Differences in cellular function and viral protein expression between IgM\textsuperscript{high} and IgM\textsuperscript{low} B-cells in bovine leukemia virus-infected cattle

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Bovine leukemia virus (BLV) induces abnormal B-cell proliferation and B-cell lymphoma in cattle, where the BLV provirus is integrated into the host genome. BLV-infected B-cells rarely express viral proteins in vivo, but short-term cultivation augments BLV expression in some, but not all, BLV-infected B-cells. This observation suggests that two subsets, i.e. BLV-silencing cells and BLV-expressing cells, are present among BLV-infected B-cells, although the mechanisms of viral expression have not been determined. In this study, we examined B-cell markers and viral antigen expression in B-cells from BLV-infected cattle to identify markers that may discriminate BLV-expressing cells from BLV-silencing cells. The proportions of IgM\textsuperscript{high} B-cells were increased in blood lymphocytes from BLV-infected cattle. IgM\textsuperscript{high} B-cells mainly expressed BLV antigens, whereas IgM\textsuperscript{low} B-cells did not, although the provirus load was equivalent in both subsets. Several parameters were investigated in these two subsets to characterize their cellular behaviour. Real-time PCR and microarray analyses detected higher expression levels of some proto-oncogenes (e.g. Maf, Jun and Fos) in IgM\textsuperscript{low} B-cells than those in IgM\textsuperscript{high} B-cells. Moreover, lymphoma cells obtained from the lymph nodes of 14 BLV-infected cattle contained IgM\textsuperscript{low} or IgM\textsuperscript{−} B-cells but no IgM\textsuperscript{high} B-cells. To our knowledge, this is the first study to demonstrate that IgM\textsuperscript{high} B-cells mainly comprise BLV-expressing cells, whereas IgM\textsuperscript{low} B-cells comprise a high proportion of BLV-silencing B-cells in BLV-infected cattle.

INTRODUCTION

Bovine leukemia virus (BLV) is a member of the genus Deltaretrovirus (subfamily Orthoretrovirinae, family Retroviridae) and is genetically related to human T-cell leukemia virus type 1 (HTLV-1) (Sagata et al., 1985). HTLV-1 infects CD4\textsuperscript{+} T-cells in humans, whereas BLV infects B-cells in cattle, but both viruses integrate into the genome as a provirus (Mirsy et al., 1996; Schwartz et al., 1994). The majority of BLV-infected cattle exhibit no clinical symptoms, but a proportion of latently infected cattle develop a disease that is characterized by abnormal B-cell proliferation and B-cell lymphoma (BL) 5–10 years after infection. Epidemics of BLV infection have caused extensive economic losses in the dairy and beef industries (Gillet et al., 2007) because there is no effective immunization against this untreatable disease in cattle.

BLV-infected B-cells and BL cells frequently express IgM, IgG, CD5, CD11b, MHCII and CD25 (Gillet et al., 2007; Meirmon et al., 1997), but the markers expressed exclusively in BLV-infected B-cells that contain the provirus remain unknown. Thus, it is necessary to identify specific markers for BLV-infected B-cells to facilitate a better understanding of the kinetics of the excessive proliferation and transformation of B-cells.

It is not possible to identify all BLV-infected B-cells using an antibody specific to BLV antigen because BLV expression is observed rarely in vivo or in freshly isolated lymphocytes (Asquith et al., 2005; Gillet et al., 2007; Hanon et al., 2000; Powers & Radke, 1992). However, short-term ex vivo cultivation at 37 \textdegree C leads to viral antigen expression, such as gp51 and p24, which are the viral membrane protein and core protein, respectively. Florins et al. (2012) reported that viral antigen expression caused high in vivo death rates of B-cells when BLV-infected lymphocytes were cultivated and transplanted into BLV-infected donor cattle. These data suggest that the silenced expression of viral antigens appears to be a strategy for reducing immunogenicity, evading immune surveillance and promoting efficient propagation in BLV-infected cattle.

Previous reports based on the detection of BLV antigens by flow cytometry and microscopy after ex vivo cultivation...
demonstrated that some, but not all, B-cells expressed BLV antigens (Fulton et al., 2006; Gillet et al., 2007; Ikebuchi et al., 2013). Moreover, a silent provirus was integrated into B-cell tumours isolated from BLV-infected sheep and the cell lines derived from these tumours (Merimi et al., 2007a, b; Van den Broeke et al., 1988). These reports suggested that ‘BLV-silencing’ B-cells are present among lymphocytes in infected cattle. Thus, it is hypothesized that at least two subsets are present among B-cells with genomes that incorporate BLV provirus: ‘BLV-expressing’ B-cells that can transmit BLV to BLV-uninfected cells, which may be detected by the immune surveillance system to activate immune cells, thereby leading to their eradication, and ‘BLV-silencing’ B-cells, which can proliferate without being attacked by immune cells (Gillet et al., 2007).

In the present study, we investigated the expression of surface markers and viral antigens on the B-cells of BLV-infected cattle to identify markers that may distinguish BLV-expressing B-cells from BLV-silencing B-cells. We found that IgM\textsuperscript{high} B-cells were likely to express BLV antigen, whereas IgM\textsuperscript{low} B-cells were not, although the provirus loads in both subsets were similar. Thus, we performed gene expression profiling and several cellular function analyses in both subsets, and speculate on the functional differences between IgM\textsuperscript{high} and IgM\textsuperscript{low} B-cells in vivo.

RESULTS

Surface IgM (sIgM) expression is increased in B-cells isolated from BLV-infected cattle

First, we analysed the expression levels of sIgM and B-cell-associated surface markers CD19-like (bovine WC4) and CD21-like, which are molecules analogous to human CD19 and CD21, respectively (Mukwedeya et al., 1993; Naessens et al., 1997), in B-cells isolated from BLV-infected cattle at different disease stages. The percentages of IgM\textsuperscript{-} B-cells among PBMCs increased in BLV-infected cattle as the number of lymphocytes increased in the blood (Fig. 1a and S1a available in the online Supplementary Material). In BLV-uninfected cattle, IgM\textsuperscript{+} B-cells comprised up to 50\% of PBMCs. The mean fluorescence index (MFI) of sIgM expression was also higher in IgM\textsuperscript{+} B-cells from BLV-infected cattle compared with BLV-uninfected cattle (Fig. 1b). In contrast, dual staining with anti-IgM and anti-CD19-like or anti-CD21-like showed that the proportions of both CD19-like\textsuperscript{-} and CD21-like\textsuperscript{-} cells were higher than those from BLV-uninfected cattle (Figs 1c and S1b). The increased IgM expression and decreased CD19-like and CD21-like expression in B-cells from BLV-infected cattle were lower than those from BLV-uninfected cattle (Figs 1c and S1b). The increased IgM expression and decreased CD19-like and CD21-like expression in B-cells were correlated with the proportions of IgM\textsuperscript{+} B-cells in PBMCs (Fig. 1d). The IgM\textsuperscript{-} B-cells were divided into two subsets: IgM\textsuperscript{high} (>4000 MFI) and IgM\textsuperscript{low} (<3000 MFI) B-cells, and the percentage of IgM\textsuperscript{high} B-cells in BLV-infected cattle was significantly higher than that of IgM\textsuperscript{low} B-cells in BLV-infected cattle as well as IgM\textsuperscript{high} and IgM\textsuperscript{low} B-cells in BLV-uninfected cattle (Fig. 1e). The results indicated that the surface marker expressions of B-cells were significantly different between BLV-infected and -uninfected cattle.

\textbf{IgM\textsuperscript{high} B-cells, but not IgM\textsuperscript{low} B-cells, are likely to express BLV antigen}

To investigate the relationship between surface markers and the expression of BLV antigens by B-cells after \textit{ex vivo} cultivation, PBMCs were cultivated to allow BLV expression and stained with anti-IgM, anti-WC4 (CD19-like) and anti-gp51 or anti-p24 antibodies. \textit{Ex vivo} cultivation allowed us to distinguish IgM\textsuperscript{high} B-cells from IgM\textsuperscript{low} B-cells as compared with fresh lymphocytes (Figs S1a and S2a). In the freshly isolated lymphocytes, CD19-like expression was higher in IgM\textsuperscript{low} B-cells than in IgM\textsuperscript{high} B-cells (Figs S1a and S2a), whereas, after overnight cultivation, the CD19-like expression was lower in IgM\textsuperscript{low} B-cells (Fig. 2a). The CD19-like expression in IgM\textsuperscript{high} B-cells did not differ between freshly isolated and cultivated lymphocytes (Fig. 2a). A mean of ~40\% of IgM\textsuperscript{high} B-cells expressed BLV gp51 (Fig. 2b), whereas IgM\textsuperscript{low} B-cells rarely expressed gp51. A higher percentage of BLV p24\textsuperscript{+} cells in IgM\textsuperscript{high} B-cells than in IgM\textsuperscript{low} B-cells was observed (Fig. 2c), and the percentage of B-cells that expressed both gp51 and p24 was also higher in IgM\textsuperscript{high} B-cells than in IgM\textsuperscript{low} B-cells (Figs 2d and S2b). Few gp51\textsuperscript{+} p24\textsuperscript{-} B-cells were present in both IgM\textsuperscript{high} and IgM\textsuperscript{low} B-cells. These results suggested that IgM\textsuperscript{high} B-cells included a high proportion of BLV-expressing cells, whereas IgM\textsuperscript{low} B-cells included a high proportion of BLV-silencing cells. Thus, BLV-expressing and BLV-silencing B-cells could be roughly discriminated based on differences in the sIgM expression levels.

\textbf{IgM and WC4 stimulation upregulate gp51 expression}

To identify whether sIgM and CD19-like molecules have a role in the expression of BLV antigens, we measured the changes in BLV gp51 expression in B-cells treated with anti-IgM, anti-WC4 (CD19-like) or phosphatidylinositol 3-kinase (PI3-kinase) inhibitors, which inhibit the signalling associated with CD19 (So & Fruman, 2012). Anti-IgM treatment upregulated gp51 expression in B-cells (Fig. S3a), which agreed with the results of a previous study (Kerkhofs et al., 1996). There was no difference in the gp51 expression levels of B-cells treated with goat IgG or PBS in some samples (data not shown). The percentages of gp51\textsuperscript{+} B-cells were increased by anti-WC4 treatment compared with mouse IgG1 treatment (Fig. S3b), but they were decreased by treatment with two types of PI3-kinase inhibitor (Fig. S3c). Moreover, the treatment of LY294002 which is a more specific PI3-kinase inhibitor than Wortmannin PI3-kinase abolished the effect of anti-WC4 treatment (Fig. S3d), thereby indicating that CD19 signalling as well as B-cell receptor (BCR) signalling may have important roles in BLV antigen expression.
Provirus loads and expression levels of BLV-binding receptors are almost the same in IgM<sup>high</sup> and IgM<sup>low</sup> B-cells

The lower expression of BLV antigen in IgM<sup>low</sup> B-cells than in IgM<sup>high</sup> B-cells might be attributable to a lower proportion of BLV-infected B-cells in the subset. Thus, to investigate the proportions of BLV-infected B-cells in IgM<sup>high</sup> and IgM<sup>low</sup> B-cells, we measured the provirus loads in both subsets. There were no differences in the provirus loads of the IgM<sup>high</sup> and IgM<sup>low</sup> B-cells (Fig. 3a), although IgM<sup>high</sup> B-cells were more likely to express BLV antigen (Fig. 2b, c).

GLUT-1 and neuropilin-1 have been identified as the cellular receptors required for HTLV-1 entry (Ghez et al., 2000).
2006; Manel et al., 2003). The receptors required for BLV entry remain unidentified, but staining using BLV envelope–Ig fusion protein (BLV-env–Ig) facilitates analysis of the expression of BLV-binding receptors, which are expected to be BLV receptors (Lavanya et al., 2008). We found that IgM<sup>high</sup> and IgM<sup>low</sup> B-cells expressed equal amounts of BLV-binding receptors in BLV-infected cattle (Figs 3b and S4). However, lower amounts of BLV-env–Ig bound to IgM<sup>low</sup> B-cells in BLV-uninfected cattle than to IgM<sup>low</sup> B-cells in BLV-infected cattle and IgM<sup>high</sup> B-cells in both the cattle. These results suggest that the infection rate and the relative susceptibility to BLV infection may be in the same range in the IgM<sup>high</sup> and IgM<sup>low</sup> B-cells of BLV-infected cattle.

**Characterization of cellular function in IgM<sup>high</sup> and IgM<sup>low</sup> B-cells**

Previous studies have reported a correlation between sIgM expression levels in B-cells and calcium influx in response to BCR stimulation (Quách et al., 2011; Zikherman et al., 2012). In the present study, more cells that responded to stimulation by polyclonal anti-bovine IgM were present among IgM<sup>high</sup> B-cells from BLV-infected cattle than among IgM<sup>high</sup> and IgM<sup>low</sup> B-cells from BLV-uninfected cattle (Figs 4a and S5a). The percentages of IgM<sup>low</sup> B-cells from BLV-infected cattle that responded to BCR stimulation were also higher than those of IgM<sup>low</sup> B-cells isolated from BLV-uninfected cattle. Unexpectedly, there was no significant difference in the responsiveness to BCR stimulation between IgM<sup>high</sup> and IgM<sup>low</sup> B-cells (Fig. 4a). Furthermore, the responsiveness to WC4 stimulation was almost the same in all B-cell subsets.

Previous studies have shown that BLV infection in sheep protected their PBMCs from ex vivo apoptosis (Dequiedt et al., 1997) and that human CD21<sup>low</sup> B-cells were sensitive to apoptosis (Charles et al., 2011; Isnardi et al., 2010). Thus, we investigated the susceptibility of B-cells from BLV-infected cattle to cell death and found that they expressed lower levels of CD21-like than B-cells from BLV-uninfected cattle (Fig. 1c). A higher frequency of dead cells and a lower frequency of live cells were observed in IgM<sup>low</sup>
B-cells than in IgM<sup>high</sup> B-cells from BLV-infected and BLV-uninfected cattle (Figs 4b and S5b). Moreover, IgM<sub>low</sub> B-cells from BLV-infected cattle were more susceptible to cell death than those from BLV-uninfected cattle.

In our previous study, we showed that the proportion of PD-L1<sup>+</sup> B-cells [programmed death ligand 1 (PD-L1) is one of the immunoinhibitory molecules that induced T-cell anergy in cattle] was higher in advanced-disease-stage BLV-infected cattle than in BLV-uninfected cattle (Ikebuchi et al., 2011). In the present study, the percentages of PD-L1<sup>+</sup> cells were higher among IgM<sub>low</sub> B-cells than among IgM<sub>high</sub> B-cells (Figs 4c and S5c). However, the fluorescence intensity of PD-L1 expression could not be compared between IgM<sub>high</sub> and IgM<sub>low</sub> B-cells because the autofluorescence differed between these subsets (data not shown).

To investigate the gene expression profiles of IgM<sub>low</sub> B-cells, which were likely to be silent with respect to BLV expression, we performed a microarray analysis using RNA isolated from IgM<sup>high</sup> and IgM<sup>low</sup> B-cells, which were sorted from the cultivated PBMCs of three BLV-infected cattle. The expression levels of 164 genes were upregulated whereas those of 28 genes were downregulated in IgM<sub>low</sub> B-cells compared with IgM<sub>high</sub> B-cells isolated from all three cattle (Tables S1 and S2). The expression levels of proto-oncogenes such as MafB (Eyche`ne et al., 2008), Jun (Jochum et al., 2001), Fos/FosB (Jochum et al., 2001; Milde-Langosch, 2005), Frat1 (Jonkers et al., 1997), Bcl9L (Anastas & Moon, 2013) and Fyn (Saito et al., 2010) were higher in IgM<sub>low</sub> B-cells than in IgM<sub>high</sub> B-cells. Gene ontology analysis of the differentially expressed genes showed that the genes with increased expression in IgM<sub>low</sub> B-cells were associated with several different functions, including chemokine activity, chemotaxis and inflammatory responses (Table S3), thereby indicating that the IgM<sub>low</sub> B-cells may have been in an inflammatory state. The expression levels of MafB, Jun, Fos, FosB, Frat1, Fyn, Ccl3, Ccl4, IL-8 and Ccr1 were also confirmed by quantitative real-time PCR to be upregulated in IgM<sub>low</sub> B-cells from nine other BLV-infected cattle (Table 1).

Fig. 4. Functional analyses of IgM<sub>high</sub> and IgM<sub>low</sub> B-cells. (a) Percentages of responding cells among IgM<sub>high</sub> and IgM<sub>low</sub> B-cells. IgM<sup>+</sup> B-cells isolated from BLV-infected (BLV<sup>+</sup>) and BLV-uninfected (BLV<sup>−</sup>) cattle were stimulated with anti-IgM (BLV-uninfected and BLV-infected cattle; n = 9 and 13, respectively) and anti-WC4 (n = 5 and 10, respectively). (b) Percentages of dead (annexin-V<sup>−</sup> 7-aminoactinomycin D (7-AAD)<sup>−</sup>) and live (annexin-V<sup>−</sup> 7-AAD<sup>+</sup>) cells among IgM<sub>high</sub> and IgM<sub>low</sub> B-cells. PBMCs isolated from BLV-infected and BLV-uninfected cattle were cultivated overnight and stained with annexin-V and 7AAD. (c) The percentages of PD-L1<sup>+</sup> cells among IgM<sub>high</sub> and IgM<sub>low</sub> B-cells from BLV-infected cattle. Each line indicates the mean value for each group. Statistical comparisons were performed using a one-way ANOVA with Tukey’s test and a Wilcoxon matched pairs test. Differences were considered statistically significant at P < 0.05 (*P < 0.05; **P < 0.01).
**Table 1. Differentially expressed genes confirmed by quantitative real-time PCR in IgM\(^{\text{low}}\) B-cells from nine BLV-infected cattle**

The expression levels of all these genes were significantly higher in IgM\(^{\text{low}}\) B-cells than in IgM\(^{\text{high}}\) B-cells (\(P<0.01\)). Statistical comparisons between IgM\(^{\text{high}}\) and IgM\(^{\text{low}}\) B-cells were performed using a Wilcoxon matched-pairs test.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Mean fold difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>v-Maf musculoaponeurotic fibrosarcoma oncogene homologue B (avian)</td>
<td>MAFB</td>
<td>196.62</td>
</tr>
<tr>
<td>Jun proto-oncogene</td>
<td>JUN</td>
<td>66.95</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue</td>
<td>FOS</td>
<td>51.84</td>
</tr>
<tr>
<td>FBJ murine osteosarcoma viral oncogene homologue B</td>
<td>FOSB</td>
<td>23.00</td>
</tr>
<tr>
<td>Frequently rearranged in advanced T-cell lymphomas</td>
<td>FRAT1</td>
<td>132.25</td>
</tr>
<tr>
<td>FYN oncogene related to SRC, FGR, YES</td>
<td>FYN</td>
<td>17.66</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 3</td>
<td>CCL3</td>
<td>2.62</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 4</td>
<td>CCL4</td>
<td>5.94</td>
</tr>
<tr>
<td>IL-8</td>
<td>IL-8</td>
<td>155.43</td>
</tr>
<tr>
<td>Chemokine (C-C motif) receptor 1</td>
<td>CCR1</td>
<td>39.38</td>
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**IgM\(^{\text{high}}\) B-cells are not observed among BL cells from lymph nodes (LNs)**

The expression level of slgM was measured in BL cells isolated from enlarged LNs in BLV-infected cattle, where BL was diagnosed based on CD5, CD19-like and CD21-like expression (data not shown). BL cells from 8 of 14 cattle expressed low levels of slgM (Fig. 3, Table 2), whereas IgM\(^{\text{high}}\) BL cells were not observed in LNs from BLV-infected cattle with BL in the present study. The blood cells comprised IgM\(^{\text{high}}\) or IgM\(^{\text{low}}\) B-cells in cattle with IgM\(^{\text{low}}\) BL. IgM\(^{+}\) B-cells were also observed in BL cells isolated from five cattle. Heterogeneous IgM expression was detected in BL cells from one animal. These results indicate that LN cells from BLV-infected cattle with BL mainly comprised IgM\(^{\text{low}}\) or IgM\(^{-}\) B-cells.

**DISCUSSION**

B-cell activation by mitogens upregulates *ex vivo* BLV expression (Kidd & Radke, 1996; Lagarias & Radke, 1989). In the present study, we demonstrated that IgM\(^{\text{high}}\) B-cells from BLV-infected cattle were likely to have elevated levels of intracellular calcium in response to BCR stimulation compared with IgM\(^{-}\) B-cells from BLV-uninfected cattle. This high responsiveness to antigen stimulation may contribute to cellular activation and BLV antigen expression in IgM\(^{\text{high}}\) B-cells, which includes a high proportion of BLV-expressing B-cells. Thus, IgM\(^{\text{high}}\) B-cells appear to have an important role, by expanding BLV infection to BLV-uninfected B-cells *in vivo*.

Most IgM\(^{\text{low}}\) B-cells appeared to be BLV-silencing cells, which were able to evade immune surveillance (Gillet *et al.*, 2003). In fact, p24 antigen was expressed by 29.3% of IgM\(^{\text{low}}\) B-cells (mean value in Fig. 2c), but only 7.1% of IgM\(^{\text{low}}\) B-cells expressed gp51 antigen. The expression of gp51 is essential for cell-to-cell infection because of the unstable nature of BLV particles outside the cell membrane (Derse *et al.*, 2001; Gatot *et al.*, 1998; Igakura *et al.*, 2003; Johnston *et al.*, 1996). Thus, p24\(^{+}\) IgM\(^{\text{low}}\) B-cells appear to be an inefficient population for the expansion of BLV infection. In future research, it will be necessary to identify markers other than slgM to facilitate studies of the mechanism of BLV silencing because it is still difficult to distinguish strictly BLV-expressing cells from BLV-silencing cells based on the detection of slgM expression.

The increased number of IgM\(^{\text{high}}\) B-cells in blood from BLV-infected cattle could be explained by an increase in the production of immature B-cells in primary lymphoid organs after BLV infection. Many studies have detected high slgM expression levels in the immature B-cells that emigrate from ileal Peyer's patches in sheep (Griebel *et al.*, 1992; Yasuda *et al.*, 2006) and in immature transitional type 1 B-cells in mice models (Cambier *et al.*, 2007; Teague *et al.*, 2007). Furthermore, Fulton *et al.* (2006) detected the emergence of IgM\(^{\text{high}}\) B-cells only in the blood and not in the efferent lymph after BLV infection in sheep. These reports suggest that the increase in IgM\(^{\text{high}}\) B-cells in the blood of BLV-infected cattle may be attributable to non-recirculating immature B-cells derived from either bone marrow or Peyer's patches, which are the primary lymphoid organs responsible for B-cell development and B-cell diversity in cattle and sheep (Yasuda *et al.*, 2006). In HTLV-1 infection, immature thymocytes are the targets for infection, and the emergence of malignant clones in the thymus may be selected over time in HTLV-1-infected patients (Feuer *et al.*, 1996; Hasegawa *et al.*, 2006). Thus, the progenitor cells of B-cells could be infected with BLV in the primary lymphoid organs. Further studies of the mechanism of bovine B-cell development are required to test this hypothesis.

CD19 expression by B-cells is essential for inducing the germinal centre (GC) reaction in LNs after the recognition of a cognate antigen by follicular dendritic cells (Depoil *et al.*, 2008). The GC reaction is considered to be an important step in the induction of BL by Epstein–Barr virus (Küppers, 2003). Furthermore, CD19 promotes oncogene expression and tumour growth in human BL, where a longer survival period was observed in patients...
with low CD19 expression levels (Chung et al., 2012). In the present study, high expression levels of CD19-like molecule were observed in IgM\textsuperscript{low} B-cells among the lymphocytes that were freshly isolated from BLV-infected cattle. Thus, IgM\textsuperscript{low} B-cells that strongly express CD19-like may have a tendency to expand and develop into lymphoma cells through the GC reaction.

The results obtained in this study demonstrated that the IgM\textsuperscript{high} and IgM\textsuperscript{low} B-cell subsets have an inherent capacity to survive in vivo (Table S4). The proportions of live and dead cells in cultivated PBMCs also suggest that IgM\textsuperscript{high} B-cells exhibit superior survival. However, a capacity for in vivo expansion was also detected in IgM\textsuperscript{low} B-cells. The higher expression levels of PD-L1 and lower expression levels of gp51 in this subset are related to immune escape from virus-specific T-cells. Higher expression levels of CD19-like molecule also appear to favour antigen recognition and cellular activation during antigen presentation by dendritic cells (Depoil et al., 2008). Moreover, the expression levels of some proto-oncogenes (MafB, Jun, Fos, FosB, Frat1 and Fyn) were increased in IgM\textsuperscript{low} B-cells, whereas IgM\textsuperscript{high} BL cells were not observed in LNs from any of the 14 BLV-infected cattle with BL, thereby raising the possibility that either IgM\textsuperscript{low} B-cells or clones derived from these cells are more likely to become neoplastic B-cells compared with IgM\textsuperscript{high} B-cells. This possibility was also supported by the microarray data, where chemokine genes and genes associated with chemotaxis, such as CCL3, CCL4, IL-8 and CCR1, were upregulated in IgM\textsuperscript{low} B-cells. In chronic lymphocytic leukaemia (CLL) patients, the recirculation and spread of CLL cells in the body are preferentially induced by the CCL3 and CCR1 system (Trentin et al., 2004). Moreover, CCL3, CCL4 and IL-8

![Fig. 5. sIgM expression by BL cells. Lower plots: Representative dot plots of IgM\textsuperscript{high/low}, IgM\textsuperscript{low} and IgM\textsuperscript{−} BL cells in LNs isolated from BLV-infected cattle with BL. Top plots: lymphocytes in blood collected from BLV-infected cattle with or without BL. The dashed lines indicate the boundary between the fluorescence intensities of IgM\textsuperscript{high} and IgM\textsuperscript{low} B-cells.](image)

### Table 2. sIgM expression in BL cells isolated from 14 cattle with BL

<table>
<thead>
<tr>
<th>IgM expression in enlarged LNs</th>
<th>IgM\textsuperscript{high}</th>
<th>IgM\textsuperscript{high/low}</th>
<th>IgM\textsuperscript{low}</th>
<th>IgM\textsuperscript{−}</th>
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<tbody>
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<td>Number</td>
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<td>1</td>
<td>8</td>
<td>5</td>
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<th>IgM expression in blood</th>
<th>ND</th>
<th>High/low</th>
<th>Low</th>
<th>-</th>
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<th>High/low</th>
<th>Low</th>
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<tbody>
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<td>Number</td>
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ND, Not determined because of sample limitations.
derived from lymphoma cells induce the homing of accessory cells, which creates a supportive microenvironment for the maintenance and expansion of neoplastic cells (Burger, 2010). In a similar manner, the IgM<sup>low</sup> B-cells in BLV-infected cattle could induce the trafficking and migration of other B-cells and accessory cells into lymphoid organs, resulting in the development of enlarged LNs in BLV-infected cattle with BL. Future research should investigate in vivo immune escape, migration and transformation in IgM<sup>low</sup> B-cells.

IgM<sup>-</sup> BL cells were observed in LNs from five BLV-infected cattle with BL in the present study, and in some cases of B-cells transformed by Epstein–Barr virus expressed no surface immunoglobulin (Küppers, 2003). One of the hypothetical mechanisms is that destructive immunoglobulin gene mutations, such as frameshift mutations, induce the loss of BCR expression during the GC reaction. B-cells that acquire this mutation normally tend to undergo apoptosis, but IgM<sup>-</sup> BL cells can overcome the apoptosis signal by transformation events. Thus, IgM<sup>-</sup> BL cells from BLV-infected cattle may have developed after antigen recognition and somatic hypermutation in LNs.

In contrast to BCR expression by BLV-infected B-cells, the expression level of the T-cell receptor, CD3, in HTLV-1-infected T-cells decreased gradually with disease progression. Progressive losses of CD3 expression have been observed in both HTLV-1-infected cell lines and CD4<sup>+</sup> T-cells from HTLV-1-infected patients (Ald et al., 2007; Kobayashi et al., 2013). A study using CD4<sup>+</sup> T-cell lines experimentally infected with HTLV-1 also showed that CD3<sup>high</sup> and CD3<sup>low</sup> T-cells had the same capacity to express the HTLV-1 core protein (Willard-Gallo et al., 2001). Moreover, despite the structural and functional homology between HTLV-1 and BLV, treatment with a calcium ionophore reduced viral expression in HTLV-1-infected T-cells (Copeland et al., 1994), whereas it was upregulated in BLV-infected B-cells (Kerkhofs et al., 1996). Previous reports and the results obtained in the present study suggest that HTLV-1-infected T-cells and BLV-infected B-cells may use different signalling pathways and strategies to express viral antigens and to expand their infection to uninfected cells.

The present study showed that the number of IgM<sup>high</sup> B-cells increased in the blood of BLV-infected cattle and that they were likely to express BLV antigen after ex vivo cultivation, suggesting their role as sources of BLV infection. By contrast, IgM<sup>low</sup> B-cells included a high proportion of BLV-silencing cells, which expressed high levels of some proto-oncogenes. To our knowledge, this is the first study to investigate IgM<sup>high</sup> and IgM<sup>low</sup> B-cells in BLV-infected cattle. Our analyses of these two B-cell subsets provide insights into the mechanisms of viral silencing, the target cells of antiviral treatments or antitumour therapy, and disease progression and transformation during BLV infection.

### METHODS

**Ethics statement.** All animal experiments were conducted in accordance with the Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan) and all protocols were approved by the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval no. 11-0059). Informed consent was obtained from clinical veterinarians and farmers before sample collection.

**Samples from cattle, BLV diagnosis and cell preparation.** In total, 166 blood samples were obtained from Holstein-Friesian and Japanese Black breed (BLV-infected: n = 117, BLV-uninfected: n = 49) bred on several farms in Japan. All animals had been diagnosed with BLV infection and BL by nested PCR and flow cytometry as described previously (Ikebuchi et al., 2011, 2013). Enlarged LNs and blood samples from 14 BLV-infected cattle with BL were provided by several farms and veterinarians. The number of lymphocytes in the blood was counted using an MEK-6450 Celltac (Nihon Kohden). PBMCs were purified from heparinized blood by density-gradient centrifugation on Percoll (GE Healthcare).

B-cells were purified from PBMCs using an autoMACS Pro (Miltenyi Biotech) with anti-bovine IgM (clone BIG73A; VMRD) and anti-mouse IgG1 MicroBeads (Miltenyi Biotec), according to the manufacturer’s instructions. IgM<sup>high</sup> and IgM<sup>low</sup> B-cells were sorted using a MoFlo Astrios (Beckman Coulter) from cultivated PBMCs, which were stained with anti-bovine IgM pre-labelled with Zenon Alexa Fluor 488 (Life Technologies), anti-WC4 (CC55; CD19-like; AbD Serotec) and Alexa Fluor 647-conjugated anti-mouse IgG (Life Technologies). The high purities (90–98%) of the IgM<sup>+</sup>, IgM<sup>high</sup> and IgM<sup>low</sup> B-cells were confirmed using a FACS Verse system (BD Biosciences).

**Expression of recombinant BLV-env–Ig.** Soluble BLV-env–Ig was expressed in CHO-DG44 cells (provided by Dr Y. Suzuki, Hokkaido University, Japan) that were stably transfected with pCAGGS (provided by Dr J. Miyazaki, Osaka University, Japan) (Niwa et al., 1991), which encoded a mouse CD150 signal sequence, the extracellular region of BLV env and the Cc fragment of rabbit IgG, as described previously (Arase et al., 2002; Ikebuchi et al., 2013). The concentration of BLV-env–Ig in supernatants was measured using a Rabbit IgG ELISA Quantification Set (Bethyl Laboratories).

**Flow cytometry.** To analyse the expression levels of B-cell markers, BLV antigens, BLV-binding receptors and PD-L1 by flow cytometry, we used the following antibodies and recombinant protein: anti-bovine IgM (clone BIG73A; VMRD) and anti-bovine IgG1 MicroBeads (Miltenyi Biotec), according to the manufacturer’s instructions. IgM<sup>high</sup> and IgM<sup>low</sup> B-cells were sorted using a MoFlo Astrios (Beckman Coulter) from cultivated PBMCs, which were stained with anti-bovine IgM pre-labelled with Zenon Alexa Fluor 488 (Life Technologies), anti-WC4 (CC55; CD19-like; AbD Serotec) and Alexa Fluor 647-conjugated anti-mouse IgG (Life Technologies). The high purities (90–98%) of the IgM<sup>+</sup>, IgM<sup>high</sup> and IgM<sup>low</sup> B-cells were confirmed using a FACS Verse system (BD Biosciences).

**Flow cytometry.** To analyse the expression levels of B-cell markers, BLV antigens, BLV-binding receptors and PD-L1 by flow cytometry, we used the following antibodies and recombinant protein: anti-bovine IgM (clone BIG73A; anti-WC4 (CC55; CD19-like), anti-B-B7 (clone GR25A; CD21-like; VMRD), anti-BLV-gp51 (BLV1; VMRD), anti-BLV-p24 (BLV3; VMRD), BLV-env–Ig and anti-bovine PD-L1 (clone 4G12) (Ikebuchi et al., 2014). To induce BLV antigen expression in BLV-infected cells, PBMCs were cultivated overnight in RPMI 1640 (Sigma-Aldrich), which was supplemented with 10% FBS (Cell Culture Technologies) and a mixture of 2 mmol l<sup>-1</sup> glutamine, 100 U penicillin ml<sup>–1</sup> and 100 μg streptomycin ml<sup>–1</sup> (Life Technologies; complete RPMI 1640). Detailed information about staining is provided in the Supplementary Methods.

**Real-time PCR.** IgM<sup>high</sup> and IgM<sup>low</sup> B-cells were isolated from PBMCs, which were cultivated overnight to express BLV antigens, as described above. To measure the provirus load, the total cellular DNA was purified from IgM<sup>high</sup> and IgM<sup>low</sup> B-cells with a Nucleospin Tissue XS (Macherey Nagel). The provirus and total cellular DNA were amplified by quantitative real-time PCR with SYBR Premix DimerEraser (Takara-Bio), as described previously (Ikebuchi et al., 2013; So & Fruman, 2012). The results were expressed as the provirus copy number per 100 cells. Detailed information on
the amplification procedure is provided in the Supplementary Methods.

**Microarray analysis.** Microarray analysis was performed using a bovine oligo DNA microarray (Agilent Technologies) in the DNA Tip Laboratory (Kanagawa, Japan). PBMCs were isolated from three BLV-infected cattle and IgM<sup>high</sup> and IgM<sup>low</sup> B-cells were sorted from Laboratory (Kanagawa, Japan). PBMCs were isolated from three expressed as percentages of FITC described previously (Ikebuchi Moloney murine leukemia virus reverse transcriptase (Takara-Bio) in complete RPMI 1640 and stained with annexin V–FITC (Beckman

**Detection of live and dead cells.** PBMCs were cultivated overnight in complete RPMI 1640 and stained with annexin V–FITC (Beckman Coulter) and 7-aminoactinomycin D (7-AAD; BD Biosciences), as described previously (Ikebuchi et al., 2013). The results were expressed as percentages of FITC<sup>+</sup> 7-AAD<sup>+</sup> cells as dead cells and FITC<sup>−</sup> 7-AAD<sup>−</sup> cells as live cells among the total IgM<sup>+</sup> B-cells.

**Statistical analysis.** Spearman’s rank-correlation test, one-way ANOVA with Tukey’s test, a Mann–Whitney test and a Wilcoxon matched-pairs test were performed using GraphPad Prism version 5.0. *P* values of <0.05 were considered statistically significant.

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