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Perspective

A TLR7 agonist activates bovine Th1 response and exerts antiviral activity against bovine leukemia virus

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ABSTRACT

Bovine leukemia virus (BLV) infection is a bovine chronic infection caused by BLV, a member of the genus *Deltaretrovirus*. In this study, we examined the immunomodulatory effects of GS-9620, a toll-like receptor (TLR) 7 agonist, in cattle (*Bos taurus*) and its therapeutic potential for treating BLV infection. GS-9620 induced cytokine production in peripheral blood mononuclear cells (PBMCs) as well as CD80 expression in CD11c$^+$ cells and increased CD69 and interferon (IFN)-γ expressions in T cells. Removing CD11c$^+$ cells from PBMCs decreased CD69 expression in T cells in the presence of GS-9620. These results suggest that TLR7 agonism promotes T-cell activation via CD11c$^+$ cells. Analyses using PBMCs from BLV-infected cattle revealed that *TLR7* expression in CD11c$^+$ cells was upregulated during late-stage BLV infection. Furthermore, GS-9620 increased IFN-γ and TNF-α production and inhibited syncytium formation *in vitro*, suggesting that GS-9620 may be used to treat BLV infection.

1. Introduction

Toll-like receptors (TLRs) are pattern recognition receptors that stimulate innate immune responses. TLRs enhance the adaptive immune response by promoting cytokine secretion, dendritic cell (DC) maturation, and antigen presentation (Akira and Takeda, 2004). TLR7 recognizes viral single-stranded RNA (Lund et al., 2004), and is predominantly expressed in the endosomal compartment of DCs and B cells in humans (Jarrossay et al., 2001; Kadowaki et al., 2001; Ito et al., 2005). Previous papers showed TLR7 agonists induce antiviral effects via the activation of immune responses (Horscroft et al., 2011). TLR7 agonists promote DC maturation and type I interferon (IFN), such as IFN-α, production (Gibson et al., 2002; Tsai et al., 2017). The secretion of type I IFNs promotes antiviral defense through interferon-stimulated genes (Samuel, 2001). Additionally, type I IFNs act as a bridge between innate and adaptive immunity, activating antiviral effects via the responses of cytotoxic T cells (Schiavoni et al., 2013). Therefore, TLR7 agonists have the potential to treat several chronic infections, such as hepatitis B virus infection and human immunodeficiency virus infection (Lanford et al., 2013; Menne et al., 2015; Bam et al., 2016; Tsai et al., 2017). In bovine studies, several reports have demonstrated the association between TLR7 and the development of bovine viral infections (Zhang et al., 2006; Marin et al., 2014). Additionally, a previous study has reported that *TLR7* expression is upregulated in the peripheral blood mononuclear cells (PBMCs) of bovine leukemia virus (BLV)-infected cattle (*Bos taurus*), especially among those with high proviral loads (Farias et al., 2016). Therefore, TLR7 agonists have the potential to treat bovine viral infections. However, in bovine research, the effects of TLR7 agonists on bovine immune cells have not been fully elucidated.

BLV is a member of the genus *Deltaretrovirus*, and is closely related to human T-cell leukemia virus type 1 (Sagata et al., 1985). BLV infects bovine B cells and causes enzootic bovine leukosis (EBL). Most BLV-infected cattle are asymptomatic carriers, called aleukemic (AL). Meanwhile, around 30% of BLV-infected cattle show persistent

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lymphocytosis (PL), which is characterized by the nonmalignant polyclonal expansion of infected B cells in the peripheral blood. Between 1% and 5% of BLV-infected cattle develop EBL, which is characterized by fatal lymphoma or lymphosarcoma after a long latent period (Schwartz and Levy, 1994). The prevalence of BLV infection is high in many countries including Japan. A previous epidemiology investigation has shown that the seroprevalence of BLV infection among dairy cattle is more than 40% in Japan (Murakami et al., 2013). The lack of an effective treatment or vaccine is one of the reasons for the high prevalence of BLV in many countries. Thus, the establishment of a novel control strategy against BLV infection is strongly required. To develop a novel strategy, our previous studies focused on immune checkpoint molecules, such as programmed death 1 (PD-1) and PD-ligand 1 (PD-L1) (Konnai et al., 2017). PD-1 is an immunoinhibitory receptor expressed on T cells, and its upregulation plays a key role in T-cell exhaustion via the interaction with its ligand PD-L1 (Okazaki and Honjo, 2007). In bovine studies, previous reports have demonstrated that the PD-1/PD-L1 pathway regulates Th1 cytokine production and is associated with BLV infection progression (Ikebuchi et al., 2010, 2011). The inhibition of the PD-1/PD-L1 pathway using specific antibodies (Abs) activates Th1 responses, such as IFN-γ production and T-cell proliferation (Ikebuchi et al., 2011; Nishimori et al., 2017). Additionally, the administration of anti-PD-1/PD-L1 Abs to BLV-infected cattle, which were classified as AL, significantly reduced BLV proviral loads (Nishimori et al., 2017; Okagawa et al., 2017; Sajiki et al., 2019), suggesting a potential of the immunotherapy targeting PD-1/PD-L1 as a novel control method against BLV infection. However, a previous paper has shown that the administration of anti-PD-L1 Abs does not reduce BLV proviral loads in BLV-infected cattle with PL (Sajiki et al., 2019). Therefore, developing a therapeutic strategy that enhances the efficacy of anti-PD-1/PD-L1 Abs is needed to overcome this situation. A previous paper has reported that TLR7 agonists have the potential to be included in combination immunotherapies. Nishii et al. showed the potential of TLR7 agonists to overcome resistance to anti-PD-L1 Abs through the modification of DCs in murine tumor models (Nishii et al., 2018). However, little information is available on the efficacy of combining anti-PD-L1 Abs with TLR7 agonists to treat viral infections.

In the present study, we found that a TLR7 agonist, GS-9620, activated Th1 responses via $CD11c^+$ cells in cattle. Additionally, analyses using samples from BLV-infected cattle showed that the expression levels of *TLR7* in $CD11c^+$ cells were upregulated in BLV-infected cattle, especially in PL cattle. GS-9620 treatment induced Th1 cytokine production and inhibited syncytium formation *in vitro*. Moreover, we examined the therapeutic potential of combining the TLR7 agonist with anti-PD-L1 Abs using PBMCs from BLV-infected cattle.

2. Materials and methods

2.1. Blood samples and BLV diagnosis

The blood samples from BLV-infected and BLV-uninfected cattle were obtained from several farmers and veterinarians in Japan. BLV infection was confirmed via the detection of the anti-BLV antibodies using a commercially available ELISA kit (JNC, Tokyo, Japan) and the provirus using quantitative real-time polymerase chain reaction (qPCR) using a LightCycler 480 System II (Roche Diagnostic, Mannheim, Germany) with BLV Detection Kit (Takara Bio). Lymphocytes in blood samples from BLV-infected cattle were counted using a Celltac alpha MEK-6450 automatic hematology analyzer (Nihon Kohden, Tokyo, Japan), and BLV-infected cattle were classified as AL or PL according to a previous paper (Ohira et al., 2016).

2.2. Cell preparation and culture

As described in our previous paper (Sajiki et al., 2018), PBMCs from blood samples were purified using density-gradient centrifugation on Percoll (GE Healthcare, Little Chalfont, UK). PBMCs were cultured in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Thermo Fisher Scientific), 100 μg/mL streptomycin (Thermo Fisher Scientific), and 2 mM L-glutamine (Thermo Fisher Scientific), and were grown in 96-well plates (Corning Inc., Corning, NY, USA). CC81 cells were cultured for syncytium assay in RPMI 1640 medium (Sigma-Aldrich) containing the same supplements as shown above, and were grown in 24-well plates (Corning Inc.).

2.3. Functional analyses of GS-9620 using PBMCs

To examine whether TLR7 agonists activate the immune responses in cattle, PBMCs from uninfected cattle were cultured with 1 μM of GS-9620 (Chemscene, Monmouth Junction, NJ, USA) for three days. Dimethyl sulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) was used as a vehicle control. After the incubation, culture supernatants were collected, and the concentrations of IFN-α, IFN-γ, and TNF-α were measured using Bovine IFN-α Do-It-Yourself ELISA (Kingfisher Biotech, St. Paul, MN, USA), Bovine IFN-γ ELISA development kit (Mabtech, Nacka Strand, Sweden), and Bovine TNF-α Do-It-Yourself ELISA (Kingfisher Biotech), respectively. After the incubation, the cells were harvested, and then, analyzed by flow cytometry and qPCR. For flow cytometry, Fc blocking was performed by incubating PBMCs in phosphate buffered saline (PBS) containing 10% heat-inactivated goat serum (Thermo Fisher Scientific) for 15 min at 25 °C to prevent nonspecific reactions. After Fc blocking, cells were stained with the following Abs: Alexa Fluor 647-labeled anti-CD11c mAb (BAQ151A; Washington State University Monoclonal Antibody Center, Pullman, WA, USA) and PerCP/Cy 5.5-conjugated anti-CD80 mAb (IL-A159; Bio-Rad, Hercules, CA, USA). BAQ151A was prelabeled using the Zenon Alexa Fluor 647 Mouse IgG_1 Labeling Kit (Thermo Fisher Scientific). IL-A159 was conjugated by using a Lightning-Link antibody labeling kit (Innova Biosciences, Cambridge, England, UK). After the staining for 20 min at 25 °C, the cells were washed twice with PBS, and analyzed immediately by using FACS Verse (BD Biosciences, San Jose, CA, USA). For qPCR, RNA extraction and cDNA synthesis were performed as described in a previous paper (Sajiki et al., 2019). To measure the mRNA expression levels of *IFN-γ*, *TNF-α*, *IL-1β*, *TGF-β1*, and *Foxp3*, qPCR was performed using a thermal cycler (LightCycler 480 System II) with SYBR Premix DimerEraser (Takara Bio), following the manufacturers' instructions. *β-actin* (*ACTB*) was used as a reference gene. Primers were 5′-ATA ACC AGG TCA TTC AAA GG-3′ and 5′-ATT CTG ACT TCT CTT CCG CT-3′ for *IFN-γ*, 5′-TAA CAA GCC AGT AGC CCA CG-3′ and 5′-GCA AGG GCT CTT GAT GGC AGA-3′ for *TNF-α*, 5′-ACC TTC ATT GCC CAG GTT TCT-3′ and 5′-CTG TTT AGG GTC ATC AGC CTC AA-3′ for *IL-1β*, 5′-CTG CTG AGG CTC AAG TTA AAA GTG-3′ and 5′-CAG CCG GTT GCT GAG GTA G-3′ for *TGF-β1*, 5′-CAC AAC CTG AGC CTG CAC AA-3′ and 5′-TCT TGC GGA ACT CAA ACT CAT C-3′ for *Foxp3*, and 5′-TCT TCC AGC CTT CCT TCC TG-3′ and 5′-ACC GTG TTG GCG TAG AGG TC-3′ for *ACTB*. To examine the effects of GS-9620 on CD69 expression in T cells, PBMCs from uninfected cattle were cultivated with 1 μM of GS-9620 for two days. After the incubation, the cells were collected, and the expression levels of CD69 were measured by flow cytometry. After Fc blocking, the cells were stained with the following Abs: PerCP/Cy 5.5-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 Alexa Fluor 647-labeled anti-CD69 mAb (KTSN7A; Kingfisher Biotech). MM1A and IL-A30 were conjugated using Lightning-Link antibody labeling kits. KTSN7A was prelabeled using the Zenon Alexa Fluor 647 Mouse IgG_1 Labeling Kit. After the staining for 20 min at 25 °C, PBMCs were washed twice with PBS, and analyzed immediately by FACS Verse. To examine the effect of GS-9620 on IFN-γ production in T cells, PBMCs from uninfected cattle were cultivated with 1 μM of GS-9620 for three days. Cultures were stimulated by adding 1

μg/mL of anti-CD3 mAb (MM1A) and 1 μg/mL of anti-CD28 mAb (CC220; Bio-Rad) to each well. Brefeldin A (10 μg/mL; Sigma-Aldrich) was added for the final 6 h to enhance intracellular cytokine staining. After the incubation, the cells were collected and Fc blocking was performed as described above. Then, surface staining was performed using the following Abs: FITC-conjugated anti-CD4 mAb (CC8), PE-conjugated anti-CD8 mAb (CC63), and PE/Cy7-conjugated anti-IgM mAb (IL-A30). After the surface staining for 20 min at 25 °C, intracellular cytokine staining was performed using the following reagents: FOXP3 Fix/Perm kit (BioLegend, San Diego, CA, USA), Biotinylated anti-bovine IFN-γ mAb (MT307; Mabtech), and APC-conjugated streptavidin (BioLegend). After final staining, the cells were analyzed immediately by FACS Verse.

PBMCs from BLV-infected cattle were cultured with 1 μM of GS-9620 for six days. Cultures were stimulated by adding a BLV antigen (fetal lamb kidney (FLK)-BLV; 2% heat-inactivated culture supernatant of FLK-BLV cells). As described in a previous paper, the FLK-BLV supernatant contained BLV antigens to activate BLV-specific T cells in PBMC cultures (Mager et al., 1994). After the incubation, culture supernatants were collected and the concentrations of IFN-γ and TNF-α were measured by ELISA as described above. The cells were also collected and CD69 expression in $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry as described above.

2.4. Functional analysis of GS-9620 using $CD11c^+$ cell-depleted PBMCs

To investigate whether GS-9620 induces the activation of T cells via $CD11c^+$ cells, $CD11c^+$ cells were depleted from the PBMCs of uninfected cattle. PBMCs were cultured with anti-bovine CD11c mAb (BAQ151A) for 30 min at 4 °C. Then, the cells were incubated with Anti-Mouse IgG_1 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 15 min

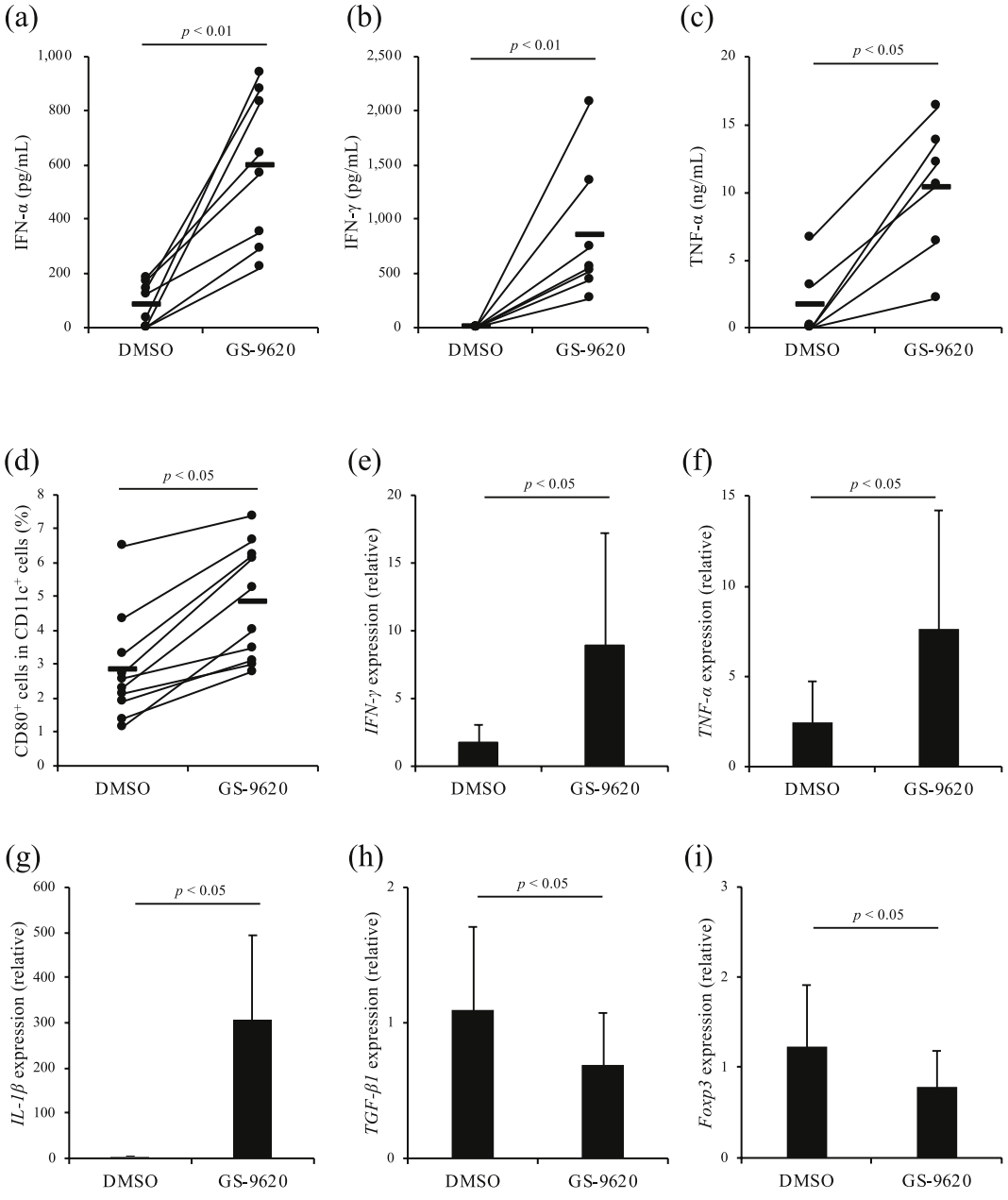

Fig. 1. Functional analysis of GS-9620 using PBMCs. (a–i) PBMCs from uninfected cattle were incubated with GS-9620. (a–c) The concentrations of IFN-α (a, $n = 8$), IFN-γ (b, $n = 7$), and TNF-α (c, $n = 6$) in culture supernatants were determined by ELISA. (d) The expression levels of CD80 in $CD11c^+$ cells were measured by flow cytometry ($n = 10$). (e–i) The gene expression levels of *IFN-γ* (e), *TNF-α* (f), *IL-1β* (g), *TGF-β1* (h), and *Foxp3* (i) were quantitated by qPCR ($n = 6$). (a–i) Statistical significance was determined by the Wilcoxon signed-rank test.

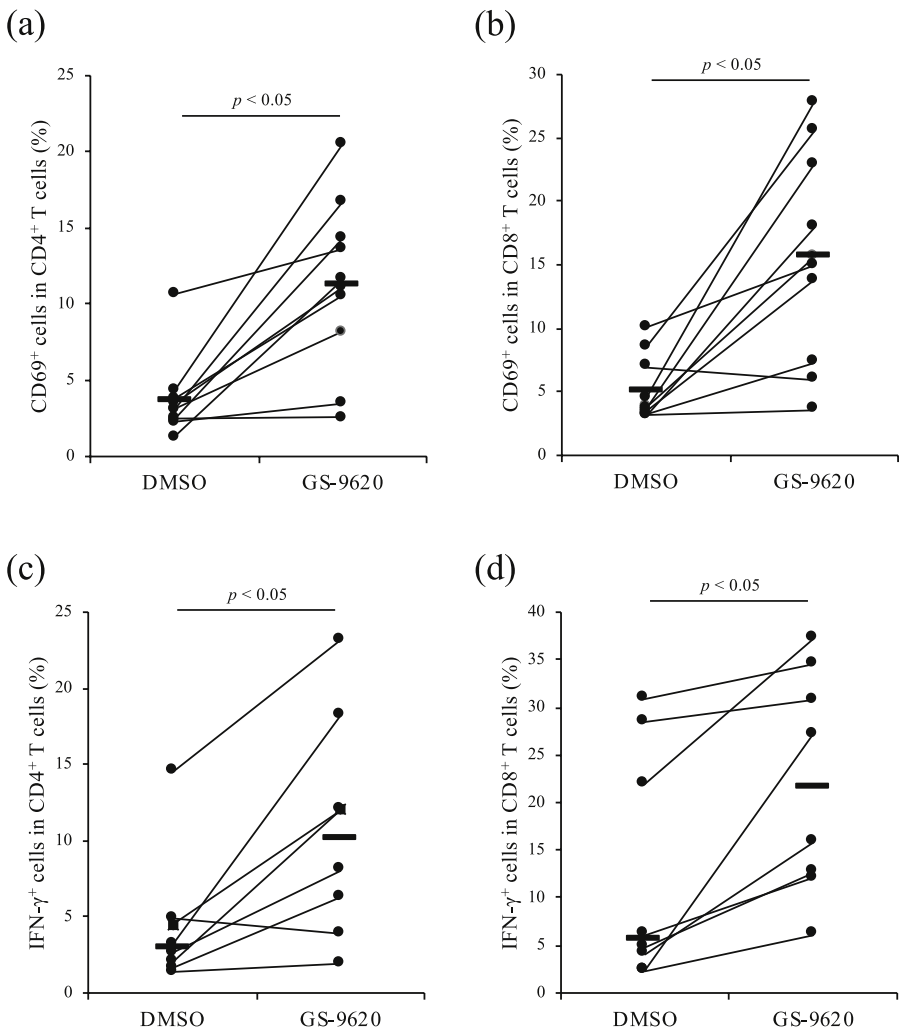

Fig. 2. The effects of GS-9620 on T cell activation (a and b) PBMCs from uninfected cattle were incubated with GS-9620 ($n = 10$). CD69 expression in $CD4^+$ (a) and $CD8^+$ (b) T cells was assayed by flow cytometry. (c and d) PBMCs from BLV-uninfected cattle were incubated with GS-9620 in the presence of anti-CD3 mAb and anti-CD28 mAb ($n = 8$). The expression levels of IFN-γ in $CD4^+$ (c) and $CD8^+$ (d) cells were quantified by flow cytometry. (a–d) Statistical significances were determined by the Wilcoxon signed-rank test.

at 4 °C. After the incubation, $CD11c^+$ cell sorting was performed using an autoMACS Pro (Miltenyi Biotec) according to the manufacturer's protocol. Negative fractions were used as $CD11c^+$ cell-depleted PBMCs, and positive fractions as $CD11c^+$ cells. The purity of the fractions was confirmed using FACS Verse. $CD11c^+$ cell-depleted PBMCs contained less than 3% of $CD11c^+$ cells. The purity of $CD11c^+$ cells was more than 95%. $CD11c^+$ cell-depleted PBMCs were cultivated with 1 μM of GS-9620 for two days, and then, the expression of CD69 in T cells was measured by flow cytometry as described above.

2.5. Carboxyfluorescein diacetate succinimidyl ester (CFSE) staining

$CD11c^+$ cell-depleted PBMCs and $CD11c^+$ cells were prepared from PBMCs of uninfected cattle using the autoMACS Pro as described above. $CD11c^+$ cell-depleted PBMCs were labeled with CFSE (Sigma-Aldrich) and cultivated in culture medium for 24 h. Simultaneously, $CD11c^+$ cells were cultured with 1 μM of GS-9620 for 24 h. Then, $CD11c^+$ cells were washed with culture medium, and co-cultured with $CD11c^+$ cell-depleted PBMCs for 24 h. After the incubation, cells were harvested and Fc blocking was performed as described above. The cells were stained with the following Abs: Alexa Fluor 647-labeled anti-CD4 mAb (CC30; Bio-Rad), PerCP/Cy 5.5-conjugated anti-CD8 mAb (CC63), and PE/Cy7-conjugated anti-IgM mAb (IL-A30). CC30 was prelabeled using the Zenon Alexa Fluor 647 Mouse IgG_1 Labeling Kit. CC63 was conjugated by using a Lightning-Link antibody labeling kit. After staining for 20 min at 25 °C, the cells were analyzed immediately by FACS Verse. After the incubation, culture supernatants were also collected and the concentration of IFN-γ was measured by ELISA as described above.

2.6. Identification of subpopulations in PBMCs

To analyze the percentage of T cells and B cells in uninfected and PL cattle, after Fc blocking, the cells were stained with the following Abs: PerCP/Cy 5.5-conjugated anti-CD3 mAb (MM1A), FITC-conjugated anti-CD4 mAb (CC8), PE-conjugated anti-CD8 mAb (CC63), and PE/Cy7-conjugated anti-IgM mAb (IL-A30). After staining for 20 min at 25 °C, PBMCs were washed twice with PBS, and analyzed immediately using the FACS Verse. To analyze the percentage of monocytic cells in uninfected and PL cattle, PBMCs were stained with the following Abs after Fc blocking: PerCP/Cy 5.5-conjugated anti-CD14 mAb (CAM36A; Washington State University Monoclonal Antibody Center) and Alexa Fluor 647-labeled anti-CD11c mAb (BAQ151A) for 20 min at 25 °C. CAM36A was conjugated using the Lightning-Link antibody labeling kit. After the staining, PBMCs were washed twice with PBS, and analyzed immediately by FACS Verse.

2.7. Expression analysis of TLR7 gene in $CD11c^+$ cells

To quantify the gene expression of *TLR7*, $CD11c^+$ cells were isolated from the PBMCs of BLV-infected and uninfected cattle as described above. *TLR7* expression was measured by qPCR as described above. Primers were 5′-ACT CCT TGG GGC TAG ATG GT-3′ and 5′-GCT GGA GAG ATG CCT GCT AT-3′ for *TLR7*.

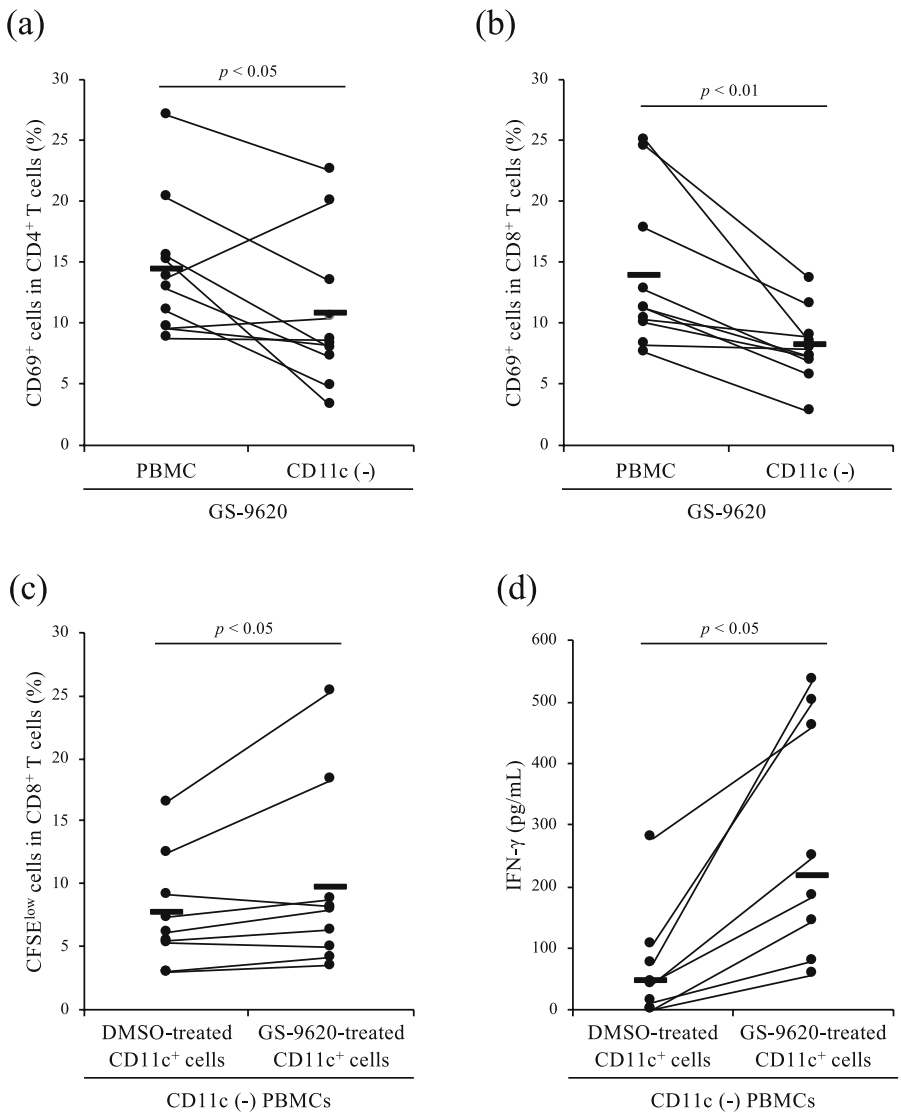

Fig. 3. T-cell activation by GS-9620 via $CD11c^+$ cells. (a and b) PBMCs from BLV-uninfected cattle were incubated with GS-9620 ($n = 10$). The expression of CD69 in $CD4^+$ (a) and $CD8^+$ (b) T cells was assayed by flow cytometry. (c and d) $CD11c^+$ cell-depleted PBMCs from BLV-uninfected cattle were cocultured with GS-9620 treated-$CD11c^+$ cells. (c) $CD8^+$ cell proliferation was assayed by flow cytometry ($n = 9$). (d) IFN-γ concentrations in culture supernatants were determined by ELISA ($n = 8$). (a–d) Statistical significance was determined by the Wilcoxon signed-rank test. CD11c (−), $CD11c^+$ cell-depleted PBMCs.

2.8. Functional analysis of the combined treatment of GS-9620 with anti-PD-L1 Ab

PBMCs from BLV-infected cattle were cultured with 1 μM of GS-9620 and/or 10 μg/mL of anti-PD-L1 Ab (Boch4G12; Nishimori et al., 2017) for six days. DMSO and/or bovine IgG (Sigma-Aldrich) were used as negative controls. Cultures were stimulated with FLK-BLV. After the incubation, culture supernatants were collected and the concentrations of IFN-γ and TNF-α were measured by ELISA as described above. The cells were also collected and CD69 expression in $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry as described above.

2.9. Syncytium assay

CC81 cells (0.6×10^5 cells/mL) were pre-cultured and used as indicator cells (Ferrer et al., 1981). After 24 h, PBMCs (5×10^5 cells/mL) from BLV-infected cattle were added to each well, and co-cultured with CC81 cells in the presence of 1 μM of GS-9620 and/or 10 μg/mL of anti-PD-L1 Ab (Boch4G12) for 70 h. DMSO and/or bovine IgG were used as negative controls. After the incubation, the cells were fixed with methanol (Sigma-Aldrich), and fixed cells were then stained with Giemsa's azur eosin methylene blue solution (Merck KGaA, Darmstadt, Germany). Syncytium formation was observed using a ZOE Fluorescent Cell Imager (Bio-Rad). The syncytium numbers represented the means from three independent culture wells.

2.10. Statistics

In Fig. 4a–e, differences were identified using the Mann-Whitney *U* test. In the other figures, statistical differences were identified using the Wilcoxon signed-rank test for comparing two-group and the Steel-Dwass test for comparing multiple groups. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. The activation of immune responses by GS-9620

To examine whether TLR7 agonists activate immune the responses in cattle, we analyzed the function of GS-9620 in cattle. PBMCs from uninfected cattle were cultivated with the TLR7 agonist, and the levels of cytokines in culture supernatants were measured using ELISA. As shown in Fig. 1a–c, GS-9620 significantly increased the production of IFN-α, IFN-γ, and TNF-α (Fig. 1a–c). Additionally, GS-9620 upregulated the expression of CD80, a maturation marker, in $CD11c^+$ cells (Fig. 1d) and significantly increased the gene expression levels of *IFN-γ*, *TNF-α*, and *IL-1β* in PBMCs (Fig. 1e–g). In contrast, GS-9620 decreased the expression of *TGF-β1* and *Foxp3* in PBMCs (Fig. 1h and i). These results suggest that

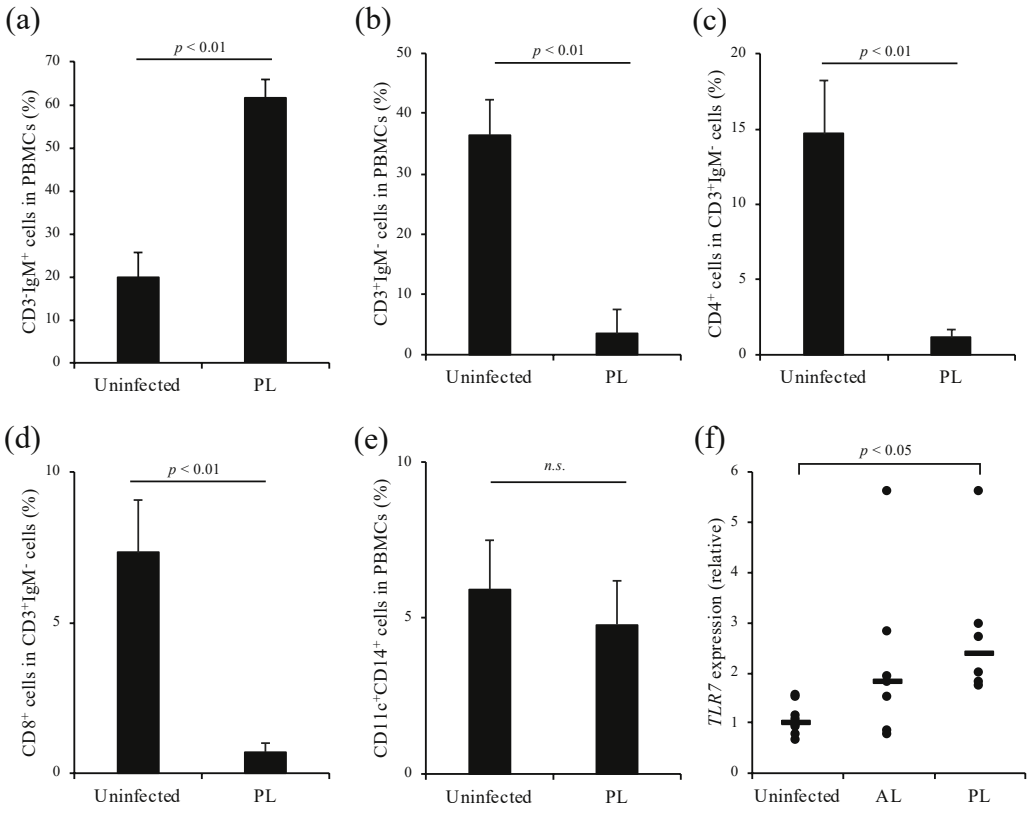

Fig. 4. The percentages of T cells, B cells, and CD11c$^+$ cells in PBMCs and *TLR7* expression in CD11c$^+$ cells from uninfected and BLV-infected cattle. The percentages of B cells (a, CD3$^-$ IgM$^+$), T cells (b, CD3$^+$ IgM$^-$), CD4$^+$ T cells (c, CD3$^+$ IgM$^-$ CD4$^+$), CD8$^+$ T cells (d, CD3$^+$ IgM$^-$ CD8$^+$), and CD11c$^+$ cells (e) in PBMCs from uninfected and PL cattle were analyzed by flow cytometry ($n = 6$ each). (f) CD11c$^+$ cells were isolated from PBMCs of uninfected and BLV-infected cattle (uninfected, $n = 8$; AL, $n = 7$; PL, $n = 6$). *TLR7* expression was quantitated by qPCR. (a–f) Statistical significance was determined by the Mann-Whitney *U* test (a–e) and the Steel-Dwass test (f). Uninfected, cattle not infected with BLV; PL, persistent lymphocytosis; n. s., not significant; AL, aleukemic.

GS-9620 treatment activates immune responses, in cattle, including inflammatory responses and Th1 responses. In general, IFN-γ and TNF-α are known as Th1-related cytokines. Therefore, we determined whether GS-9620 activates Th1 responses in cattle. As shown in Fig. 2a and b, GS-9620 induced CD69 expression, an activation marker, in CD4$^+$ and CD8$^+$ T cells (Fig. 2a and b). Additionally, GS-9620 significantly increased the percentage of IFN-γ$^+$ cells in CD4$^+$ and CD8$^+$ T cells (Fig. 2c and d). Taken together, GS-9620 treatment has the potential to activate Th1 responses *in vitro*.

3.2. The induction of Th1 responses by the TLR7 agonist via CD11c$^+$ cells

To examine whether GS-9620-induced upregulation of the Th1 response is caused by CD11c$^+$ cell activation, CD11c$^+$ cell-depleted PBMCs were cultured with GS-9620. Flow cytometric analyses revealed that CD69 expression in CD4$^+$ and CD8$^+$ T cells was lower in the CD11c$^+$ cell-depleted PBMCs compared to PBMCs containing CD11c$^+$ cells (Fig. 3a and b). Additionally, CD11c$^+$ cells were pre-cultured with GS-9620 or DMSO, and pretreated CD11c$^+$ cells were co-cultured with CD11c$^+$ cell-depleted PBMCs. The pretreatment of CD11c$^+$ cells increased CD8$^+$ cell proliferation (Fig. 3c) and IFN-γ production in culture supernatants (Fig. 3d). Collectively, these results suggest that GS-9620 enhances the Th1 responses, at least in part, via CD11c$^+$ cells.

3.3. The percentages of T cells, B cells, and CD11c$^+$ cells in PBMCs from BLV-uninfected and PL cattle

Th1 response plays an important role for the control of bovine chronic infections including BLV infection (Orlik and Splitter, 1996; Kabeya et al., 2001). During late-stage of BLV infection, the number of circulating B cells is dramatically increased, whereas the number of circulating T cells is dramatically decreased (Nieto Farias et al., 2018). In this study, we confirmed the percentages of these cells in PBMCs derived from PL cattle. As expected, the percentage of B cells (CD3$^-$IgM$^+$ cells) in PBMCs from PL cattle was significantly higher than the percentage of B cells in PBMCs from uninfected cattle (Fig. 4a). In contrast, the percentages of T cells (CD3$^+$IgM$^-$ cells), CD4$^+$ T cells, and CD8$^+$ T cells in PL cattle were dramatically lower than the percentages in uninfected cattle (Fig. 4b–d). In addition, we analyzed the percentage of monocytic cells in PBMCs from PL cattle. Interestingly, the percentage of CD11c$^+$CD14$^+$ cells in PBMCs was not significantly different between BLV-uninfected and PL cattle (Fig. 4e). Furthermore, *TLR7* expression was significantly upregulated in CD11c$^+$ cells of PL cattle (Fig. 4f).

3.4. The effects of the combined treatment with the TLR7 agonist and anti-PD-L1 Abs on Th1 responses and syncytium formation

We then examined the effects of GS-9620 on the activation of Th1 responses using PBMCs from BLV-infected cattle *in vitro*. The treatment with GS-9620 significantly increased IFN-γ and TNF-α production from PBMCs of BLV-infected cattle in the presence of FLK-BLV, a BLV antigen (Fig. 5a and b). Additionally, CD69 expression in CD4$^+$ and CD8$^+$ T cells was upregulated by the treatment with GS-9620 (Fig. 5c and d). To examine whether the combined treatment with GS-9620 enhances the efficacy of anti-PD-L1 Abs, PBMCs from BLV-infected cattle were cultured with anti-PD-L1 Abs and GS-9620 in the presence of FLK-BLV. The combined treatment with GS-9620 and anti-PD-L1 Abs increased the production of IFN-γ and TNF-α to a greater extent compared to treatment with anti-PD-L1 antibody or GS-9620 alone (Fig. 5e and f). In addition, the combined treatment tended to upregulate CD69 expression in CD4$^+$ and CD8$^+$ T cells (Fig. 5g and h). Finally, we examined the effects of the combined treatment on syncytium formation. Representative pictures of syncytium formation in each treatment were shown in Fig. 6a. The treatment with GS-9620 significantly inhibited syncytium formation by BLV compared to the control group (Fig. 6b, Table 1). Moreover, the combined treatment tended to inhibit syncytium formation compared to treatment with anti-PD-L1 antibody or GS-9620 alone

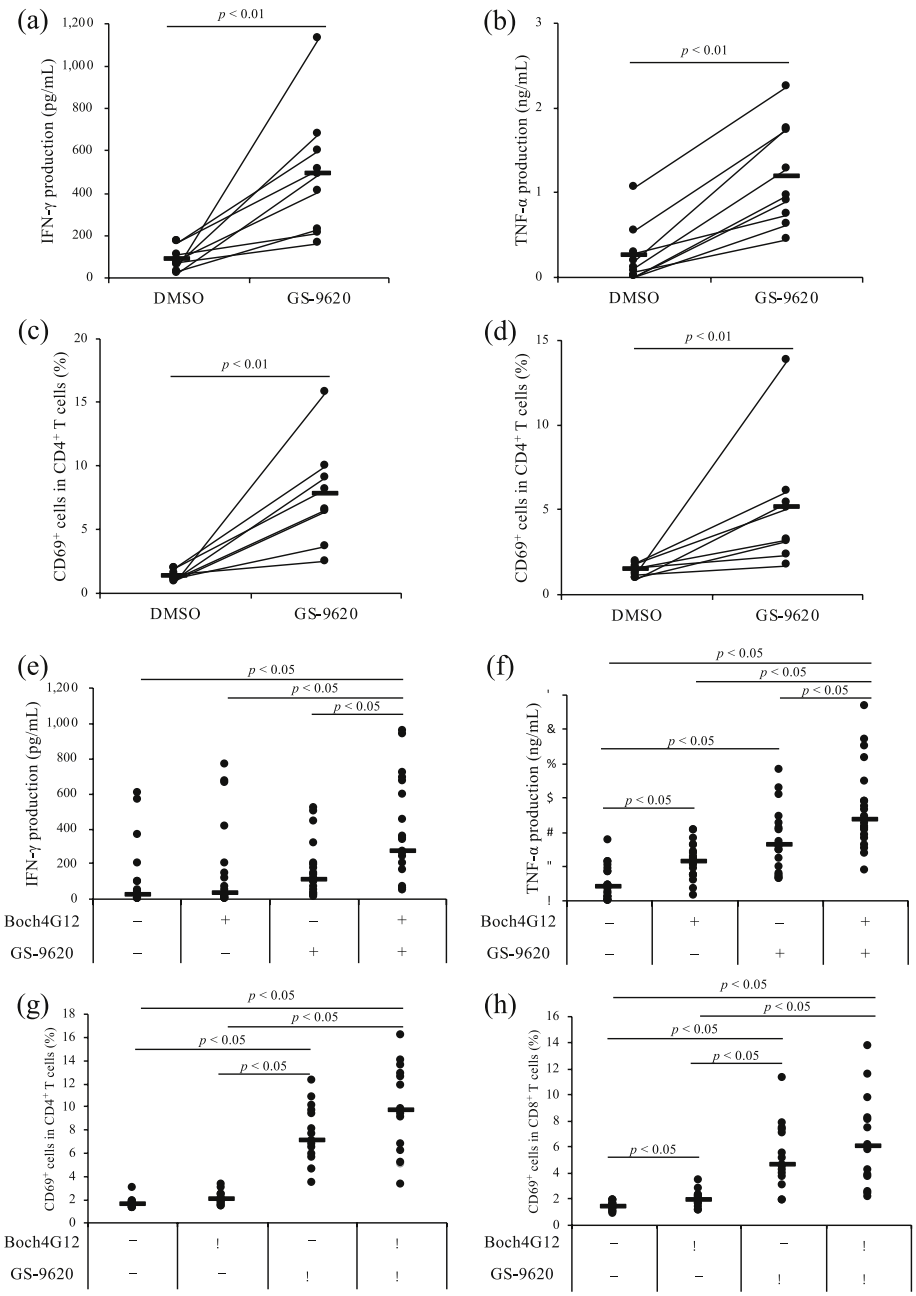

Fig. 5. Functional analysis of the combined treatment of GS-9620 with anti-PD-L1 Abs. (a–d) PBMCs from BLV-infected cattle were cultured with GS-9620 in the presence of FLK-BLV. IFN-γ (a) and TNF-α (b) concentrations in culture supernatants were measured by ELISA ($n = 9$). CD69 expression in $CD4^+$ (c) and $CD8^+$ (d) T cells was measured by flow cytometry ($n = 8$). Statistical significance was determined by the Wilcoxon signed-rank test. (e–h) PBMCs from BLV-infected cattle were cultured with GS-9620 and/or anti-PD-L1 Abs in the presence of FLK-BLV. IFN-γ (e) and TNF-α (f) concentrations in culture supernatants were measured by ELISA ($n = 20$). CD69 expression in $CD4^+$ (g) and $CD8^+$ (h) T cells was measured by flow cytometry ($n = 15$). Statistical significance was determined by the Steel-Dwass test.

(Fig. 6b, Table 1). These data suggest that the combined treatment with anti-PD-L1 Ab and the TLR7 agonist could be a novel control strategy for treating BLV infection.

4. Discussion

During BLV infection, the suppression of BLV-specific Th1 responses, including T-cell proliferation and cytotoxic T cell responses against BLV antigens, is associated with disease progression (Orlik and Splitter, 1996; Kabeya et al., 2001). Therefore, Th1 responses are important for controlling BLV infection. In this study, we found that the percentage of $CD11c^+$ cells in PBMCs was not changed between uninfected cattle and PL cattle, and *TLR7* expression in $CD11c^+$ cells was upregulated in PL cattle. In addition, functional analyses of the TLR7 agonist revealed that the treatment of GS-9620 increased T-cell activation, including Th1 cytokine production, at least in part, through $CD11c^+$ cells. Furthermore, GS-9620 treatment reduced the number of syncytia compared to the control group. A previous study has shown the antiviral activity of recombinant bovine IFN-γ against BLV using syncytium assay (Sentsui et al., 2001). Thus, the antiviral effect of GS-9620 is caused presumably due to the induction of IFN-γ production. These data suggest the potential of TLR7 agonism as a novel control method for controlling BLV infection. However, it is still unclear whether the TLR7 agonist activates BLV-specific Th1 responses *in vivo*. Further clinical studies on the efficacy of the TLR7 agonist in BLV-infected cattle will be needed.

Our previous studies have shown that anti-PD-L1 Ab administration significantly reduces BLV proviral loads in AL cattle (Nishimori et al., 2017; Sajiki et al., 2019). However, the anti-PD-L1 Ab did not reduce BLV proviral loads in PL cattle (Sajiki et al., 2019). During late-stage of BLV infection, Th1 responses are not only suppressed by the PD-1/PD-L1 pathway but also by other immunosuppressive factors such as PGE_2 and regulatory T cells (Tregs) (Ohira et al., 2016; Konnai et al., 2017; Sajiki et al., 2019). Thus, PL cattle might be resistant to the anti-PD-L1 Abs alone due to Th1 suppression via several immunoinhibitory factors. To

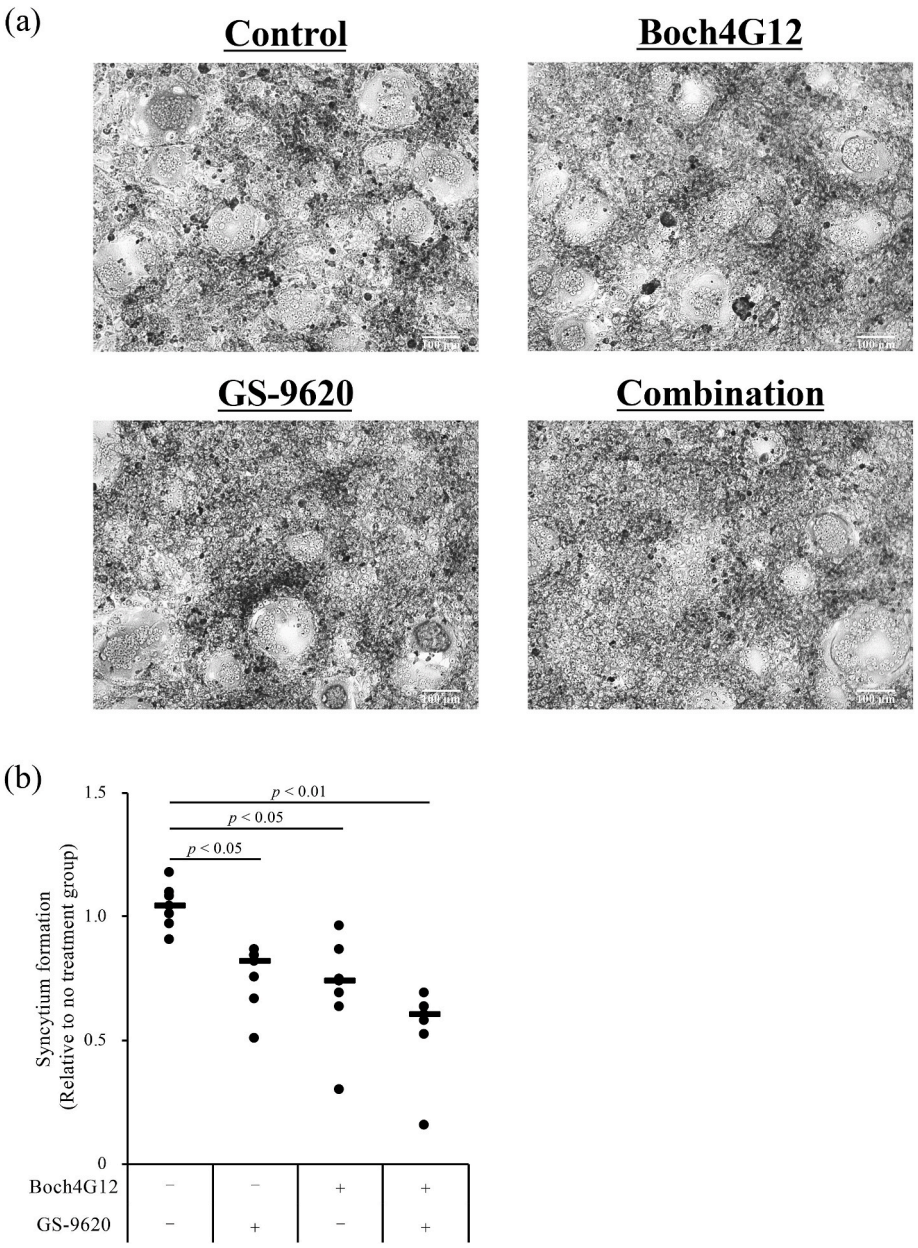

Fig. 6. The inhibition of syncytium formation by the combined treatment of GS-9620 with anti-PD-L1 Abs. (a and b) CC81 cells and PBMCs of BLV-infected cattle (n = 7) were co-cultured in the presence of GS-9620 and/or anti-PD-L1 Abs, and the number of syncytia was counted. (a) Representative pictures of syncytium formation in each treatment. (b) The results were shown as relative changes to no treatment group. Statistical significance was determined by the Steel-Dwass test.

Table 1
The number of syncytia in each treatment group.

Cattle	Number of syncytia (/cm^2)				
	No treatment	Control	GS-9620	Boch4G12	Combination
#1	1118.6	1313.0	769.5	940.1	702.1
#2	1178.1	1293.1	741.8	880.6	674.3
#3	53.6	55.5	51.6	35.7	27.8
#4	779.5	785.4	674.3	654.5	468.1
#5	345.1	333.2	103.1	174.5	51.6
#6	487.9	527.6	357.0	420.5	309.4
#7	380.8	345.1	281.6	309.4	261.8
Median	487.9	527.6	357.0	420.5	309.4

overcome this issue, combining anti-PD-L1 Abs with other drugs that enhance the immune responses may be an effective strategy. A previous study has reported that PD-L1 blockade combined with TLR7 agonists enhances the antitumor immune response in murine tumor models (Nishii et al., 2018). In the present study, we demonstrated that the combined treatment with anti-PD-L1 Ab and GS-9620 significantly enhanced BLV-specific Th1 cytokine production *in vitro*. In addition, the combined treatment tended to inhibit syncytium formation compared to the single treatment with GS-9620 or anti-PD-L1 Abs. These data suggest that this combined treatment could be an effective strategy for overcoming the resistance to PD-L1 blockade in BLV-infected cattle. To the best of our knowledge, this is the first study showing that a TLR7 agonist could be used in combination therapies to overcome the resistance to PD-L1 blockade in bovine infections.

Tregs are immune cell populations which limit immune responses by producing inhibitory cytokines, such as TGF-β and IL-10, and maintain host immune homeostasis (Sakaguchi et al., 2008). Tregs dampen T-cell immunity against cancers and infectious diseases, being an obstacle that tempers successful immunotherapy (Belkaid and Rouse, 2005; Zou,

2006). It is reported that inducing TGF-β secretion from Tregs in BLV-infected cattle reduces Th1 cytokine production (Ohira et al., 2016). Thus, the inhibition of Tregs would be important for the control of BLV infection. Interestingly, previous reports have demonstrated that TLR7 stimulation suppresses the function of Tregs via DC activation (Hackl et al., 2011; Wang et al., 2015). Also, the anti-PD-L1 Abs with TLR7 agonists overcome the resistance to anti-PD-L1 immunotherapy by attenuating Tregs via DC modification (Nishii et al., 2018). Our results showed that the treatment with GS-9620 decreased the gene expression of *TGF-β1* and *Foxp3* in bovine PBMCs. Foxp3 is a transcription factor that is required for the development of Tregs (Hori et al., 2003). Therefore, these results suggest that GS-9620 activates the Th1 immune response via the inhibition of Tregs. Additional experiments are needed to reveal the effect of TLR7 agonists on Treg function in cattle.

5. Conclusion

Our data in the present study showed that TLR7 is a potential therapeutic target for enhancing antiviral immunity through $CD11c^{+}$ cell activation in cattle. In addition, the combined treatment of the TLR7 agonist with anti-PD-L1 Abs enhanced Th1 cytokine production from PBMCs of BLV-infected cattle *in vitro*. Future studies, which further analyze the efficacy of the treatment of the TLR7 agonist with or without anti-PD-L1 Abs in BLV-infected cattle, will open up new avenues for the treatment of BLV infection.

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Author contributions

YSa, SK, SM, and KO: designed the work; YSa, TO, NM, and HN: performed the experiments; YSa, SK, TO, NM, and HN: acquired, analyzed, and interpreted the data; YK and YSu: provided laboratory materials and reagents; YSa and SK: wrote the manuscript; TO, NM, YK, YSu, SM, and KO: revised the manuscript. All authors reviewed and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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