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PATHOGENESIS AND IMMUNITY

Combined Immune Checkpoint Blockade Enhances Antiviral Immunity against Bovine Leukemia Virus

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ABSTRACT Bovine leukemia virus (BLV) is a retrovirus that causes enzootic bovine leukosis (EBL) in cattle and is widespread in many countries, including Japan. Recent studies have revealed that the expression of immunoinhibitory molecules, such as programmed death-1 (PD-1) and PD-ligand 1, plays a critical role in immunosuppression and disease progression during BLV infection. In addition, a preliminary study has suggested that another immunoinhibitory molecule, T-cell immunoglobulin domain and mucin domain-3 (TIM-3), is involved in immunosuppression during BLV infection. Therefore, this study was designed to further elucidate the immunoinhibitory role of immune checkpoint molecules in BLV infection. TIM-3 expression was upregulated on peripheral $CD4^+$ and $CD8^+$ T cells in BLV-infected cattle. Interestingly, in EBL cattle, $CD4^+$ and $CD8^+$ T cells infiltrating lymphomas expressed TIM-3. TIM-3 and PD-1 were upregulated and coexpressed in peripheral $CD4^+$ and $CD8^+$ T cells from BLV-infected cattle. Blockade by anti-bovine TIM-3 monoclonal antibody increased CD69 expression on T cells and gamma interferon (IFN-γ) production from peripheral blood mononuclear cells from BLV-infected cattle. A syncytium formation assay also demonstrated the antiviral effects of TIM-3 blockade against BLV infection. The combined inhibition of TIM-3 and PD-1 pathways significantly enhanced IFN-γ production and antiviral efficacy compared to inhibition alone. In conclusion, the combined blockade of TIM-3 and PD-1 pathways shows strong immune activation and antiviral effects and has potential as a novel therapeutic method for BLV infection.

IMPORTANCE Enzootic bovine leukosis caused by bovine leukemia virus (BLV) is an important viral disease in cattle, causing severe economic losses to the cattle industry worldwide. The molecular mechanisms of BLV-host interactions are complex. Previously, it was found that immune checkpoint molecules, such as PD-1, suppress BLV-specific Th1 responses as the disease progresses. To date, most studies have focused only on how PD-1 facilitates escape from host immunity in BLV-infected cattle and the antiviral effects of the PD-1 blockade. In contrast, how T-cell immunoglobulin domain and mucin domain-3 (TIM-3), another immune checkpoint molecule, regulates anti-BLV immune responses is rarely reported. It is also unclear why PD-1 inhibition alone was insufficient to exert anti-BLV effects in previous clinical studies. In this study, the expression profile of TIM-3 in T cells derived from BLV-infected cattle suggested that TIM-3 upregulation is a cause of immunosuppression in infected cattle. Based on these results, anti-TIM-3 antibody was used to experimentally evaluate its function in influencing immunity against BLV. Results indicated that TIM-3 upregulation induced by BLV infection suppressed T-cell activation and antiviral

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cytokine production. Some T cells coexpressed PD-1 and TIM-3, indicating that simultaneous inhibition of PD-1 and TIM-3 with their respective antibodies synergistically restored antiviral immunity. This study could open new avenues for treating bovine chronic infections.

KEYWORDS bovine leukemia virus, TIM-3, PD-L1, lymphoma, cattle

In chronic infections and malignancies, T cells exposed to persistent antigenic stimuli and inflammation become exhausted T cells that are unable to perform their natural immune functions even after antigen presentation due to decreased production of cytokines, such as interferon-γ (IFN-γ), and decreased cellular proliferation (1). Exhausted T cells exhibit increased expression of immunosuppressive factors, such as programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), and T-cell immunoglobulin and mucin domain-3 (TIM-3). Exhausted T cells cannot respond to activation from T-cell receptors (TCRs) and costimulatory receptors via inhibition of activation signals, thereby suppressing T-cell effector functions (1). T-cell exhaustion mediated by immunosuppressive factors is involved in the progression and maintenance of pathological conditions in various chronic infections and malignancies. Interestingly, T-cell exhaustion interferes with immune responses against infections and tumors, but inhibiting immunosuppressive factors with specific antibodies can reverse this exhaustion and activate immune responses (2, 3). Thus, inhibitory antibodies against immunosuppressive factors may be an effective treatment for chronic infections and malignancies that have been difficult to treat.

TIM-3 is a transmembrane protein on the surface of cell membranes belonging to the TIM family and has been identified as a suppressor of $CD4^+$ Th1 cells (4). TIM-3 is expressed on Th1 cells, $CD8^+$ cytotoxic T cells (5), dendritic cells and monocytes (6), and natural killer cells in humans and mice (7). The intracellular region of TIM-3 contains tyrosine kinase phosphorylation sites; TIM-3 functions as a tyrosine kinase-type receptor (4, 8). Various factors have been reported as ligands for TIM-3, including galectin-9 (Gal-9) and carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam-1) (9–12). When TIM-3 binds to Gal-9 or Ceacam-1, it phosphorylates tyrosine residues in the intracellular region and suppresses TCR signaling via FYN, another tyrosine kinase (12–14). As a result, production of cytokines (Th1 cytokines, such as IFN-γ and interleukin-2 [IL-2], and inflammatory cytokines, such as tumor necrosis factor alpha [TNF-α] and IL-6) and cell proliferation are inhibited, and T-cell death is induced (9, 15, 16). Interestingly, the inhibitory effects of TIM-3 are ineffective when Ceacam-1 is deficient (12, 17). Because TIM-3 is highly expressed, especially in IFN-γ-secreting Th1 cells, cellular immunity is suppressed by selectively inducing cell death in Th1 cells (9). Studies using knockout mice have reported that Th1 cell-specific TIM-3 suppresses autoimmune diseases and transplant immune responses (18). Thus, TIM-3 is considered to be a suppressor of T-cell function.

In lymphocytic choriomeningitis virus (LCMV)-infected mice, a chronic infection model, TIM-3 is highly expressed in LCMV-specific $CD8^+$ T cells (19). In HIV-infected $CD4^+$ and HIV-specific $CD8^+$ T cells, TIM-3 expression, which increases with disease progression, promotes T-cell exhaustion (5). During hepatitis C virus (HCV) infection, suppression of $CD4^+$ and HCV-specific $CD8^+$ T-cell function by TIM-3 is associated with pathogenesis (20, 21). In contrast, the production of Gal-9, a ligand for TIM-3, is upregulated in liver Kupffer cells during HCV infection. Gal-9 induces the expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) and apoptosis of HCV-specific $CD8^+$ T cells (22). Such disease progression involving elevated TIM-3 expression has been reported not only in infectious diseases but also in tumor diseases, such as malignant melanoma (23). Studies in mouse tumor models and human tumor patients have shown that increased TIM-3 expression in tumor-infiltrating T cells (TILs) suppresses cellular immune responses, including cytokine production (24, 25). These findings suggest that the TIM-3 pathway may contribute to immune evasion mechanisms in chronic infectious and tumor diseases.

Bovine leukemia virus (BLV) infection is one of the most devastating bovine infectious diseases in Japan. BLV has a genetic structure most closely related to human T-cell leukemia virus (HTLV) (26) and persistently infects bovine lymphocytes, primarily B cells (27, 28). It causes enzootic bovine leukosis (EBL), leading to systemic lymphosarcoma formation, poor prognosis, and death (29). Although viral factors and the host immune response have been implicated in the development and progression of EBL (30, 31), the exact pathogenesis of the disease has not yet been elucidated. Comparative analysis of BLV-infected cattle by disease state revealed that persistently lymphocytotic (PL) cattle with increased B-cell lymphocyte counts had decreased expression of Th1 cytokines such as IFN-γ, IL-2, and IL-12 compared to alymphocytotic (AL) cattle with normal lymphocyte counts (32–35). $CD4^+$ T cells from EBL and PL cattle exhibit a decreased T-cell proliferative response to BLV antigens (33) and increased IL-10 mRNA expression, indicating a shift to humoral immunity (32). These findings suggest that immunosuppression, primarily a decrease in cellular immunity, is a major factor in disease progression in BLV-infected cattle.

Immunosuppression during BLV infection is associated with PD-1/PD-ligand 1 (PD-L1) (36–42), LAG-3 (43–45), and CTLA-4 (46, 47) expression; inhibition of each of these factors can have an immunostimulatory effect. In addition, the proportion of $PD\text{-}1^+$ $LAG\text{-}3^+$ $CD4^+$ and $PD\text{-}1^+$ $LAG\text{-}3^+$ $CD8^+$ T cells is increased in EBL-affected cattle; simultaneous inhibition of PD-1/PD-L1 and LAG-3 pathways in peripheral blood mononuclear cells (PBMCs) from BLV-infected cattle has an immunostimulatory effect (45). *TIM-3* mRNA expression increases in $CD4^+$ and $CD8^+$ T cells as BLV infection progresses, and inhibition of the TIM-3 pathway increases IFN-γ and IL-2 mRNA expression (48). Thus, these data suggest that TIM-3 is involved in immunosuppression during BLV infection; the TIM-3 inhibition and simultaneous inhibition of TIM-3 and other immunosuppressive factors may have overall immunostimulatory effects. In this study, an anti-bovine TIM-3 monoclonal antibody (MAb) was generated to investigate whether TIM-3-targeted therapies effectively improve immune function in BLV infection. First, TIM-3 expression on T cells from BLV-infected cattle was analyzed, and the association between bovine TIM-3 and immunosuppression and the immunostimulatory effects of TIM-3 pathway inhibition were examined using an anti-TIM-3 antibody. The immunostimulatory and antiviral effects of inhibiting both pathways were examined using anti-TIM-3 and anti-PD-L1 antibodies.

RESULTS

Increased TIM-3 expression on T cells in BLV-infected cattle. First, TIM-3 expression on $CD4^+$ and $CD8^+$ T cells in PBMCs from uninfected and BLV-infected cattle was analyzed by flow cytometry. Flow cytometric analyses showed that the percentage of $TIM\text{-}3^+$ $CD4^+$ T cells was significantly higher in BLV-infected cattle than in uninfected cattle (Fig. 1A). The percentage of $TIM\text{-}3^+$ $CD8^+$ T cells was also significantly higher in BLV-infected cattle than in uninfected cattle (Fig. 1A), indicating that TIM-3 expression was elevated in $CD4^+$ and $CD8^+$ T cells in peripheral blood of BLV-infected cattle. TIM-3 expression on infiltrating $CD4^+$ and $CD8^+$ T cells in the lymph nodes of uninfected cattle and cattle with EBL was analyzed. The percentages of $TIM\text{-}3^+$ $CD4^+$ and $TIM\text{-}3^+$ $CD8^+$ T cells were significantly higher in tumorigenic, superficial cervical lymph nodes (Fig. 1B) and mesenteric lymph nodes (Fig. 1C) derived from cattle with EBL than in those from uninfected cattle.

Immune activation by TIM-3 blockade. To clarify the effects of TIM-3 blockade on bovine T-cell responses, bioassays were first conducted using PBMCs from healthy cattle. PBMCs cultured *in vitro* with anti-TIM-3 antibody increased the percentage of CD69-expressing cells in $CD4^+$ and $CD8^+$ T cells (Fig. 2A). The percentage of $CD4^+$ and $CD8^+$ T cells expressing IFN-γ (Fig. 2B) increased with *in vitro* culture with anti-TIM-3 antibody. Consistent with the increase in cells expressing intracellular IFN-γ, blockade with anti-TIM-3 antibody increased IFN-γ production in the supernatant (Fig. 2C).

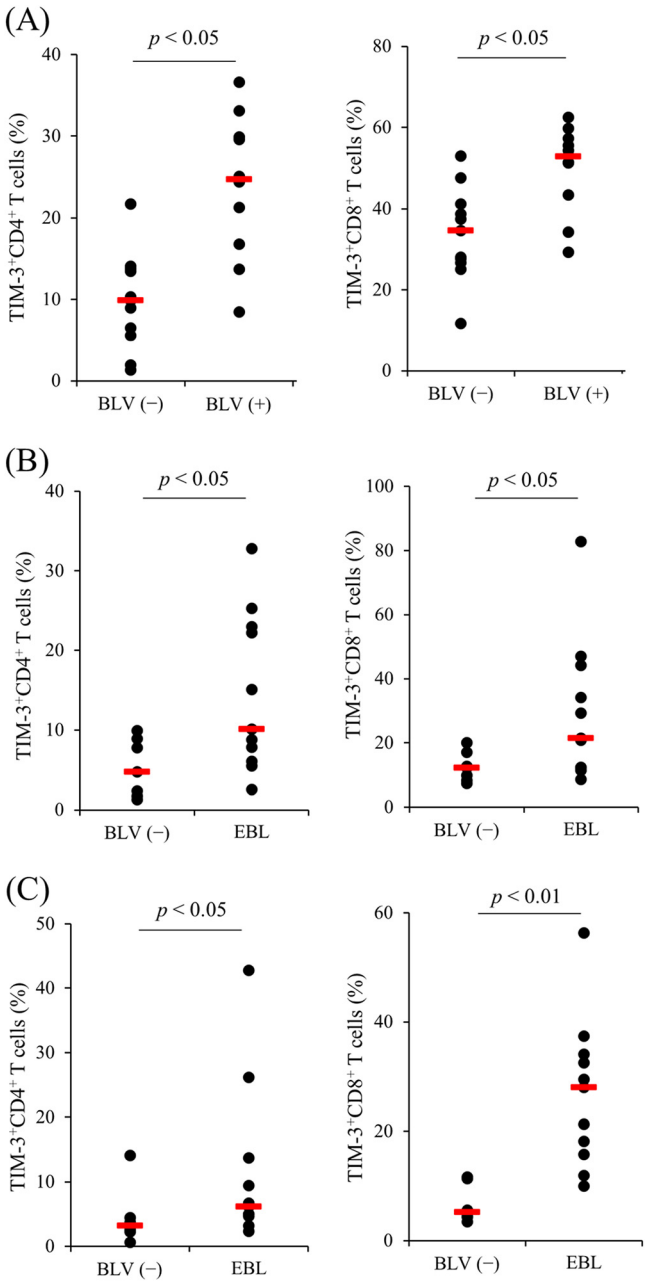

FIG 1 Percentages of TIM-3$^+$ CD4$^+$ or TIM-3$^+$ CD8$^+$ T cells from BLV-infected and uninfected cattle. (A) TIM-3 expression on CD4$^+$ and CD8$^+$ T cells in PBMCs from uninfected cattle [BLV (−); n = 11] and BLV-infected cattle [BLV (+); n = 10] was measured by flow cytometry. (B and C) TIM-3 expression was measured in infiltrating CD4$^+$ or CD8$^+$ T cells from tumorigenic shallow cervical lymph nodes (B) or tumorigenic mesenteric lymph nodes (C) in EBL cattle (n = 12). TIM-3 expression on CD4$^+$ or CD8$^+$ T cells in superficial cervical lymph nodes (B) or mesenteric lymph nodes (C) from uninfected bovines is shown as controls [BLV (−); n = 7]. Statistical significance was determined using the Mann-Whitney U test. The median values for each group are indicated by red bars.

These results indicated that inhibition of the TIM-3 pathway activates bovine T cells and increases IFN-γ production.

Next, PBMCs from BLV-infected cattle were used to examine the effects of the TIM-3 inhibition on the activation of T-cell responses to BLV antigen. PBMCs derived from BLV-infected cattle were cultured under BLV antigen stimulation along with anti-TIM-3 antibody. CD69 expression and IFN-γ production were analyzed. *In vitro* culture of PBMCs from BLV-infected cattle with anti-TIM-3 antibody increased the percentage of CD69-expressing CD4$^+$ and CD8$^+$ T cells (Fig. 3A) and IFN-γ production in the supernatant (Fig. 3B). These

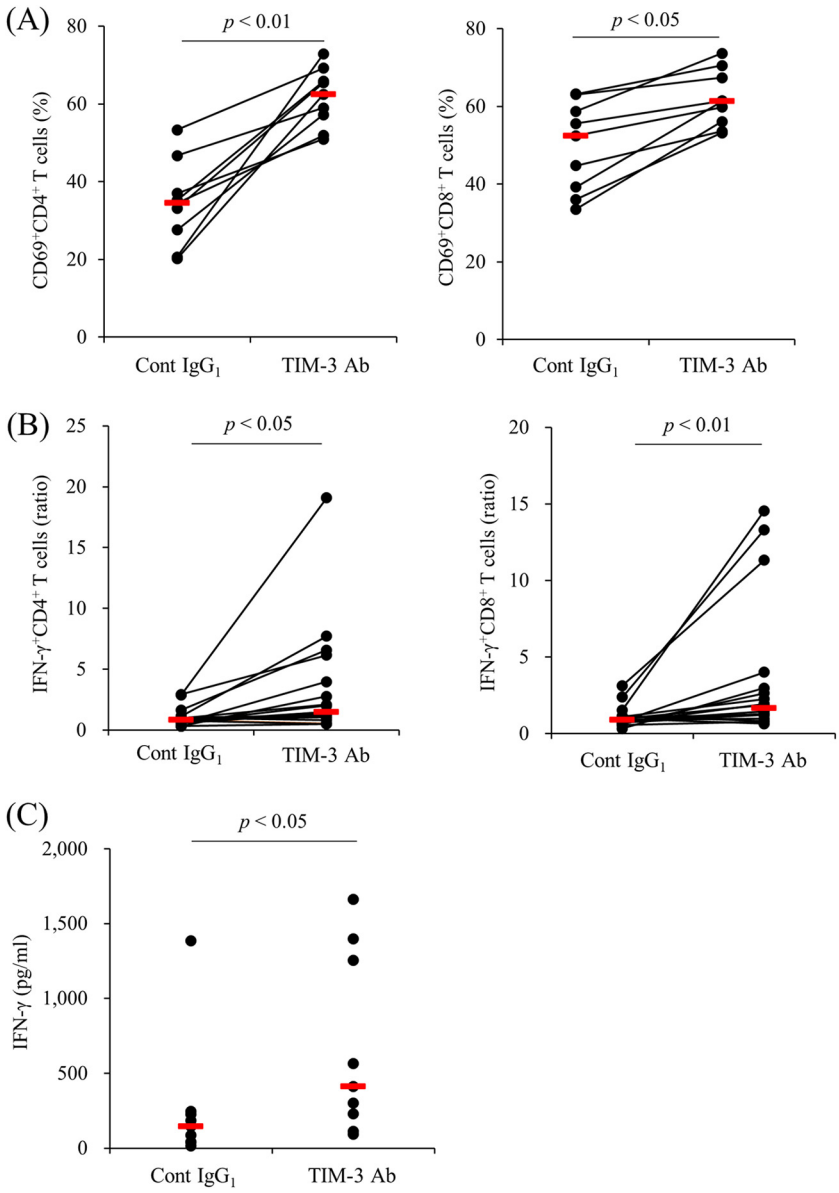

FIG 2 Effects of T-cell activation by inhibiting the TIM-3 pathway using PBMCs derived from healthy cattle. Bovine PBMCs stimulated with anti-bovine CD3 and CD28 MAbs were cultured with anti-TIM-3 antibody for 24 h. CD69 expression (A; $n = 9$) and IFN-γ intracellular expression (B; $n = 18$) were measured by flow cytometry. The number of IFN-γ^+ CD4$^+$ T cells was reported relative to T-cell stimulation alone. After 3 days of cultivation, IFN-γ in the culture supernatant was measured by ELISA (C; $n = 9$). Mouse IgG1 antibody was used as a negative control (Cont.). Each antibody was added to a final concentration of 10 μg/mL. Statistical significance was determined by the Wilcoxon signed-rank test.

results indicated that inhibition of the TIM-3 pathway could reactivate T-cell responses to BLV antigens in exhausted T cells from BLV-infected cattle.

Enhancement of antiviral immunity against BLV by combined immune checkpoint blockade. In previous studies, PD-1 expression was upregulated in BLV infection. In this study, TIM-3 and PD-1 expression levels in CD4$^+$ and CD8$^+$ T cells in PBMCs from BLV-infected cattle were simultaneously analyzed by flow cytometry (Fig. 4A). The percentage of TIM-3$^+$ PD-1$^+$ CD4$^+$ T cells was significantly higher in BLV-infected cattle than in uninfected cattle (Fig. 4B). The percentage of TIM-3$^+$ PD-1$^+$ CD8$^+$ T cells was also significantly higher in PBMCs from BLV-infected cattle than in uninfected cattle (Fig. 4B).

The percentage of T cells coexpressing TIM-3 and PD-1 increased in BLV-infected cattle. Thus, it was expected that the simultaneous inhibition of TIM-3 and PD-1/PD-L1

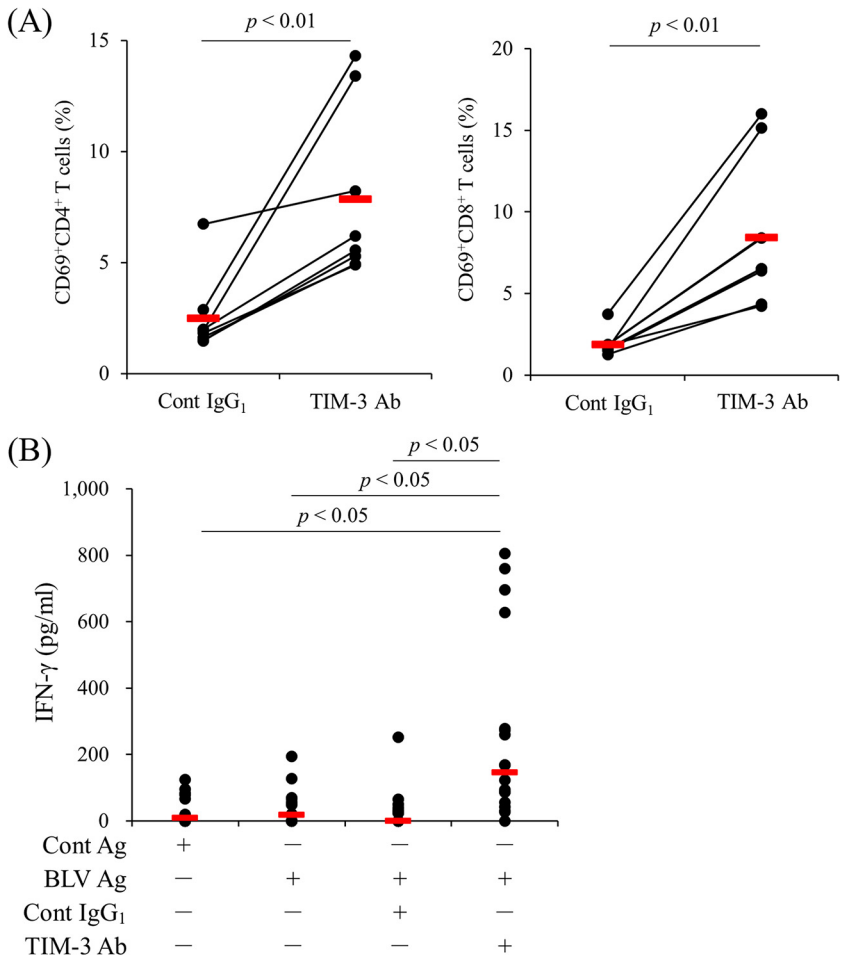

FIG 3 Effects of T-cell activation by inhibiting the TIM-3 pathway using PBMCs derived from BLV-infected cattle. PBMCs derived from BLV-infected cattle were incubated with anti-TIM-3 antibody in the presence of BLV antigen (Ag) for 24 h. CD69 expression on $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry (A; $n = 8$). IFN-γ production in the culture supernatant was measured by ELISA (B; $n = 18$). Mouse IgG_1 antibody was used as a negative control. Each antibody was added to a final concentration of 10 μg/mL.

pathways would enhance the immunostimulatory effects. Therefore, PBMCs derived from BLV-infected cattle were cultured with anti-TIM-3 and anti-PD-L1 antibodies under BLV antigen stimulation. The production of Th1 cytokines, IFN-γ and TNF-α, was analyzed by enzyme-linked immunosorbent assay (ELISA). These results indicated that the addition of anti-TIM-3 and anti-PD-L1 antibodies as single agents significantly increased IFN-γ production compared to control antibodies (Fig. 5A). The combined treatment with anti-TIM-3 and anti-PD-L1 antibodies resulted in a further significant increase in IFN-γ production compared to treatment with each alone (Fig. 5A). In addition, TNF-α production increased after combined treatment with anti-TIM-3 and anti-PD-L1 antibodies, although there was no significant difference from that after the treatment with each alone (Fig. 5B).

The simultaneous inhibition of TIM-3 and PD-1/PD-L1 pathways in BLV-infected cattle can reactivate exhausted T cells and strongly enhance T-cell responses to BLV. Therefore, the antiviral effects of TIM-3 and PD-1/PD-L1 pathway inhibition were evaluated by syncytium formation assay (Fig. 6A). Treatment with anti-TIM-3 or anti-PD-L1 antibodies significantly inhibited BLV-induced syncytium formation compared to controls; the combination of antibodies resulted in a stronger antiviral effect than single-agent treatment (Fig. 6B). These results indicated that inhibition of TIM-3 and PD-1/PD-L1 pathways has antiviral effects against BLV.

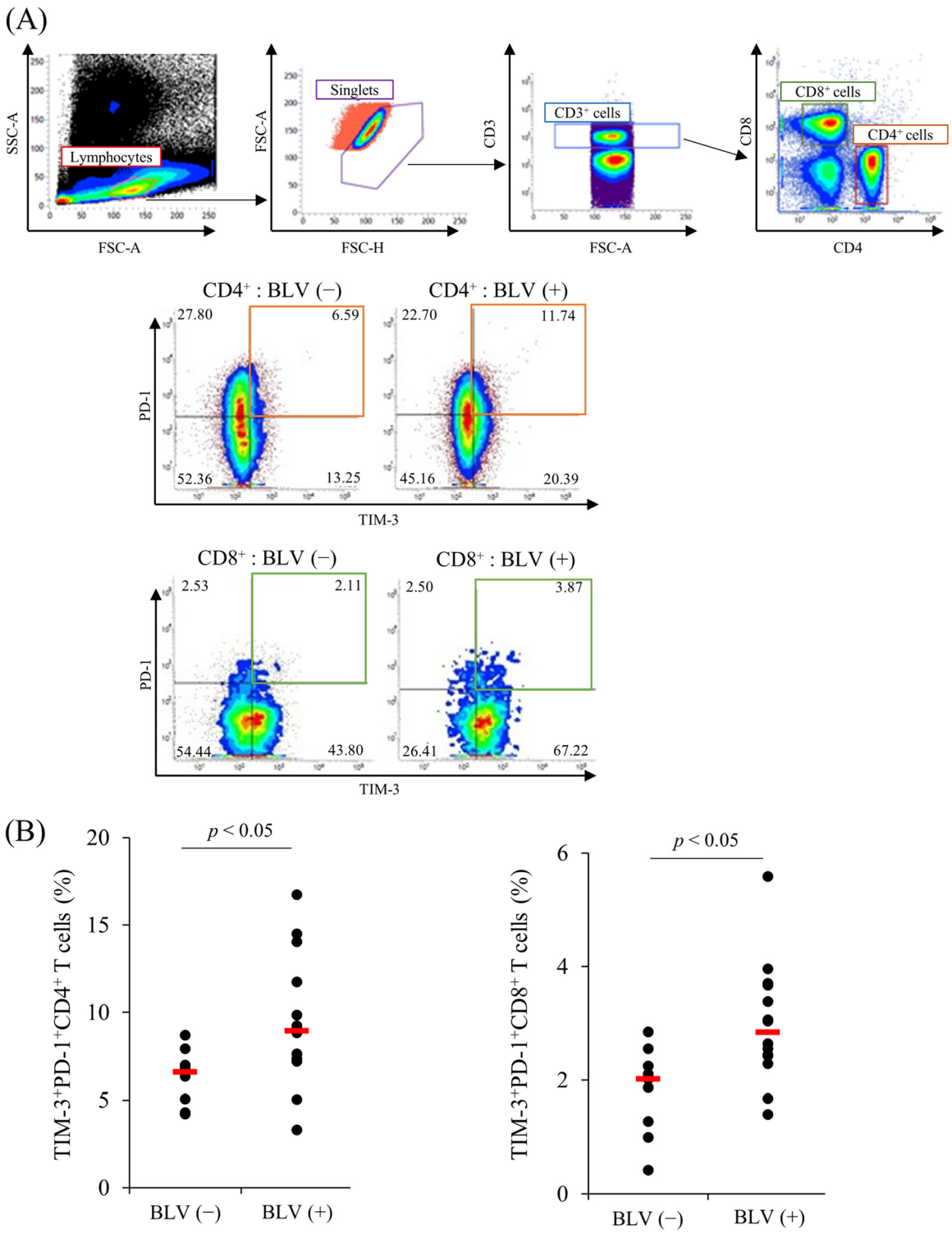

FIG 4 TIM-3/PD-1 coexpression in $CD4^+$ and $CD8^+$ T cells in PBMCs derived from BLV-infected cattle. (A) Gating strategy used for TIM-3 and PD-1 staining. Total lymphocytes were first gated in a forward scatter (FSC)/side scatter (SSC) plot and then in a single population. Cells were subsequently gated for the $CD3^+$ population and further gated for CD4 or CD8 expression. Gated $CD4^+$ and $CD8^+$ T cells were analyzed for TIM-3 and PD-1 coexpression, respectively. (B) Percentage of TIM-3^+ PD-1^+ $CD4^+$ or TIM-3^+ PD-1^+ $CD8^+$ T cells in uninfected cattle [BLV (−); n = 10] and BLV-infected cattle [BLV (+); n = 13]. The Steel-Dwass test was used for statistical analysis.

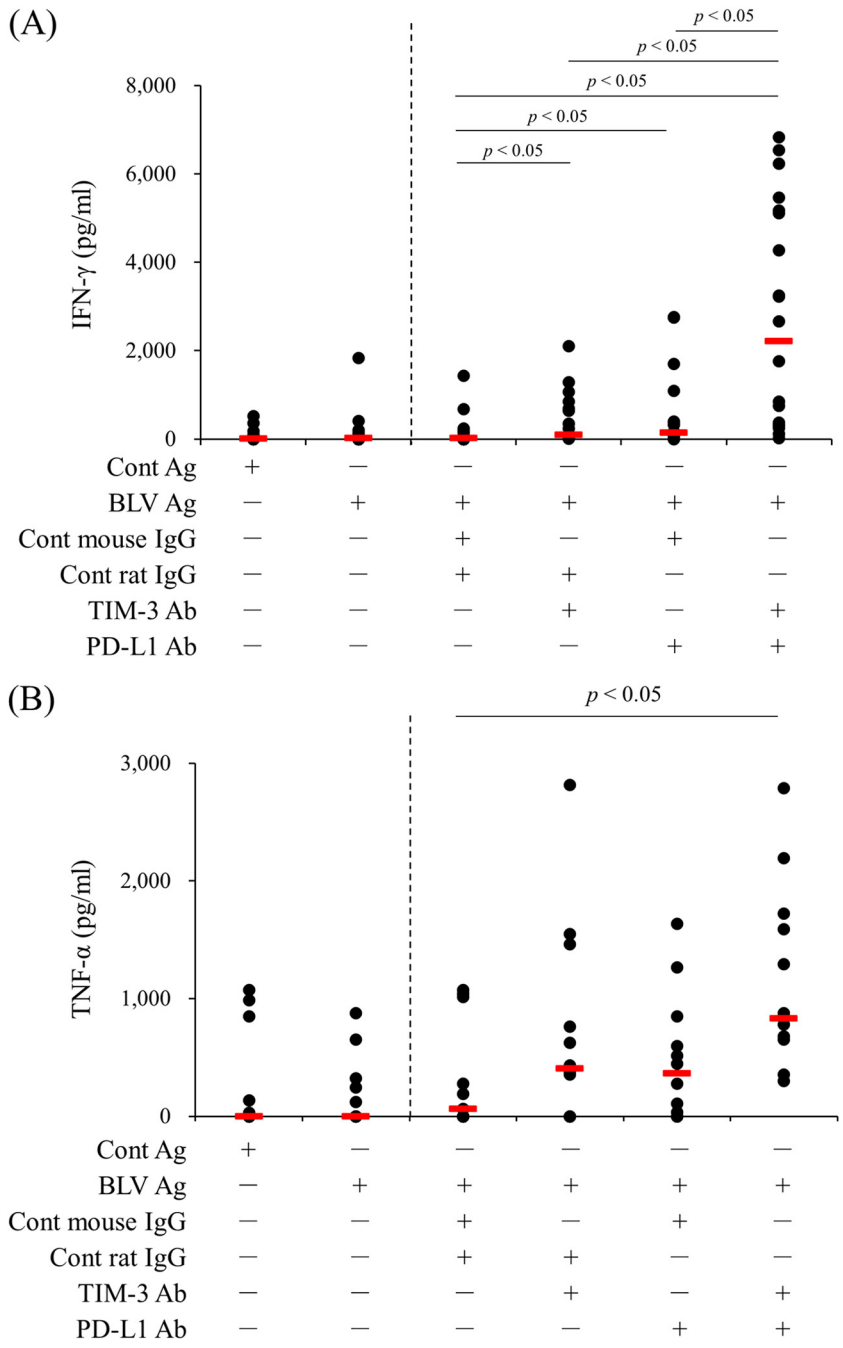

FIG 5 Effects of the dual inhibition of TIM-3 and PD-1 pathways on antiviral cytokine production using PBMCs derived from BLV-infected cattle. PBMCs from BLV-infected cattle (n = 20) were cultured with anti-TIM-3 and/or anti-PD-L1 antibodies in the presence of BLV antigen. IFN-γ (A) and TNF-α (B) concentrations in culture supernatants were measured by ELISA. Statistical significance was determined by the Steel-Dwass test.

DISCUSSION

Previous studies have shown that immunosuppressive receptors, such as PD-1, LAG-3, and CTLA-4, suppress cellular immunity during BLV infection (36–47). Another immunosuppressive receptor, TIM-3, has also been identified in cattle. A preliminary study showed that *TIM-3* mRNA expression increases in $CD4^+$ and $CD8^+$ T cells in the blood as BLV infection progresses (49). However, the details of TIM-3-mediated immunosuppressive mechanisms in BLV-infected cattle were unknown. This study analyzed

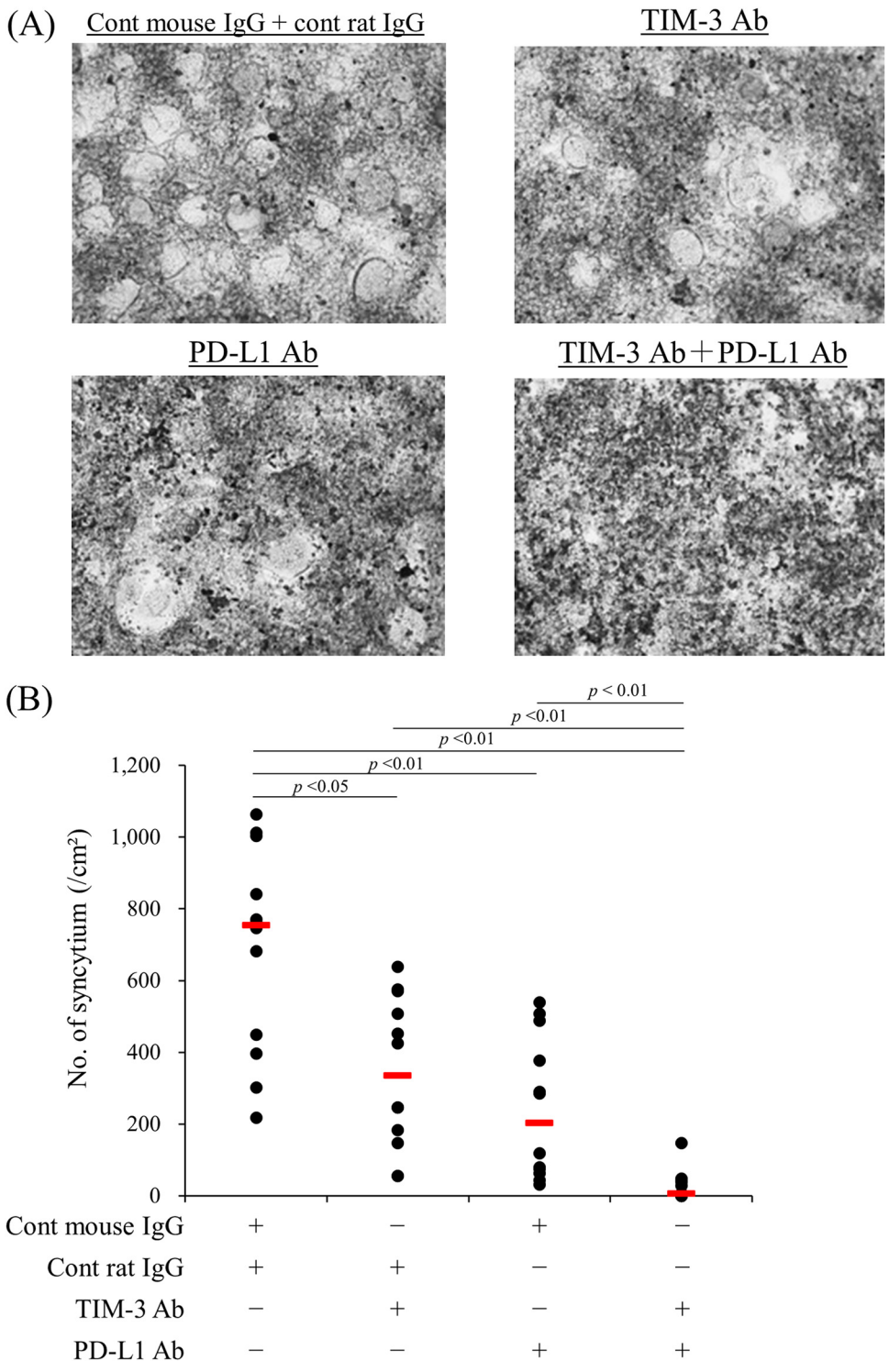

FIG 6 Antiviral effects of the dual inhibition of TIM-3 and PD-1 pathways using PBMCs derived from BLV-infected cattle. Indicator cells ($CC81^+$; 0.6×10^5) were cultured at 37°C for 24 h in the presence of 5% CO_2. Anti-TIM-3 and anti-PD-L1 antibodies were added simultaneously with PBMCs derived from BLV-infected cattle ($n = 12$; 5.0×10^5 cells) and cultured for 3 days. Mouse and rat IgG antibodies were used as negative controls. Each antibody was added at a final concentration of 10 μg/mL. After incubation, Giemsa staining was performed, and the number of syncytia containing >10 nuclei was measured. (A) Representative pictures of syncytium formation in each treatment. (B) Number of syncytia in each treatment. Three wells were cultured with PBMCs from each specimen bovine, and results were expressed as the mean from three wells. The Steel-Dwass test was used for statistical analysis. Median values for each group are indicated by red bars.

the expression and function of TIM-3 protein in BLV-infected cattle and its association with immunosuppression during the pathological progression of BLV infection.

TIM-3 protein expression in $CD4^+$ and $CD8^+$ T cells in peripheral blood of BLV-infected cattle was higher than in uninfected cattle, consistent with previous *TIM-3* mRNA kinetic analysis (49). A study that analyzed TIM-3 expression in patients infected with HIV, a member of the same family, *retroviridae*, as BLV, also reported that $CD4^+$ and $CD8^+$ T cells in peripheral blood had expressed higher levels of TIM-3 protein (5). This suggested that TIM-3 may also be involved in suppressing cellular immunity in BLV infection. In contrast, TIM-3 expression decreased in patients infected with HTLV, a virus closely related to BLV (49, 50). All BLV-infected cattle analyzed in this study were naturally infected; the timing of BLV infection and the stage of infection at the time of this analysis were unknown. Therefore, the detailed role of TIM-3 in BLV pathogenesis from infection to the onset of EBL requires further investigation. Interestingly, analysis of TIM-3 expression in BLV-infected cattle with EBL revealed increased TIM-3 protein expression in $CD4^+$ and $CD8^+$ T cells infiltrating tumorigenic lymph nodes. Similar to this finding, increased PD-1 expression was also observed in $CD4^+$ and $CD8^+$ T cells in peripheral blood and lymph nodes of EBL cattle (38). Studies in mouse models of colon adenocarcinoma and human renal cell carcinoma reported increased TIM-3^+ tumor-infiltrating lymphocytes in tumors and decreased production of Th1 cytokines, such as IFN-γ, in TIM-3^+ TILs (24, 25). These findings suggested that T cells with high expression of TIM-3 are exhausted, and Th1 responses are reduced in tumorigenic lymph nodes in EBL and other cancers in mice and humans. In this study, the number of collected TILs was limited, and functional analysis was not possible. It remains to be elucidated whether TIM-3^+ TILs, like TIM-3^+ T cells in peripheral blood, decrease the production of Th1 cytokines, such as IFN-γ, and inhibit tumor clearance.

Although the mechanism of elevated TIM-3 expression in BLV infection is unknown in this study, prostaglandin E_2 (PGE_2) may trigger the induction of TIM-3 expression. Previous studies showed that the PD-1/PD-L1 pathway suppresses cellular immunity in bovine chronic infectious diseases, such as BLV infection (36–42), Johne's disease (51–53), bovine anaplasmosis (54), and bovine mycoplasmosis (55, 56). In addition to the function of the PD-1/PD-L1 pathway in cattle, PGE_2 is involved in suppressing Th1 responses in bovine chronic infections. Specifically, this study focused on the function of PGE_2 because serum PGE_2 levels are elevated in bovine chronic infectious diseases, including BLV infection. PGE_2 induces PD-L1 expression in bovine immune cells via its receptor, E-prostanoid 4, thereby suppressing Th1 responses. More recently, PGE_2 induced the expression of not only PD-1 and PD-L1 but also LAG-3 and CTLA-4 (42, 57). Based on these data thus far, it is possible that PGE_2 induced by BLV also induces TIM-3 expression, which will be verified in future investigations.

Previously, several clinical studies were performed using an anti-bovine PD-L1 or PD-1 rat-bovine chimeric antibody (chAb) administered to BLV-infected cattle (40–42). The administration of these chAbs decreased the BLV provirus load in cattle. However, certain BLV-infected cattle did not respond to this treatment, suggesting the presence of immunosuppressive mechanisms using molecules other than those in the PD-1/PD-L1 pathway. In this study, increased TIM-3 expression was observed in T cells in peripheral blood and tumorigenic lymph nodes of BLV-infected cattle. Interestingly, the percentage of PD-1^+ TIM-3^+ $CD4^+$ and PD-1^+ TIM-3^+ $CD8^+$ T cells increased in peripheral blood of BLV-infected cattle. These results suggested that PD-1^+ TIM-3^+ T cells may be involved in pathological progression in BLV infection by inducing stronger immune exhaustion. In addition, Tregs that suppress immunity may be involved. Previous studies have suggested that Tregs increase with the progression of BLV infection, causing immunosuppression (35, 46). In mouse models of colorectal cancer, PD-1 expression increased on TIM-3^+ Tregs, and expression of inhibitory factors, such as IL-10 and CTLA-4, is elevated compared to that in TIM-3-negative Tregs (58, 59). In this study, a significant increase in the percentage of PD-1^+ TIM-3^+ $CD4^+$ T cells was observed in BLV-infected cattle. This suggested that BLV infection may increase PD-1 and TIM-3

expression on Tregs and suppress the immune function of effector T cells. Further analysis of PD-1 and TIM-3 expression in Tregs is needed to clarify the immunomodulatory function of Tregs in BLV infection.

In a previous report, IFN-γ and IL-2 mRNA expression was upregulated when PBMCs from BLV-infected cattle were cultured with Cos-7 cells expressing TIM-3 to inhibit the TIM-3/Gal-9 pathway (48). Moreover, the combined blockade of TIM-3/Gal-9 and PD-1/PD-L1 pathways significantly promoted IFN-γ mRNA expression compared to the blockade of the PD-1/PD-L1 pathway alone (48). Although preliminary results suggested that TIM-3 is involved in suppressing T-cell function during BLV infection, they also suggested that TIM-3 may be a therapeutic target for restoring antiviral efficacy. Indeed, TIM-3 blockade by anti-bovine TIM-3 MAb restored T-cell activation and IFN-γ production in PBMCs from BLV-infected cattle in this study. Dual inhibition of bovine TIM-3 and PD-1/PD-L1 pathways by their respective antibodies enhanced IFN-γ and TNF-α production compared to that after inhibition of either pathway alone, resulting in potent antiviral activity against BLV. Presumably, dual inhibition of TIM-3 and PD-1/PD-L1 pathways effectively activated severely exhausted $CD4^+$ and $CD8^+$ T cells, enhancing their antiviral activity. However, in human cancers, inhibition of the PD-1/PD-L1 pathway produces dramatic antitumor effects in some patients but is partial or ineffective in most patients, requiring new strategies to overcome resistance to PD-1/PD-L1 therapy (60, 61). Similarly, when chAbs to PD-L1 were administered to BLV-infected cattle, certain BLV-infected cattle were found not to respond to the treatment (42). Interestingly, PD-1/PD-L1-targeted therapy increases TIM-3 expression in peripheral T cells and TILs in mouse models of lung adenocarcinoma and squamous cell carcinoma (62, 63). TNF-α, an antiviral cytokine, increases TIM-3 expression (64). In this study, enhanced TNF-α production was observed when both bovine TIM-3 and PD-1/PD-L1 pathways were inhibited either alone or simultaneously. Recently, anti-PD-L1 Ab treatment induced TNF-α production, and TNF-α blockade by TNF-RII-Ig reduces PGE_2 production in the presence of anti-PD-L1 Ab, suggesting that anti-PD-L1 antibody-induced TNF-α impairs T-cell activation by PGE_2 upregulation (65). Thus, TNF-α, although important as an antiviral cytokine, may be responsible for the immunosuppression associated with TIM-3. It is possible that cytokines (including TNF-α) increased via PD-1/PD-L1 pathway inhibition induce increased TIM-3 expression, leading to resistance to PD-1/PD-L1-targeted therapy through compensatory immunosuppression of the TIM-3 pathway. Therefore, combination therapy with anti-TIM-3 and anti-PD-L1 antibodies is also expected to be an effective strategy to overcome resistance to PD-L1-targeted therapies. It is possible that the reason for the limited antiviral efficacy of anti-bovine PD-L1 or PD-1 rat-bovine chAb in previous clinical trials against BLV was the compensatory induction of TIM-3. This point should be investigated in the future.

These results suggested that bovine TIM-3 is upregulated in T cells during BLV infection and plays a role in immunosuppressive mechanisms in pathogenesis. In addition, targeting the TIM-3 pathway in cattle, as well as in humans and mice, has immunostimulatory effects, as there are many immunosuppressive factors associated with T-cell exhaustion, such as TIM-3 and PD-1 (1, 66). Inhibition of these immunosuppressive pathways restores and enhances the function of exhausted T cells and thus has potential therapeutic applications in chronic infectious diseases and tumor diseases. This study demonstrated the potential of targeting TIM-3 as a new control strategy against bovine chronic infections. Future studies need to determine the relationship between TIM-3-mediated immunosuppressive mechanisms and pathological progression in various bovine chronic diseases and apply this new immunoregulation method to bovine diseases.

MATERIALS AND METHODS

Bovine blood and tumor samples. This study was conducted at the Faculty of Veterinary Medicine, Hokkaido University, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care International. All experimental procedures were conducted with the approval of the Hokkaido University Laboratory Animal Committee (approval no. 17-0024). Blood samples were

collected from cattle (Holstein breed, adult cattle) at the Field Science Center for the Northern Biosphere, Hokkaido University, and dairy farmers in Hokkaido. The samples were submitted for analysis within 2 days. PBMCs were isolated from the collected blood by density gradient centrifugation using Percoll (GE Healthcare, Chicago, IL, USA) for flow cytometry and cell bioassays.

Shallow cervical and mesenteric lymph nodes from cattle (Holstein breed, Japanese Black breed, and crossbreed) with or without lymphoma were donated by the Hokkaido Hayakita Meat Inspection Center (Abira, Hokkaido). Those lymph nodes were randomly collected for this study. The lymph nodes were shredded with scissors, separated into a single-cell suspension using a 40-μm cell strainer (BD Biosciences, Franklin Lakes, NJ, USA), and washed twice with phosphate-buffered saline (PBS; pH 7.2) supplemented with 0.5 mg/mL disodium EDTA (Dojindo Molecular Technologies, Kumamoto, Japan). The isolated single cells were suspended in deactivated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% dimethyl sulfoxide (Nacalai Tesque, Inc., Kyoto, Japan) and stored at −80°C until analysis.

BLV infection was confirmed by detection of anti-BLV antibodies in serum using a commercial ELISA kit (JNC, Tokyo, Japan) and quantitative real-time PCR using a LightCycler 480 System II (Roche Diagnostics, Mannheim, Germany) with a BLV detection kit (TaKaRa Bio, Shiga, Japan). BLV-associated lymphomas were further diagnosed via immunophenotyping using flow cytometry (40) to determine if EBL was due to BLV infection.

Analysis of TIM-3 and PD-1 expression by flow cytometry. TIM-3 and PD-1 expression on $CD4^+$ and $CD8^+$ T cells in PBMCs or EBL-derived cells was measured by flow cytometry using the antibodies listed in Table 1. To inhibit the nonspecific binding of antibodies to Fc receptors, PBMCs (1×10^6) or cells derived from lymph nodes (5×10^6) from cattle were pretreated with PBS supplemented with 10% inactivated goat serum (Thermo Fisher Scientific) at 25°C for 15 min. Pretreated cells were reacted with anti-bovine TIM-3 mouse MAb or mouse IgG_1 isotype control antibody at 37°C for 20 min. After the reaction, cells were reacted with Alexa Fluor 647-conjugated anti-mouse IgG(H+L) goat polyclonal $F(ab')_2$ (Thermo Fisher Scientific) at 25°C for 20 min. Cells were washed twice and reacted with fluorescein isothiocyanate-conjugated anti-bovine CD4 mouse MAb, phycoerythrin (PE)-conjugated anti-bovine CD8 mouse MAb, peridinin chlorophyll protein-cyanin 5.5 (PerCp/Cy5.5)-labeled anti-bovine CD3 mouse MAb, and PE/Cy7-conjugated anti-bovine IgM MAb for 20 min at 25°C. Anti-bovine CD3 mouse MAb and anti-bovine IgM mouse MAb were labeled with PerCp/Cy5.5 or PE/Cy7 using Lighting-Link antibody labeling kits (Novus Biological, Centennial, CO, USA). After the reactions, cells were washed twice and immediately analyzed by flow cytometry (FACS Verse; BD Biosciences). PBS supplemented with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used for antibody dilution and cell washing.

Dual TIM-3 and PD-1 expression on $CD4^+$ and $CD8^+$ T cells was also measured by flow cytometry. PBMCs were pretreated with inactivated goat serum as described above and reacted with anti-bovine TIM-3 mouse MAb and anti-bovine PD-1 rabbit MAb at 37°C for 20 min. Mouse IgG_1 and rabbit IgG isotype control antibodies were used as negative-control antibodies. After the reaction, cells were washed twice and reacted with Alexa Fluor 488-conjugated anti-mouse IgG(H+L) goat polyclonal $F(ab')_2$ (Thermo Fisher Scientific) and Alexa Fluor 647-conjugated anti-rabbit IgG(H+L) goat polyclonal $F(ab')_2$ (Thermo Fisher Scientific) at 25°C for 20 min. Cells were reacted with MAbs against CD4, CD8, and CD3, as listed in Table 1, and analyzed by flow cytometry, as described above.

Blockade assay by TIM-3 antibody. PBMCs (1×10^6) from BLV-infected or uninfected cattle were cultivated with 10 μg/mL anti-bovine TIM-3 mouse MAb (1H2-E2) or mouse IgG_1 isotype control antibody (15H6; Southern Biotech) at 37°C for 1 to 6 days in the presence of 5% CO_2. PBMCs from uninfected cattle were incubated with 1 μg/mL anti-bovine CD3 mouse MAb (MM1A; Washington State University Monoclonal Antibody Center, Pullman, WA, USA) and 1 μg/mL anti-bovine CD28 mouse MAb (CC220; Bio-Rad, Hercules, CA, USA). PBMCs derived from BLV-infected cattle were cultivated in the presence of BLV antigen, 2% heat-inactivated culture supernatant of BLV-infected FLK cell line, as described previously (42).

To evaluate lymphocyte activation, 24-h-cultivated PBMCs were analyzed for CD69 expression by flow cytometry, as described previously (42). Cell staining was performed as described above, except that Alexa Fluor 647-conjugated anti-bovine CD69 mouse MAb (Table 1) was also used.

IFN-γ production was analyzed by flow cytometry and ELISA, as described previously (42). When measuring IFN-γ intracellular expression of 24-h-cultivated cells, 10 μg/mL brefeldin A (Sigma-Aldrich) was added 5 h before cell collection. Cell staining for CD4, CD8, and CD3 was performed as described above, and stained cells were permeabilized using Foxp3 Perm buffer (BioLegend, San Diego, CA, USA) at 25°C for 15 min. After treatment, cells were washed twice and reacted with biotin-conjugated anti-bovine IFN-γ mouse MAb at 25°C for 20 min (Table 1). After the reaction, cells were treated with allophycocyanin-labeled streptavidin (BioLegend) for 20 min and immediately analyzed by flow cytometry (FACS Verse; BD Biosciences) after washing. Cells were cultured under the culture conditions for 6 days, as described above, and IFN-γ production in the culture supernatant was measured using the bovine IFN-γ ELISA development kit (Mabtech, Nacka Strand, Sweden). Absorbance was measured at 450 nm using a microplate reader (MTP-900; Corona Electric, Hitachinaka, Japan).

Dual blockade assay by TIM-3 and PD-L1 antibodies. PBMCs (1×10^6) from BLV-infected cattle were treated with anti-bovine TIM-3 mouse MAb (1H2-E2) and anti-bovine PD-L1 rat MAb (4G12-C1) in the presence of BLV antigen, as described above. The blockade antibody was added at 10 μg/mL to each well and incubated at 37°C for 7 days in the presence of 5% CO_2. Mouse serum-derived IgG (Sigma-Aldrich) or rat serum-derived IgG (Sigma-Aldrich) was used as a negative-control antibody. IFN-γ and TNF-α production in the culture was measured using the bovine IFN-γ ELISA development kit and the bovine TNF-α Do-It-Yourself ELISA (Kingfisher Biotech, Saint Paul, MN, USA).

TABLE 1 Primary antibodies used in flow cytometric analyses of this study[a]

Type of cells analyzed by flow cytometry and target	Isotype	Clone	Source	Fluorochrome	Conjugation or labeling
TIM-3$^+$ CD4$^+$ T cells and TIM-3$^+$ CD8$^+$ T cells					
CD3	Mouse IgG1	MM1A	WSU	PerCp/Cy5.5	Lightning-Link PerCp/Cy5.5 conjugation kit (Novus Biosciences)
CD4	Mouse IgG2a	CC8	Bio-Rad	FITC	(Conjugated primary antibody)
CD8	Mouse IgG2a	CC63	Bio-Rad	PE	(Conjugated primary antibody)
IgM	Mouse IgG1	IL-A30	Bio-Rad	PE/Cy7	Lightning-Link PE/Cy7 conjugation kit (Novus Biosciences)
TIM-3	Mouse IgG1	1H2-E2	This study		2nd Ab: Alexa Fluor 647-conjugated anti-mouse IgG(H+L) goat polyclonal F(ab')$_2$ (Thermo Fisher Scientific)
Mouse IgG1 isotype control	Mouse IgG1	15H6	Southern Biotech		2nd Ab: Alexa Fluor 647-conjugated anti-mouse IgG(H+L) goat polyclonal F(ab')$_2$ (Thermo Fisher Scientific)
TIM-3$^+$ PD-1$^+$ CD4$^+$ T cells and TIM-3$^+$ PD-1$^+$ CD8$^+$ T cells					
CD3	Mouse IgG1	MM1A	WSU	PerCp/Cy5.5	Lightning-Link PerCp/Cy5.5 conjugation kit (Novus Biosciences)
CD4	Mouse IgG2a	CC8	Bio-Rad	FITC	(Conjugated primary antibody)
CD8	Mouse IgG2a	CC63	Bio-Rad	PE	(Conjugated primary antibody)
TIM-3	Mouse IgG1	1H2-E2	This study		2nd Ab: Alexa Fluor 488-conjugated anti-mouse IgG(H+ L) goat polyclonal F(ab')$_2$ (Thermo Fisher Scientific)
PD-1	Rabbit IgG	1D10F1	In house		2nd Ab: Alexa Fluor 647-conjugated anti-rabbit IgG(H+L) goat polyclonal F(ab')$_2$ (Thermo Fisher Scientific)
Mouse IgG1 isotype control	Mouse IgG1	15H6	Southern Biotech		2nd Ab: Alexa Fluor 488-conjugated anti-mouse IgG(H+ L) goat polyclonal F(ab')$_2$ (Thermo Fisher Scientific)
Rabbit IgG isotype control	Rabbit IgG	-	Southern Biotech		2nd Ab: Alexa Fluor 647-conjugated anti-rabbit IgG(H+L) goat polyclonal F(ab')$_2$ (Thermo Fisher Scientific)
CD69$^+$ CD4$^+$ T cells and CD69$^+$ CD8$^+$ T cells					
CD4	Mouse IgG2a	CC8	Bio-Rad	FITC	(Conjugated primary antibody)
CD8	Mouse IgG2a	CC63	Bio-Rad	PE	(Conjugated primary antibody)
IgM	Mouse IgG1	IL-A30	Bio-Rad	PE/Cy7	Lightning-Link PE/Cy7 conjugation kit (Novus Biosciences)
CD69	Mouse IgG1	KTSN7A	WSU	Alexa Fluor 647	Zenon Alexa Fluor 647 mouse IgG1 labeling kit (Thermo Fisher Scientific)
IFN-γ^+ CD4$^+$ T cells and IFN-γ^+ CD8$^+$ T cells					
CD3	Mouse IgG1	MM1A	WSU	PerCp/Cy5.5	Lightning-Link PerCp/Cy5.5 conjugation kit (Novus Biosciences)
CD4	Mouse IgG2a	CC8	Bio-Rad	FITC	(Conjugated primary antibody)
CD8	Mouse IgG2a	CC63	Bio-Rad	PE	(Conjugated primary antibody)
IFN-γ	Mouse IgG21	MT307	Mabtech	APC	APC-conjugated streptavidin (BioLegend)

[a]WSU, WSU Monoclonal Antibody Center; FITC, fluorescein isothiocyanate; APC, allophycocyanin.

The antiviral efficacy of the dual blockade by anti-TIM-3 and anti-PD-L1 antibodies was evaluated by the syncytium formation assay using the number of syncytia formed by BLV-infected cells as an indicator, as described previously (67). Briefly, CC81 cells (0.6×10^5) were seeded onto 24-well plates (Corning, Inc., Corning, NY, USA) and cultivated for 24 h at 37°C in the presence of 5% CO_2. Next, PBMCs (5×10^5 cells) from BLV-infected cattle, anti-bovine TIM-3 mouse MAb (1H2-E2; 10 μg/mL), and anti-bovine PD-L1 rat antibody (4G12-C1; 10 μg/mL) were added and incubated for an additional 72 h. Mouse serum-derived IgG (Sigma-Aldrich) and rat serum-derived IgG (Sigma-Aldrich) were used as negative-control antibodies. After incubation, cells were fixed in methanol (Sigma-Aldrich) and stained with Giemsa's azure-eosin-methylene blue solution (Merck, Kenilworth, NJ, USA). The number of syncytia containing $\geq$10 cell nuclei was measured in 10 fields of view in each well using the ZOE fluorescent cell imager (Bio-Rad) for stained cells. Three wells were cultured per specimen cow. The results were expressed as the average from three wells.

Statistical analysis. Statistical analysis was performed using the statistical analysis software JMP (SAS), using the Mann-Whitney U test for two matched groups, the Wilcoxon signed-rank sum test for two independent groups, and the Steel-Dwass test for more than three groups. A difference was considered statistically significant if P was <0.05.

Data availability. The data sets used and analyzed in this study are available from the corresponding author on reasonable request.

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