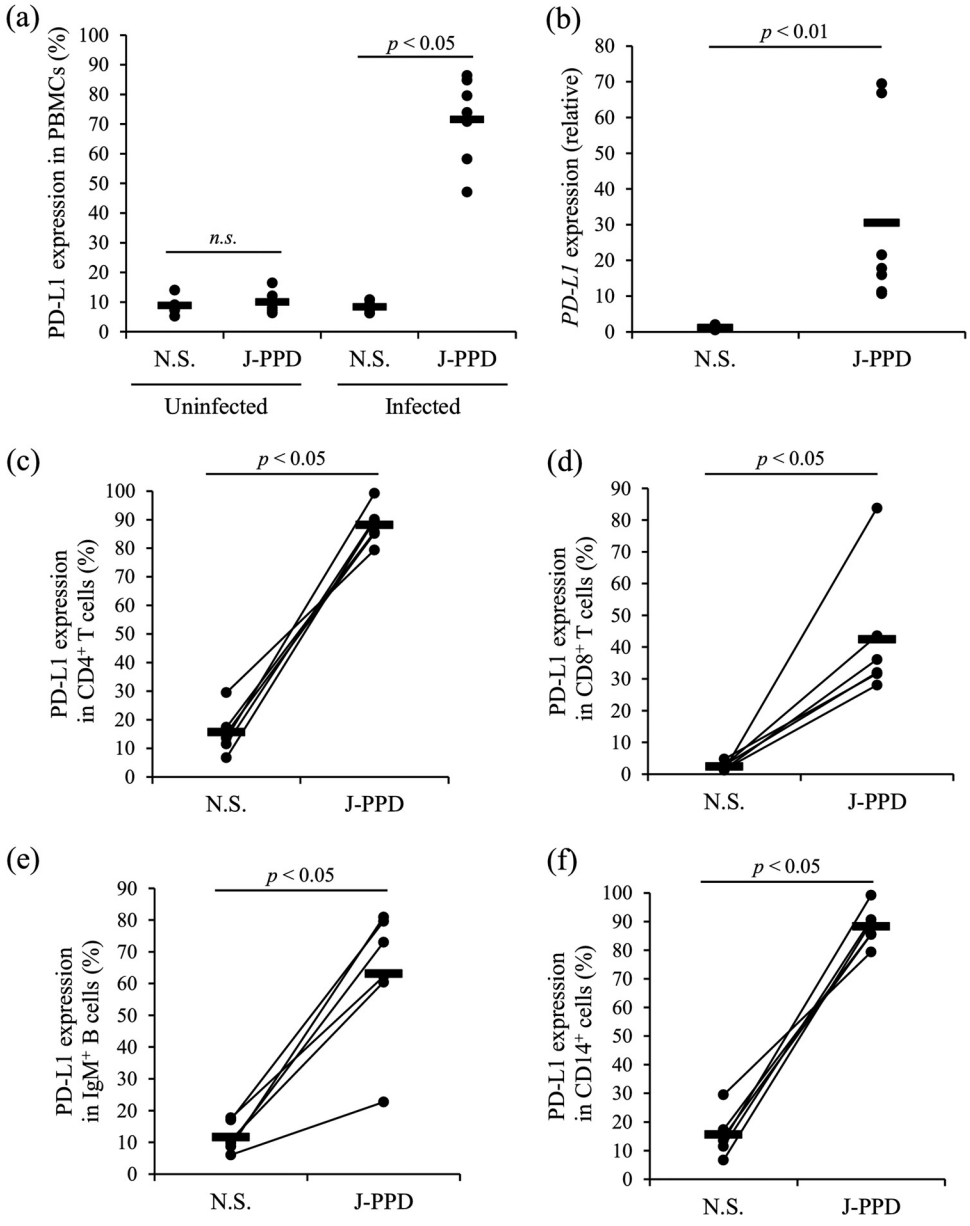


**FIG 4** Upregulation of PD-L1 expression by J-PPD. PBMCs from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ($n = 6$) were incubated with J-PPD, and the expression of PD-L1 in PBMCs (a), $CD4^+$ T cells (c), $CD8^+$ T cells (d), $IgM^+$ B cells (e), and $CD14^+$ cells (f) was assayed by flow cytometry. PBMCs of uninfected cattle ($n = 5$) were incubated with J-PPD, and PD-L1 expression in PBMCs was assayed by flow cytometry (a). Expression of PD-L1 mRNA in PBMCs of the experimentally infected cattle ($n = 7$) was assayed by real-time PCR in duplicate (b). Statistical significance was determined by the Wilcoxon signed-rank test. N.S., no stimulation.

T cells, $IgM^+$ B cells, and $CD14^+$ cells of the infected animals by J-PPD stimulation (Fig. 4c to f). The expression of PD-1 and LAG-3 was also upregulated in $CD4^+$ T cells and $CD8^+$ T cells of the infected animals by J-PPD stimulation (Fig. 5a to d). *In vitro* stimulation by Johnin antigen thus strongly induced the expression of immunoinhibitory molecules in PBMCs of cattle infected with *M. avium* subsp. *paratuberculosis*.

**Expression of $PGE_2$, EP2, and PD-L1 on *M. avium* subsp. *paratuberculosis*-infected cells of the ileum.** A previous study confirmed PD-L1 expression in macrophages infected with *M. avium* subsp. *paratuberculosis* in the ileum, with possible association with the clinical disease (27). This study investigated the expression of $PGE_2$ and EP2 in the intestinal macrophages of cattle with Johne's disease. $PGE_2$ expression was detected in cattle naturally and experimentally infected with *M. avium* subsp.

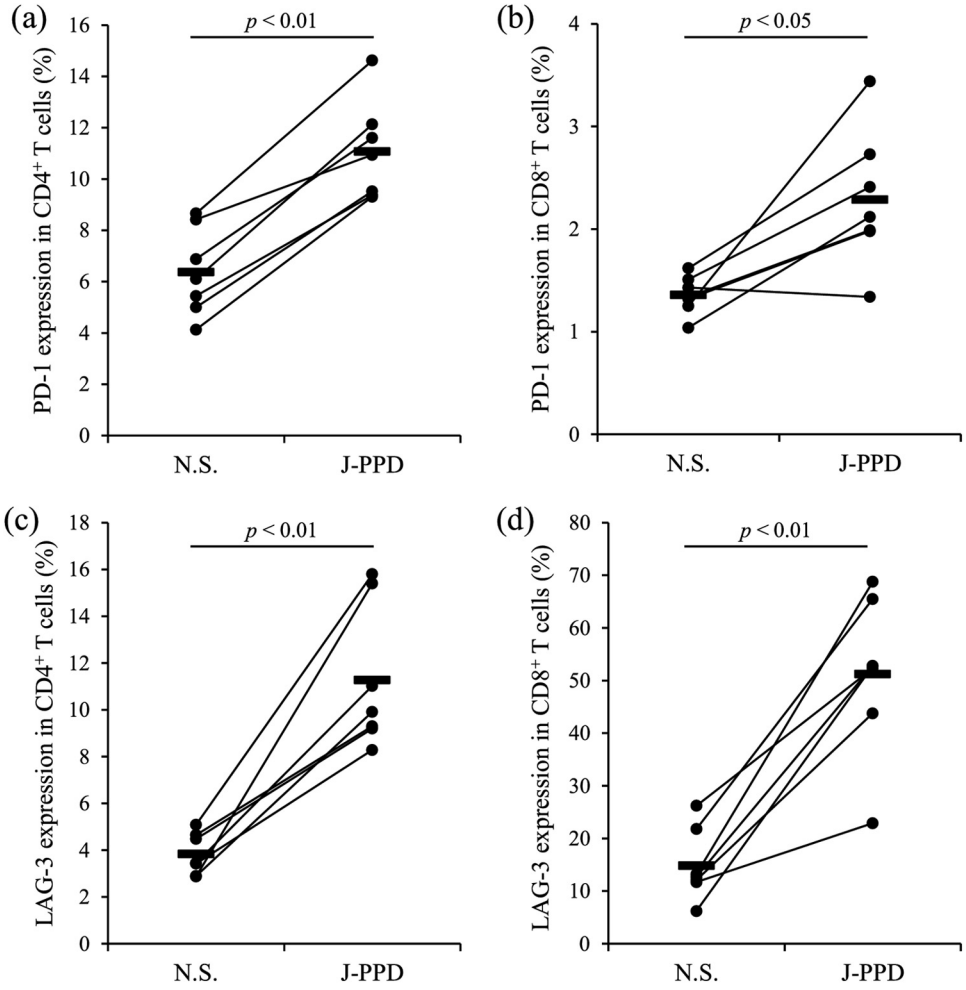


**FIG 5** Upregulation of PD-1 and LAG-3 expression by J-PPD. PBMCs from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ($n = 7$) were incubated with J-PPD, and the expression of PD-1 (a and b) and LAG-3 (c and d) on $CD4^+$ T cells (a and c) and $CD8^+$ T cells (b and d) was assayed by flow cytometry. Statistical significance was determined by the Wilcoxon signed-rank test. N.S., no stimulation.

*paratuberculosis* (1 and 65) but not in the uninfected cattle (C6) (Fig. 6a). Expression of EP2, a $PGE_2$ receptor, was detected in both the infected and uninfected animals (Fig. 6b), and both $PD\text{-}L1^+$ and *M. avium* subsp. *paratuberculosis*-infected cells were observed in the same lesion (Fig. 6c and d). The results demonstrated coexpression of $PGE_2$ and PD-L1 in the ilea of cattle with Johne's disease.

**Activation of J-PPD-specific T-cell responses by a COX-2 inhibitor.** J-PPD can induce IFN-$\gamma$ production from T cells in cattle with *M. avium* subsp. *paratuberculosis* infection (31). This study investigated whether the COX-2 inhibitor meloxicam activates cytokine production and T-cell proliferation in response to J-PPD. As shown in Fig. 7, J-PPD upregulated IFN-$\gamma$ and TNF-$\alpha$ production in meloxicam-treated cultures of PBMCs from infected cattle (Fig. 7b and c). The proliferation of $CD8^+$ cells was also induced by meloxicam treatment (Fig. 7a). Meloxicam thus activated J-PPD-specific immune responses.

**Activation of J-PPD-specific T-cell responses by PD-L1 blockade.** Blockade of the PD-1/PD-L1 pathway can activate IFN-$\gamma$ production from PBMCs in bovine chronic infections, such as bovine leukemia virus (BLV) infection and bovine mycoplasmosis (32, 33). To demonstrate the effects of PD-L1 blockade on cattle infected with *M. avium* subsp. *paratuberculosis*, PBMCs from the experimentally infected cattle were incubated with an anti-PD-L1 monoclonal antibody (MAb) in the presence of J-PPD. As expected,

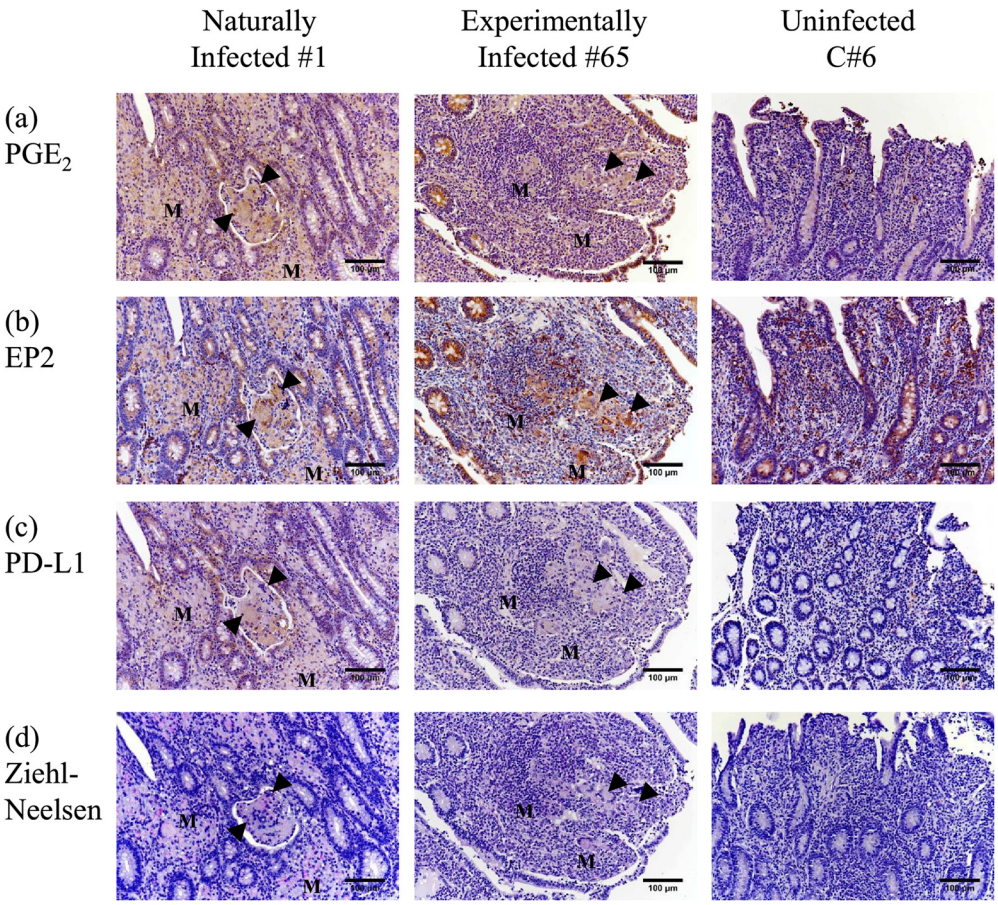


**FIG 6** $PGE_2$, EP2, and PD-L1 expression and localization of *M. avium* subsp. *paratuberculosis* in the ileal mucosa of cattle with or without Johne's disease. (a to c) Immunohistochemical staining of $PGE_2$ (a), EP2 (b), and PD-L1 (c) in ileal tissue of cattle naturally or experimentally infected with *M. avium* subsp. *paratuberculosis* (1 and 65) and uninfected cattle (C6) was performed using anti-human $PGE_2$ antibody (rabbit polyclonal), anti-human EP2 MAb [EPR8030(B)], and anti-bovine PD-L1 MAb (6C11-3A11). (d) Ziehl-Neelsen staining for acid-fast bacilli in ileal tissue of the infected and uninfected cattle was also performed. M, accumulations of *M. avium* subsp. *paratuberculosis*-infected macrophages. Arrowheads, *M. avium* subsp. *paratuberculosis*-infected Langhans giant cells.

J-PPD-specific proliferation of $CD8^+$ cells and IFN-γ production were activated by the anti-PD-L1 MAb (Fig. 7d and e).

**Combined effects of meloxicam and anti-PD-L1 antibody on activation of J-PPD-specific T-cell response.** Compared with that in negative controls, the proliferation of $CD8^+$ cells was significantly increased by the combination of meloxicam and anti-PD-L1 MAb (Fig. 8a). Stimulation by B-PPD, a negative-control antigen, had no effect in either group (Fig. 8a). IFN-γ production was increased by meloxicam and anti-PD-L1 MAb, but the difference from the results with negative controls did not reach significance (Fig. 8b). A previous study found that an anti-PD-L1 chimeric antibody (ChAb) activated immune responses in BLV infection *in vitro* and *in vivo* (34). In this study, combined treatment with the COX-2 inhibitor and anti-PD-L1 ChAb significantly increased $CD8^+$ cell proliferation compared with that for the negative controls (Fig. 8c). The results obtained by combining a COX-2 inhibitor and an anti-PD-L1 antibody support their potential as a novel treatment of Johne's disease.

## DISCUSSION

During the early phase of Johne's disease in cattle, strong Th1 responses are elicited by T cells specific to *M. avium* subsp. *paratuberculosis* that are activated by *M. avium* subsp. *paratuberculosis*-infected macrophages following antigen presentation (17–19). At the late subclinical stage, a decline in the Th1 response contributes to bacterial

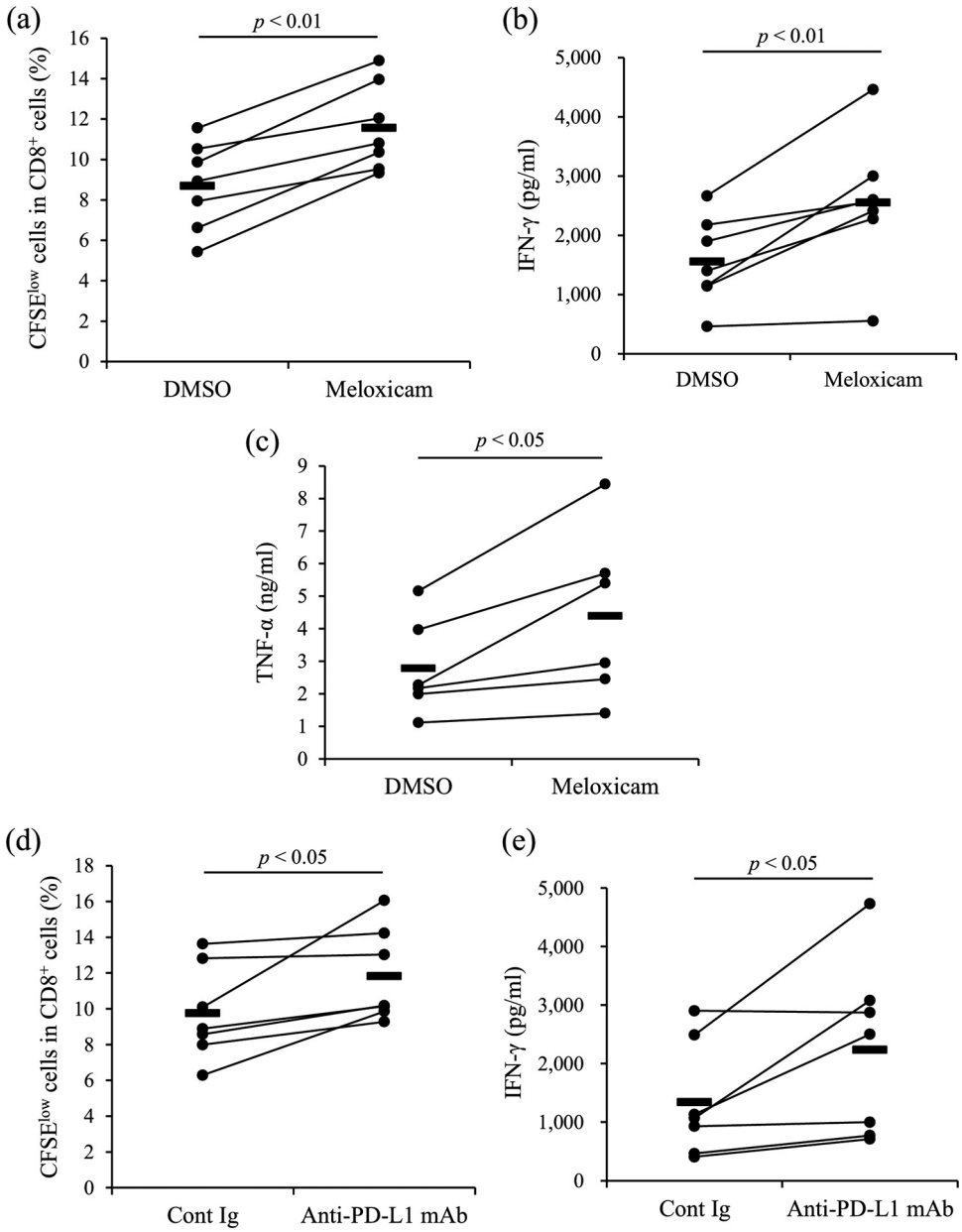


**FIG 7** Activation of T-cell responses by the COX-2 inhibitor or anti-PD-L1 MAb. (a to c) PBMCs from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ($n = 7$ [a and b] or 6 [c]) were cultured with meloxicam in the presence of J-PPD. $CD8^+$ cell proliferation (a) was assayed by flow cytometry. IFN-$\gamma$ (b) and TNF-$\alpha$ (c) production was determined by ELISA in duplicate. (d and e) PBMCs from the infected cattle ($n = 7$) were cultured with anti-PD-L1 MAb in the presence of J-PPD. $CD8^+$ cell proliferation (d) was assayed by flow cytometry. IFN-$\gamma$ production (e) was determined by ELISA in duplicate. Statistical significance was determined by the Wilcoxon signed-rank test. Cont Ig, rat control IgG.

growth and progression to clinical disease (20–22). The loss of functional Th1 responses during subclinical Johne's disease is mediated by immunoinhibitory molecules (27), but the mechanism of the upregulation of these molecules during the disease course is not clear. This study investigated the immunosuppressive effect of $PGE_2$ because its involvement in the regulation of immune responses in chronic human infections is well documented (10–12). $PGE_2$ inhibited the proliferation of $CD4^+$ and $CD8^+$ T cells and the production of the Th1 cytokines IFN-$\gamma$ and TNF-$\alpha$ in cultures of PBMCs isolated from cattle. $PGE_2$ treatment downregulated the expression of IFN-$\gamma$, IL-2, and TNF-$\alpha$ mRNAs and upregulated the expression of IL-10 and STAT3 mRNAs in cultured PBMCs. $PGE_2$

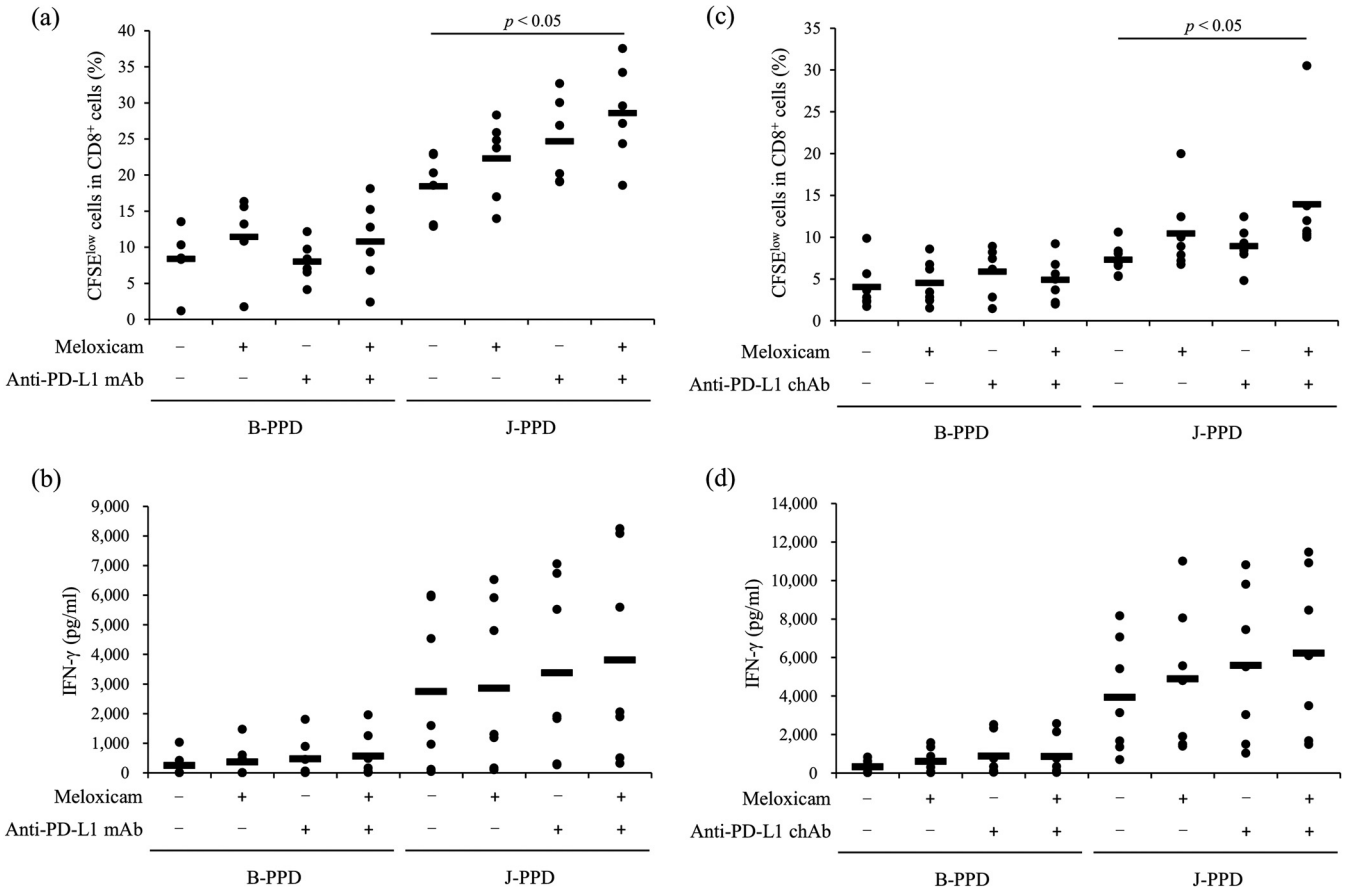


**FIG 8** Activation of T-cell responses by the combination of meloxicam and anti-PD-L1 antibody. (a and b) PBMCs from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* ($n = 6$ [a] or 7 [b]) were incubated with meloxicam and anti-PD-L1 MAb in the presence of J-PPD or B-PPD. CD8$^+$ cell proliferation was assayed by flow cytometry (a). IFN-γ production was determined by ELISA in duplicate (b). (c and d) PBMCs from the infected cattle ($n = 7$) were incubated with meloxicam and anti-PD-L1 ChAb in the presence of J-PPD or B-PPD. CD8$^+$ cell proliferation was assayed by flow cytometry (c). IFN-γ production was determined by ELISA in duplicate (d). Statistical significance was determined by the Steel-Dwass test.

production was increased in the blood of animals infected with *M. avium* subsp. *paratuberculosis*. The Th1 response plays a critical role in the control of *M. avium* subsp. *paratuberculosis* infection and disease progression, but this infection induces IL-10, which activates STAT3 via IL-10 receptor signaling, which in turn results in immunosuppression and bacterial persistence (35, 36). Our results show that $PGE_2$ contributed to the progression of Johne's disease by the suppression of Th1 responses and the activation of IL-10 production. Previous studies have associated $PGE_2$ with mycobacterial infections (37, 38), but the role of $PGE_2$ was not described. The results of this study contribute to the understanding of $PGE_2$ activity during infection with mycobacteria.

PD-L1 is upregulated in the macrophages in the peripheral blood and in the ilea of cattle with Johne's disease (27), but the mechanism underlying PD-L1 upregulation has been unknown. In this study, $PGE_2$ upregulated *in vitro* PD-L1 expression in the PBMCs of healthy cattle. Additionally, J-PPD stimulation induced $PGE_2$ production by activating COX-2 and promoting PD-L1 expression in the PBMCs of cattle infected with *M. avium* subsp. *paratuberculosis* but not in uninfected cattle. In PBMC cultures of infected animals, J-PPD-specific CD4$^+$ T cells secrete mainly IFN-γ and TNF-α by J-PPD stimulation (17–19). COX-2 transcription is known to be induced by inflammatory cytokines (TNF-α, IL-1, etc.), Toll-like receptor (TLR) signaling, and oxidative stress via the NF-κB pathway in human cells (39, 40). In addition, a previous paper has shown that IFN-γ-induced TNF activates NF-κB, which is required for full COX-2 expression in murine macrophages (41). Therefore, TNF-α produced from J-PPD-specific T cells and/or IFN-γ-stimulated macrophages might activate COX-2 transcription via the TNF–NF-κB

pathway and result in increased $PGE_2$ production in the PBMC cultivation assay with J-PPD stimulation.

In this study, *in vitro* treatment of $PGE_2$ upregulated transcription of the IL-10 and STAT3 genes in the PBMCs of healthy cattle. The inhibition of STAT3 activity has been shown to downregulate PD-L1 expression in non-small-cell lung cancer (42). The results indicated that $PGE_2$ may regulate PD-L1 expression in bovine PBMCs by activating IL-10–STAT3 signaling. PD-L1 expression in *M. avium* subsp. *paratuberculosis*-infected macrophages has previously been associated with diseases involving the ileum (27). In this study, $PGE_2$ was expressed in *M. avium* subsp. *paratuberculosis*-infected macrophages and Langhans giant cells in the ilea of animals with clinical Johne's disease. The overall findings suggest that the $PGE_2$–IL-10–STAT3–PD-L1 axis is involved in the downregulation of the Th1 response and the formation of pathological lesions in the intestine during Johne's disease. It is still unclear what types of cell subsets produce $PGE_2$ and what types of prostanoid receptors, EP1, EP2, EP3, or EP4, mediate immunosuppression by $PGE_2$ on T cells during Johne's disease. Further investigation is required to determine the mechanism involved.

Macrophages and $CD8^+$ cytotoxic T cells activated by Th1 cytokines are involved in the killing of intracellular bacteria during Johne's disease (17–19). COX-2 inhibition induced the proliferation of $CD8^+$ T cells and Th1 cytokine production in PBMCs from *M. avium* subsp. *paratuberculosis*-infected cattle following J-PPD stimulation. Combined blockade of $PGE_2$ and PD-1/PD-L1 strongly stimulated the proliferation of $CD8^+$ T cells in the infected animals. IFN-$\gamma$ production tended to be activated by dual blockade of $PGE_2$ and PD-1/PD-L1, although significance was not observed because of the limited number of samples that were evaluated. The combination of COX-2 inhibition and PD-1/PD-L1 blockade has therapeutic potential for the control of Johne's disease. Selective COX-2 inhibitors, including meloxicam, are launched and widely used as anti-inflammatory compounds in the dairy and beef industries. Previous studies have investigated the *in vivo* effect of meloxicam on T-cell subsets in healthy or vaccinated cattle (43, 44). However, the *in vivo* effect of meloxicam on the Th1 response during Johne's disease is still unclear and should be determined in further experiments in infected cattle. In addition, we are developing a method for large-scale production of bovinized chimeric antibody using a mammalian expression system (34, 45), which would enable antibody therapy even in cattle. Further study is warranted to investigate the clinical effectiveness and cost-effectiveness of these inhibitors against Johne's disease. Previous studies have demonstrated that immunoinhibitory molecules mediate T-cell exhaustion in cattle with other infectious diseases, including bovine leukosis, mycoplasmosis, and anaplasmosis (33, 46–51), but the involvement of $PGE_2$ in the immunopathogenesis of those infections has not been investigated.

This is the first study of the immunosuppressive effects of $PGE_2$ in cattle and the immunomodulatory effect of COX-2 inhibition during Johne's disease in cattle. The results suggest cotargeting $PGE_2$ and PD-L1 as a novel immunotherapy for Johne's disease. Pilot clinical trials have shown that antibodies blocking the PD-1/PD-L1 pathway have immunomodulatory and antiviral effects (34, 45). Cost-effectiveness is an obstacle to this approach because large amounts of the therapeutic antibodies are required. If the antibody dose can be reduced by combination with a COX-2 inhibitor, this approach could have a significant impact. Further study will open up new avenues for the treatment of bovine chronic infections, including Johne's disease.

## MATERIALS AND METHODS

**Animals.** Blood from Holstein cattle not infected with *M. avium* subsp. *paratuberculosis* was obtained from dairy farms in Hokkaido, Japan, which have no history of Johne's disease. Seven male Holstein calves (animals 80 to 86) between 3 and 4 weeks of age were orally inoculated with intestinal tissue homogenate from an infected cow containing *M. avium* subsp. *paratuberculosis* ($6.8 \times 10^6$ CFU) once daily for 20 days, as described previously with slight modifications (27, 52). All infected calves were kept in a biosafety level 2 animal facility at the National Institute of Animal Health (Tsukuba, Ibaraki, Japan). These cattle did not show clinical symptoms. All animal experiments were approved by the National Institute of Animal Health Ethics Committee.

**Cell preparation and culture.** Peripheral blood samples were collected from cattle that were uninfected or experimentally infected with *M. avium* subsp. *paratuberculosis*. Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples by density gradient centrifugation on Percoll (GE Healthcare, Buckinghamshire, England, UK). Cells were cultured in 200 $\mu$l RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) including 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 200 IU/ml penicillin, 200 $\mu$g/ml streptomycin, and 0.01% L-glutamine (Thermo Fisher Scientific) in 96-well culture plates (Corning Inc., Corning, NY, USA) at 37°C in a 5% $CO_2$ atmosphere. The culture assays of PBMCs from the experimentally infected animals were carried out using samples at various time points between 82 and 120 weeks after the first inoculation.

**Functional analysis of $PGE_2$.** To evaluate immunosuppressive functions of $PGE_2$, PBMCs ($4 \times 10^5$ cells/well) from uninfected cattle were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich) and incubated with 2.5 to 2,500 nM $PGE_2$ (Cayman Chemical, Ann Arbor, MI, USA) for 72 h. Cultures were stimulated by adding 1 $\mu$g/ml of anti-CD3 monoclonal antibody (MAb) (MM1A; Washington State University Monoclonal Antibody Center, Pullman, WA, USA) and 1 $\mu$g/ml of anti-CD28 MAb (CC220; Bio-Rad, Hercules, CA, USA) to each well.

To demonstrate the effects of $PGE_2$ on IFN-$\gamma$ and TNF-$\alpha$ production, 72-h culture supernatants of CFSE-labeled PBMCs were collected, and IFN-$\gamma$ and TNF-$\alpha$ were determined by enzyme-linked immunosorbent assay (ELISA) for bovine IFN-$\gamma$ (Mabtech, Nacka Strand, Sweden) and bovine TNF-$\alpha$ (Kingfisher Biotech, St. Paul, MN, USA), following the manufacturer's instructions.

To demonstrate the effects of $PGE_2$ on cell proliferation, CFSE-labeled PBMCs were harvested after 72 h and incubated in phosphate-buffered saline (PBS) with 10% goat serum (Thermo Fisher Scientific) for 15 min at room temperature to prevent nonspecific reactions. Cells were washed with PBS containing 1% bovine serum albumin (Sigma-Aldrich) (1% BSA-PBS) and stained with Alexa Fluor 647-conjugated anti-CD4 MAb (CC30; Bio-Rad), peridinin-chlorophyll-protein complex/cyanin 5.5 (PerCP/Cy 5.5)-conjugated anti-CD8 MAb (CC63; Bio-Rad), and phycoerythrin/cyanin 7 (PE/Cy7)-conjugated anti-IgM MAb (IL-A30; Bio-Rad) for 25 min at room temperature. CC30 was prelabeled with a Zenon Alexa Fluor 647 mouse IgG1 labeling kit (Thermo Fisher Scientific). CC63 and IL-A30 were conjugated with PerCP/Cy5.5 and PE/Cy7, respectively, by using Lightning-Link antibody labeling kits (Innova Biosciences, Cambridge, England, UK). The stained cells were then washed with 1% BSA-PBS and analyzed immediately by FACS Verse (BD Biosciences, San Jose, CA, USA) and FCS Express 4 (De Novo Software, Glendale, CA, USA). The primary antibodies used in this experiment are shown in Table S1 in the supplemental material.

To investigate the effect of $PGE_2$ on the transcriptional profile of genes involved in the T-cell response, PBMCs ($4 \times 10^5$ cells/well) from uninfected cattle were incubated with 2,500 nM $PGE_2$ (Cayman Chemical) for 24 h. Total RNA was extracted from the cultivated cells in TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) following the manufacturer's instructions. cDNA was synthesized from the obtained total RNA using PrimeScript reverse transcriptase (TaKaRa Bio, Otsu, Japan) following the manufacturer's instructions. The mRNA expression of COX-2, PD-L1, STAT3, and some cytokines in PBMCs was assayed by real-time PCR performed with a LightCycler 480 system II (Roche Diagnostics, Mannheim, Germany) using SYBR Premix DimerEraser (TaKaRa Bio) following the manufacturer's instructions. The GAPDH and ACTB genes were used as internal control genes. All assays were performed in duplicate. The primer sequences used in this study are shown in Table S2 in the supplemental material.

**Kinetic analysis of $PGE_2$.** The $PGE_2$ concentrations in sera of uninfected animals and animals naturally infected with *M. avium* subsp. *paratuberculosis* were measured using the Prostaglandin $E_2$ Express enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical) following the manufacturer's instructions with slight modifications. To indicate whether $PGE_2$ production was promoted by Johnin antigen stimulation, PBMCs ($4 \times 10^5$ cells/well) from the infected cattle were incubated with 1 $\mu$g/ml of Johnin purified protein derivative (J-PPD) for 5 days. J-PPD is the inactivated crude protein fraction prepared from the culture supernatant of *M. avium* subsp. *paratuberculosis*, which is an immunogenic antigen widely used to evaluate T-cell responses against the bacterial infection *ex vivo*. Total RNA then was extracted from the cultivated cells, and cDNA was synthesized from the obtained total RNA as described above. The mRNA expression of COX-2 in PBMCs was then assayed by real-time PCR as described above (Table S2). Culture supernatants were collected, and the $PGE_2$ concentration was determined by ELISA as described above.

**Flow cytometry assay of PD-L1, PD-1, and LAG-3 expression.** PBMCs ($1 \times 10^6$ cells/well) from cattle uninfected or experimentally infected with *M. avium* subsp. *paratuberculosis* were incubated with 1 $\mu$g/ml of J-PPD for 24 h. After 24 h, cells were harvested and incubated in PBS with 10% goat serum (Thermo Fisher Scientific) for 15 min at room temperature.

To analyze PD-L1 expression, the treated cells were washed with 1% BSA-PBS and stained with anti-bovine PD-L1 MAb (4G12, rat IgG2a) (32) or a rat IgG2a isotype control (R35-95; BD Biosciences) for 25 min at room temperature. To detect PD-L1 on T cells and B cells, cells were washed with 1% BSA-PBS after the reaction with primary antibody and then stained with phycoerythrin (PE)-conjugated anti-CD3 MAb (MM1A; Washington State University Monoclonal Antibody Center), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 MAb (CC8; Bio-Rad), PerCP/Cy5.5-conjugated anti-CD8 MAb (CC63; Bio-Rad), PE/Cy7-conjugated anti-IgM MAb (IL-A30; Bio-Rad), and allophycocyanin (APC)-conjugated anti-rat immunoglobulin antibody (Southern Biotech, Birmingham, AL, USA) for 25 min at room temperature. MM1A was prelabeled with the Zenon R-PE mouse IgG1 labeling kit (Thermo Fisher Scientific). CC63 and IL-A30 were conjugated with PerCP/Cy5.5 and PE/Cy7, respectively, by using Lightning-Link antibody labeling kits (Innova Biosciences). To detect PD-L1 on $CD14^+$ cells, cells were washed with 1% BSA-PBS after the reaction with primary antibody and then stained with PerCP/Cy5.5-conjugated anti-CD14 MAb (CAM36A;

**TABLE 1** Bacterial loads in fecal and tissue samples at necropsy

| Animal | Status | *M. avium* subsp. *paratuberculosis* DNA (pg) in[a]: | |
|---|---|---|---|
| | | Feces | Ileum tissue |
| C6 | Uninfected control | ND | ND |
| 65[b] | Experimentally infected | 0.589 | 26.8 |
| 1 | Naturally infected | NA | NA |

[a]ND, not detected; NA, not available.
[b]The data for animal 65 have been published previously (27).

Washington State University Monoclonal Antibody Center) and APC-conjugated anti-rat immunoglobulin antibody (Southern Biotech) for 25 min at room temperature. CAM36A was prelabeled using Lightning-Link PerCP/Cy5.5 antibody labeling kit (Innova Biosciences).

To analyze PD-1 expression, the treated cells were washed with 1% BSA-PBS and then stained with anti-bovine PD-1 MAb (5D2, rat IgG2a) (46) or a rat IgG2a isotype control (R35-95; BD Biosciences) for 25 min at room temperature. To analyze LAG-3 expression, the treated cells were washed with 1% BSA-PBS and then stained with anti-bovine LAG-3 MAb (71-2D8, rat IgG1) (27) or a rat IgG1 isotype control (R3-34; BD Biosciences) for 25 min at room temperature. Cells were washed with 1% BSA-PBS after the reaction with primary antibody and then stained with PerCP/Cy5.5-conjugated anti-CD3 MAb (MM1A; Washington State University Monoclonal Antibody Center), FITC-conjugated anti-CD4 MAb (CC8; Bio-Rad), PE-conjugated anti-CD8 MAb (CC63; Bio-Rad), PE/Cy7-conjugated anti-IgM MAb (IL-A30; Bio-Rad), and APC-conjugated anti-rat immunoglobulin antibody (Southern Biotech) for 25 min at room temperature. MM1A and IL-A30 were conjugated with PerCP/Cy5.5 and PE/Cy7, respectively, by using Lightning-Link antibody labeling kits (Innova Biosciences). The stained cells were washed with 1% BSA-PBS and analyzed immediately by FACS Verse (BD Biosciences) and FCS Express 4 (De Novo Software). The primary antibodies used in this experiment are shown in Table S1 in the supplemental material.

**Immunohistochemical assay of PD-L1, EP2, and $PGE_2$ and Ziehl-Neelsen staining.** Immunohistochemical assays were performed as previously described with slight modifications (27). Sections of ileum tissue from cattle experimentally or naturally infected with *M. avium* subsp. *paratuberculosis* were evaluated. A naturally infected animal (naturally infected animal 1) had clinical symptoms of Johne's disease. Tissue samples from experimentally infected cattle were obtained from an animal (animal 65) with bacterial shedding in feces and clinical symptoms such as diarrhea (Table 1) (27). Anti-prostaglandin $E_2$ antibody (Abcam, Cambridge, England, UK), anti-prostaglandin E receptor EP2 antibody [EPR8030(B); Abcam], or anti-PD-L1 MAb (6C11-3A11, rat IgG2a) (see Fig. S1 in the supplemental material) (32) was used for the primary antibody for immunohistochemistry. Ziehl-Neelsen staining was performed as previously described (27).

**Functional assay of COX-2 inhibition.** To evaluate the immunostimulatory effects of COX-2 inhibition, PBMCs ($4 \times 10^5$ cells/well) from cattle uninfected or experimentally infected with *M. avium* subsp. *paratuberculosis* were labeled with CFSE (Sigma-Aldrich) and incubated with 1 $\mu$M meloxicam (Sigma-Aldrich) or dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) for 3 days (uninfected cattle) or 5 days (infected cattle). For stimulation of PBMCs from the uninfected cattle, 1 $\mu$g/ml of anti-CD3 MAb (MM1A; Washington State University Monoclonal Antibody Center) and 1 $\mu$g/ml of anti-CD28 MAb (CC220; Bio-Rad) were added to cultures; 1 $\mu$g/ml of J-PPD was added to cultures of PBMCs from the infected cattle. After cultivation, IFN-$\gamma$ and TNF-$\alpha$ concentrations were measured by ELISA, and cell proliferation was measured by flow cytometry as described above.

**Functional analysis of combined COX-2 inhibition and PD-L1 blockade.** To investigate the immunostimulatory effects of combined treatment with COX-2 inhibitor and anti-PD-L1 blocking antibodies, PBMCs ($4 \times 10^5$ cells/well) from cattle experimentally infected with *M. avium* subsp. *paratuberculosis* were labeled with CFSE (Sigma-Aldrich) and incubated with 1 $\mu$M meloxicam and 10 $\mu$g/ml of anti-PD-L1 antibody in the presence of 1 $\mu$g/ml J-PPD for 5 days. DMSO (Nacalai Tesque) and rat IgG (10 $\mu$g/ml; Sigma-Aldrich) were used as negative controls. PPD purified from *M. bovis* BCG strain (B-PPD) (1 $\mu$g/ml) was used as a negative-control antigen. In this assay, two types of anti-PD-L1 blocking antibodies, anti-PD-L1 rat MAb (4G12) (32) and anti-PD-L1 rat-bovine chimeric antibody (ChAb) (Boch4G12) (34), were used to evaluate the combination effect. After 5 days of culture, the IFN-$\gamma$ concentration was determined by ELISA, and cell proliferation was measured by flow cytometry as described above.

**Statistical analysis.** Differences were evaluated by the Wilcoxon signed-rank test and the Steel-Dwass test. A $P$ value of $<0.05$ was considered statistically significant.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00910-17.

**SUPPLEMENTAL FILE 1,** PDF file, 0.2 MB.

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