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Enhanced expression of LAG-3 on lymphocyte subpopulations from persistently lymphocytotic cattle infected with bovine leukemia virus

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ABSTRACT

An immunoinhibitory receptor, lymphocyte activation gene-3 (LAG-3), which is mainly expressed in T-cells, is involved in the immune evasion of several pathogens causing chronic infections and tumors. However, unlike human or mouse LAG-3, no functional analysis of LAG-3 has been reported in domestic animals. Thus, in this study, bovine LAG-3 expression was analyzed in bovine leukemia virus (BLV)-infected cattle. In persistent lymphocytotic (PL) cattle, the numbers of LAG-3$^+$CD4$^+$ cells and LAG-3$^+$CD8$^+$ cells were conserved whilst the number of MHC class II$^+$ cells was remarkably higher than in the control animals. In contrast, the mean fluorescence intensity (MFI) for LAG-3 on PBMCs from PL cattle was significantly increased compared to control and asymptomatic (AL) cattle. Specifically, the LAG-3 expression level was significantly increased in both CD4$^+$ and CD8$^+$ T cells from PL cattle. LAG-3 expression correlated positively with increased numbers of lymphocytes and MHC class II$^+$ cells in infected animals. Preliminary results from PD-L1 and LAG-3 blockade assay revealed that IFN-γ and IL-2 expressions were significantly up-regulated by addition of anti- PD-L1 and LAG-3 antibodies in PBMCs from PL cattle. These findings suggest that LAG-3 might be involved in the inhibition of T-cell function through its binding and signaling on MHC class II molecule during BLV infection.

1. Introduction

In human and mice, lymphocyte activation gene-3 (LAG-3; CD223), a member of the immunoglobulin superfamily has been identified as a membrane protein, which contains a CD4 homolog [1] and is expressed on various immune cells such as activated CD4$^+$ and CD8$^+$ T-cells, natural killer cells, plasmacytoid dendritic cells and CD4$^+$CD25highFoxP3$^+$ regulatory T-cells [2,3]. Its intracellular domain, corresponding to residues 477–482, contains the KIEELE motif which is known to be important for LAG-3 signaling and negatively regulates T-cell proliferation [4]. Similar to the CD4 molecule, LAG-3 expressed on the surface of T-cells binds to MHC class II on antigen presenting cells or B-cells, but with 100-fold higher affinity than the CD4 molecule [5]. At the same time, LAG-3 is expressed on activated T-cells where it associates with the T-cell receptor (TCR)-CD3 complex at the cell surface and results in inhibition of signal transduction *via* TCR. Consequently, it negatively regulates proliferation, function, and homeostasis of T-cells [4,6,7].

Several inhibitory receptor molecules including the LAG-3/MHC class II pathway have been closely associated

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with immune exhaustion and disease progression in chronic infectious diseases and tumors [8–10]. LAG-3 is highly expressed by exhausted T-cells in mice with lymphocytic choriomeningitis virus (LCMV) chronic infection [11], and in human cancer such as Epstein–Barr virus (EBV)-related Hodgkin's lymphoma [12] and ovarian cancer [13]. Furthermore, LAG-3 is elevated during tumor development, resulting in expansion of $CD4^+CD25^+FoxP3^+$ regulatory T-cells that are expanded at tumor sites with subsequent induction of tumor-tolerance systems [2,14]. Thus, LAG-3 is one of the mechanisms involved in the down-regulation of immune responses during the progression of chronic diseases, as well as in facilitating immune evasion by several pathogens causing chronic infections and tumors.

Recently, blocking of the immune inhibitory pathway by specific antibodies has been shown to restore T-cell function during chronic diseases progression, and this approach novel therapeutics against chronic infectious diseases and cancer. The benefits of the approach have received widespread attention not because it has a direct effect against specific agents, but because it improves the ability of the immune system to eliminate disease agents. The blockade of an immunoinhibitory pathway, programmed death-1 (PD-1)/programmed death-ligand1 (PD-L1), is of particular interest in the development of new therapeutic strategies against several chronic diseases [15,16]. For instance, recent studies showed that dual, rather than individual, blockade of LAG-3 pathway and PD-1/PD-L1 pathway augmented anti-virus or anti-tumor cytokine production, and the proliferation of antigen-specific T-cells [11,13]. Therefore, elucidating the mechanism of dual blockade of the LAG-3 and PD-1/PD-L1 pathways in different disease models is crucial in developing effective cures for these ailments. Previously, we identified bovine LAG-3 gene and showed that it contains the immuno-inhibitory motif corresponding to residues 476–481 which is conserved as 'KTGELE' (identical to the swine LAG-3) within the intracellular region of the polypeptide. Moreover, the bovine LAG-3 contained a Serine (Ser) residue at amino acid position 467 (a possible phosphorylation site) which is conserved in all mammalian LAG-3 [17]. However, unlike human or mouse LAG-3, no functional analysis of LAG-3 has been reported in domestic animals.

In previous reports, we have shown that in infections with bovine leukemia virus (BLV), the aetiologic agent of enzootic bovine leucosis (EBL), the expression of inhibitory molecules is closely associated with disease progression in infected cattle [17–19]. Moreover, blocking of the PD-1/PD-L1 pathway by antibody specific to PD-L1 increases cytokine responses and enhances immune functions leading to a decrease in the viral load [19]. Therefore, blockade of LAG-3 pathway and PD-1/PD-L1 pathway is worth investigating to enhance the design of an effective immunotherapy that can induce cell-mediated immune responses. In the present study, to elucidate the role of LAG-3 in disease progression during BLV infection, we examined the expression of LAG-3 in BLV-infected cattle at two subclinical stages of infection, AL (aleukemia) and PL (persistently lymphocytosis), and LAG-3/PD-L1 blockade to augment the production of anti-viral cytokines IL-2 and IFN-γ in immunosuppressed PL cattle.

2. Materials and methods

2.1. BLV-infected animals

BLV-infected cattle were diagnosed at the Veterinary Teaching Hospital, Graduate School of Veterinary Medicine, Hokkaido University, between 2007 and 2011. Genomic DNA for diagnosis of BLV-infection was extracted from 0.5 ml of whole blood samples using the Wizard™ genomic DNA kit (Promega Corp., Madison, WI, USA) according to the manufacturer's instructions. BLV infection was tested by nested-PCR to amplify the BLV long terminal region (LTR) using primer pairs BLV-LTR1 5′-TGT ATG AAA GAT CAT GCC GAC-3′ and BLV-LTR533 5′-AAT TGT TTG CCG GTC TCT-3′ for the initial PCR, and BLV-LTR256 5′-GAG CTC TCT TGC TCC CGA GAC-3′ and BLV-LTR453 5′-GAA ACA AAC GCG GGT GCA AGC CAG-3′ for the second PCR. The conditions for both of the PCR were incubations at 94 °C for 5 min, followed by amplification of template for 35 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C 30 s with the final extension at 72 °C for 7 min. In BLV-positive cattle, the numbers of leukocytes were intermittently counted using Celltac α MEK-6450 (NIHON KOHDEN, Tokyo, Japan), and animals were regarded as positive for PL if at least two consecutive lymphocyte counts was more than 10,000/μl [20]. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood from BLV-infected animals by Percoll density gradient (Amersham Pharmacia Biotech, Piscataway, NJ, USA) [21] for subsequent flow-cytometric and real-time RT-PCR analyses. To investigate the degree of immunosuppression in cattle, interferon (IFN)-γ mRNA was quantified by quantitative RT real-time PCR with the specific primers (5′-ATA ACC AGG TCA TTC AAA GG-3′ and 5′-ATT CTG ACT TCT CTT CCG CT-3′) [22]. BLV-negative cattle from the same herd were used as controls.

2.2. Flow-cytometric analysis

To analyze the cells expressing LAG-3, single- and dual-color flow cytometric analyses were performed using the following antibodies; CACT138A (mouse anti-bovine CD4, VMRD, Pullman, WA, USA), IL-A51 (mouse anti-bovine CD8: a gift from International Livestock Research Institute), CAT82A (mouse anti-bovine MHC class II, VMRD) and goat anti-human LAG-3 (R&D systems, Minneapolis, MI, USA) [19]. Purified PBMCs (1×10^7 cells/mL) were incubated with the optimal concentration of CACT138A or IL-A51 for 40 min at 4 °C. The cells were then washed with PBS containing EDTA (0.5 mg/mL) and stained with either FITC-conjugated goat anti-mouse IgG (Beckman Coulter, Inc., Fullerton, CA, USA) or PE-conjugated goat anti-human LAG-3. PE-conjugated goat IgG (R&D systems) and normal mouse serum were used as isotype controls. Fluorescence of the cells was measured on an EPICS XL flowcytometry system (Beckman Coulter, Inc.), and the data analyzed using the EPICS EXPO32 ADC software (Beckman Coulter, Inc.).