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

Yamato Sajiki a, Satoru Konnai a,b,c, Tomohiro Okagawa b, Naoya Maekawa b, Hayato Nakamura a, Yukinari Kato c,d, Yasuhiro Suzuki b,d,e, Shiro Murata a,b, Kazuhiko Ohashi a,b

a Department of Disease Control, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, Japan
b Department of Advanced Pharmaceutics, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, 060-0818, Japan
c Department of Antibody Drug Development, Tohoku University Graduate School of Medicine, Sendai, 980-8575, Japan
d New Industry Creation Hatchery Center, Tohoku University, Sendai, 080-8575, Japan
e Division of Bioresources, Research Center for Zoonosis Control, Hokkaido University, Sapporo, 001-0019, Japan

ARTICLE INFO

Keywords:
Bovine leukemia virus
TLR7 agonist
CD11c+ cell
T cell
Anti PD-L1 antibody
Cattle

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 infection.
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) agonists to treat viral infections.

Nishii et al. showed the potential of TLR7 agonists to overcome resistance to anti-PD-L1 Abs through the modification of DCs (Nishii et al., 2017; Sajiki et al., 2019), suggesting a potential 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; Oka et al., 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 10%–20% (Ohira et al., 2016).

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 especially in PL cattle. GS-9620 treatment induced Th1 cytokine production and inhibited syncytium formation especially in PL 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 in serum using a commercially available ELISA kit (JNC, Tokyo, Japan) and the concentrations of IFN-γ, TNF-α, and IFN-γ were measured using an ELISA 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-10, 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′-ATG TCA CCT TCC AAA GG-3′ and 5′-ATT CTG ACT TCT CTT CCG TCT CGG-3′ for IFN-γ, 5′-TAA CAA GCC AGT AGC CCA CGG-3′ and 5′-GCA AGG GCT GAT GGC AGA-3′ for TNF-α, 5′-ACC TTC ATT GGC CAG CAG G CTG-3′ and 5′-CTT GTT AGC TTC ATG AAC CCA CAA A-3′ for IL-10, 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 ACC ATG CTG AGC CAC AA-3′ and 5′-TCT TG TGC GGA ACT CAA ACT CAT C-3′ for Foxp3, and 5′-TCT CTC AGG CCA AAG TTA AAA GTG-3′ and 5′-ACC GTT TTG GCC GTG AGG GTA G-3′ for TGF-β1. (See Table S1 for the sequences of the primers). The blood samples from BLV-infected and BLV-uninfected cattle were counted using a Celltac alpha (Kuraray Medical, Tokyo, Japan). PBMCs were cultured with 1 μM of GS-9620 for 20 min at 25 °C, the cells were washed twice with PBS, and analyzed immediately by using FACS Verse. 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: Alexa Fluor 647-labeled anti-CD11c mAb (BAQ151A; Washington State University Monoclonal Antibody Center, Pullman, WA, USA) and PerCP/Cy5.5-conjugated anti-CD80 mAb (IL-A159; Bio-Rad, Hercules, CA, USA). BAQ151A was prelabeled using the Zenon Alexa Fluor 647 Mouse IgG1 Labeling Kit (Thermo Fisher Scientific). IL-A159 was conjugated 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).

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 (Chesmecne, 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-γ, TNF-α, and IFN-γ were measured using an ELISA 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-10, 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′-ATG TCA CCT TCC AAA GG-3′ and 5′-ATT CTG ACT TCT CTT CCG TCT CGG-3′ for IFN-γ, 5′-TAA CAA GCC AGT AGC CCA CGG-3′ and 5′-GCA AGG GCT GAT GGC AGA-3′ for TNF-α, 5′-ACC TTC ATT GGC CAG CAG G CTG-3′ and 5′-CTT GTT AGC TTC ATG AAC CCA CAA A-3′ for IL-10, 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 ACC ATG CTG AGC CAC AA-3′ and 5′-TCT TG TGC GGA ACT CAA ACT CAT C-3′ for Foxp3, and 5′-TCT CTC AGG CCA AAG TTA AAA GTG-3′ and 5′-ACC GTT TTG GCC GTG AGG GTA G-3′ for TGF-β1. (See Table S1 for the sequences of the primers).
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 IgG1 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.
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 labeled with CFSE. The purity of CD11c⁻ cells was more than 95%. CD11c⁺ cell-depleted PBMCs were cultured 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.

2.6. 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; Wash-ington 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. 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.
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 × 10^6 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 responses in cattle, we analyzed the function of GS-9620 in cattle. PBMCs from uninfected cattle were incubated 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
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, including in PBMCs from BLV-infected 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 percentages 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.
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 PGE2 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
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, 2008). Tregs are also known to have regulatory activity on adaptive immune responses in the setting of viral and helminth infections (Lazarova and Forsthuber, 2009). However, the role of Tregs in the context of bovine BLV infection remains largely unexplored.

Table 1
The number of syncytia in each treatment group.

<table>
<thead>
<tr>
<th>Cattle</th>
<th>Number of syncytia (/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
</tr>
<tr>
<td>#1</td>
<td>1118.6</td>
</tr>
<tr>
<td>#2</td>
<td>1178.1</td>
</tr>
<tr>
<td>#3</td>
<td>53.6</td>
</tr>
<tr>
<td>#4</td>
<td>779.5</td>
</tr>
<tr>
<td>#5</td>
<td>345.1</td>
</tr>
<tr>
<td>#6</td>
<td>487.9</td>
</tr>
<tr>
<td>#7</td>
<td>380.8</td>
</tr>
<tr>
<td>Median</td>
<td>487.9</td>
</tr>
</tbody>
</table>

Median values are shown below.

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.
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 (Horii 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 agent 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.

Funding

This work was supported by JSPS KAKENHI grant number 19K01072 [to S.K.], grants from the Project of the NARO, Bio-oriented Technology Research Advancement Institution (Research Program on Development of Innovative Technology 20658 BC [to S.K.]) and Special Scheme Project on Regional Developing Strategy, Grant 16B17557 [to S.K.]), regulatory research projects for food safety, animal health and plant protection (JP1008617.17935709 [to S.K.]) funded by the Ministry of Agriculture, Forestry and Fisheries of Japan and AMED under grant number JP20am0101078 [to Y.K.]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

Y.Sa, SK, SM, and KO: designed the work; Y.Sa, TO, NM, and HN: performed the experiments; Y.Sa, 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.

Acknowledgments

We are grateful to Dr. Hideyuki Takahashi, Dr. Yasuyuki Mori, and Dr. Tomio Ibayashi for valuable advice and discussions. We thank Enago (http://www.enago.jp) for the English language review.

References


Y. Sajiki et al. / Developmental and Comparative Immunology 114 (2021) 103847


