

## Research paper

## Lymphocyte proliferation and apoptosis of lymphocyte subpopulations in bovine leukemia virus-infected dairy cows with high and low proviral load



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## ABSTRACT

Bovine leukemia virus (BLV) is one of the most important virus in dairy cattle. The infection behavior follows what we call the iceberg phenomenon: 60% of infected animals do not show clinical signs; 30% develop persistent lymphocytosis (PL); and the remaining 10%, die due to lymphosarcoma. BLV transmission depends on infected cell exchange and thus, proviral load is determinant. Understanding the mechanisms by which cattle governs the control of viral dissemination will be desirable for designing effective therapeutic or preventive strategies for BLV. The development of high proviral load (HPL) or low proviral load (LPL) might be associated to genetic factors and humoral immune responses, however cellular responses are not fully described. It is known that BLV affects cellular homeostasis: proliferation and apoptosis. It is also known that the BLV tropism is directed towards B lymphocytes, and that lymphocytotic animals have elevated amounts of these cells. Usually, when an animal is infected by BLV, the B markers that increase are CD21, CD5 and CD11b. This increase could be related to the modulation of apoptosis in these cells. This is the first work in which animals infected with BLV are classified according to their proviral load and the subpopulations of B and T lymphocytes are evaluated in terms of their percentage in peripheral blood and its stage of apoptosis and viability. PBMCs from HPL animals proliferated more than LPL and non-infected animals. CD11b<sup>+</sup>/CD5<sup>+</sup> lymphocytes in LPL animals presented greater early and late apoptosis than HPL animals and cells of HPL animals had increased viability than LPL animals. Our results confirm that BLV alters the mechanism of apoptosis and proliferation of infected cells.

## 1. Introduction

Bovine leukemia virus (BLV) is a deltaretrovirus, the most widespread pathogen in dairy cattle. It is the etiological agent of enzootic bovine leukemia (EBL), a disease characterized by three different presentations: asymptomatic or aleukemic (AL), persistent lymphocytosis (PL), and leukemia or lymphoma (Bartlett et al., 2013). Approximately 30% of infected animals will develop PL, while 0.1–10% of infected animals will develop either leukemia or lymphoma (Kabeya et al., 2001). PL stage is characterized by a polyclonal B-cell expansion that occurs almost exclusively within the CD5<sup>+</sup> B-cell subset, which are in turn the primary target for BLV proviral integration. This accumulation of B-lymphocytes in blood and lymphoid tissue potentially cause leukemia/lymphoma, especially in PL cattle (Gillet et al., 2007; Florins

et al., 2007).

BLV transmission occurs by exchange of cells harboring the virus, so the number of infected cells in the animal is crucial (Hopkins and DiGiacomo, 1997). Even though PL cattle are considered the most risky for transmission, asymptomatic animals may also play a role depending on their proviral load (Juliarena et al., 2007). In fact, approximately 60% of the AL animals present low proviral load (LPL), while the remaining 40% will be AL animals with high proviral load (HPL) (Juliarena et al., 2007). The ability to control viral spread in infected animals could be associated in part to host genetic factors, such as polymorphisms in the bovine leukocyte antigen (*BoLA*) gene (Juliarena et al., 2008; Polat et al., 2015) and the promoter region of tumor necrosis factor-alpha (*TNF-α*) gene (Lendez et al., 2015). However, these factors cannot fully explain LPL profile. Even though humoral immune

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**Table 1**  
Monoclonal antibodies used for differentiation and labeling of bovine peripheral blood lymphocytes applying flow cytometry.

Description	Primary antibody					Secondary antibody							
	Name	Type	Specificity	Host	Company	Isotype	cat. n.	Fluorochrome	Specificity	Host	Company	Isotype	
T Lymphocyte	MM1A	CD3	Bovine	Mouse	W.S.U. <sup>1</sup>	IgG1	A31562	Alexa Fluor 680	Mouse	Goat	Invitrogen <sup>3</sup>	IgG1	
CD4 T Lymphocyte	ILA11	CD4	Bovine	Mouse	W.S.U. <sup>1</sup>	IgG2a	565817	BV421	Mouse	Rat	BD H. TM <sup>4</sup>	IgG2a	
CD8 T Lymphocyte	MCA837PE	CD8 (PE)	Bovine	Mouse	Bio-rad <sup>2</sup>								
B Lymphocyte	MCA1424PE	CD21 (PE)	Bovine	Mouse	Bio-rad <sup>2</sup>								
CD5	B29A	CD5	Bovine	Mouse	W.S.U. <sup>1</sup>	IgG2a	565817	BV421	Mouse	Rat	BD H. TM <sup>4</sup>	IgG2a	
β <sub>2</sub> -integrin	MM12A	CD11b	Bovine	Mouse	W.S.U. <sup>1</sup>	IgG1	A31562	Alexa Fluor 680	Mouse	Goat	Invitrogen <sup>1</sup>	IgG1	

PE: R-Phycoerythrin; <sup>1</sup>Washington State University, Pullman, WA, USA; <sup>2</sup>Bio-rad, Raleigh, USA; <sup>3</sup>Invitrogen, Carlsbad, CA, USA; <sup>4</sup>BD Horizon TM, Piscataway, USA.

responses were described (Juliarena et al., 2007), cellular response against the virus has not been well characterized in animals that develop LPL or HPL. Most of the studies carried out the cell dynamics in BLV-infected ewes as a model, nonetheless there is great difference between cattle and ewes in BLV-infection (Florins et al., 2007). To the best of our knowledge, no study has been carried out yet on lymphocyte proliferation and apoptosis of different lymphocyte subpopulations in dairy cows infected with BLV with distinct proviral loads.

BLV infection alters immunological mechanisms, mainly affecting B-lymphocytes; moreover, BLV can also infect and alter other cell types, particularly T-cells (Gillet et al., 2007; Florins et al., 2007; Kabeya et al., 2001; Frie and Coussens, 2015). While polyclonal proliferation of B-cells is likely one factor leading to dramatic alterations in the relative percent of other cell types in peripheral blood mononuclear cells (PBMCs), other factors must be considered, such as the rate of proliferation and apoptosis in various immune cells. Evidence in the literature suggest that both cellular proliferation and apoptosis rates are altered in BLV-infected cattle, though there is no consensus on the direction of these changes (Klener et al., 2006; Erskine et al., 2011; Souza et al., 2011; Frie and Coussens, 2015).

To deepen our knowledge on BLV effects on immune lymphocyte responses, our study attempts to explore the proliferation and apoptosis of distinct lymphocyte subpopulations in clinically healthy BLV-infected cows that sustained different proviral load profiles.

## 2. Materials and methods

### 2.1. Animals

Twenty-four adult Holstein dairy cattle were used: eight animals were classified as BLV-negative or uninfected and 16 animals were classified as BLV-positive animals using the ELISA 108 (Gutierrez et al., 2001). All lactating dairy cows were clinically healthy and between the second and third lactations. Cows around parturition were excluded. The proviral load was determined by real-time PCR as described previously (Farias et al., 2016) and evaluated two times at six-month intervals before classifying BLV-positive animals into the high proviral load, HPL, n = 8 (> 1000 BLV copies/reaction) or into the low proviral load, LPL, n = 8 (< 100 BLV copies/reaction) group. Once animals were re-tested and classified into the different groups, trials were carried out.

### 2.2. PBMCs culture and proliferation assay

Peripheral venous blood samples were collected in tubes containing sodium citrate/EDTA. PBMCs were isolated by Ficoll-Paque™ PLUS density gradient (GE Healthcare, Sigma-Aldrich, Darmstadt, Germany). PBMCs were suspended in RPMI 1640 (Gibco, Thermo Fisher, Massachusetts, USA) with 10% heat-inactivated fetal calf serum (FCS) supplemented with penicillin and streptomycin. PBMCs (3 × 10<sup>5</sup> cells per well) were cultured for 48 h in triplicate wells of 96-well culture cluster (Corning, USA) in the presence of different mitogens:

Concanavalin A (ConA, Sigma-Aldrich, Darmstadt, Germany; 10 ng/1 × 10<sup>6</sup> PBMCs), lipopolysaccharides from *Escherichia coli* 026:B6 (LPS, Sigma-Aldrich, Darmstadt, Germany; 19 μg/1 × 10<sup>6</sup> PBMCs), Pokeweed mitogen (PWM, Sigma-Aldrich, Darmstadt, Germany; 100 ng/1 × 10<sup>6</sup> PBMCs), or without mitogen (control cells). Con-A was used for mitogen-induce proliferation of T lymphocytes (Soder and Holden, 1999; Mehrzad and Zhao, 2008), LPS was used to induce B cells (Mond and Brunswick, 2003; Mehrzad and Zhao, 2008; Latorre et al., 2009), and PWM was used to induce both T- and B- cells (Vries et al., 1980).

The methodology used for cell proliferation assay was similar to that described by Erskine et al., (2011), but with modifications in mitogens concentration, cell culture conditions and metabolite quantification. We measured cell proliferation by 3-(4,5 dimethylthiazol-2-yl) 2,5-diphenyltetrazole (MTT, Sigma-Aldrich, Darmstadt, Germany) reduction assay, quantifying the absorbance of formazan crystals in a spectrophotometer at 490 nm (Mosmann, 1983), at conditions set up in our laboratory.

### 2.3. Flow cytometry analysis

#### 2.3.1. Identification of lymphocyte subpopulations

Aliquots of blood (100 μL) were hypotonically lysed twice, using 0.2% NaCl for approximately 20 s followed by addition of 1.6% NaCl to eliminate contaminating erythrocytes. After centrifugation at 250 × g for 8 min, the cells were washed, resuspended in PBS supplemented with 1% (v/v) FCS and incubated for 30 min with a mix of the following primary monoclonal antibodies (mAbs) CD3, CD4 and CD8, and CD21, CD5 and CD11b. After incubation, 1 mL of PBS was added to the cell suspension and centrifuged at 250 × g for 8 min. Then, cells were incubated for 30 min at room temperature with the labelled secondary Abs resuspended in PBS/1% (v/v) FCS. Antibodies used are described in Table 1. After the second incubation, cells were washed with PBS with 0.1% (v/v) bovine serum albumin and promptly analyzed by flow cytometry (BD FACSCanto II, Becton Dickinson Immunocytometry System™, San Diego, USA). Here, 20,000 cells, excluding most of the cellular debris, were analyzed. An unstained control and single-stained samples were also prepared as compensation controls. Negative control samples were also stained with conjugated isotype control antibodies. In addition, cells were stained with fluorescence minus-one (FMO) controls. Our gating strategy analysis for B-lymphocytes subsets are shown in supplemental Figure S1. Obtained data was analyzed with FlowJo software (TreeStar Inc., Ashland, OR, USA). The percentages of B- and T-lymphocytes were determined within gated peripheral blood mononuclear cells, and their subpopulations were assessed within gated B- or T-cells, respectively. The T-lymphocytes were divided into: T CD4<sup>+</sup>, T CD8<sup>+</sup> and T CD4<sup>+</sup>/CD8<sup>+</sup>.

#### 2.3.2. Detection of apoptosis by flow cytometry

The viability of B and T lymphocytes, and their subpopulations, was evaluated using dual labeling with an annexin-V FITC antibody (556547, BD Biosciences, San Diego, USA) and 7-aminoactinomycin (7-AAD; 420404, Biolend, San Diego, USA). Blood cells were labelled using

mAbs as described above. To analyze the data, scatter plots were generated for the gated B and T cells, and their subpopulations. The non-apoptotic cells were negative for the FITC-labeled annexin V and 7-AAD staining. Cells that were reactive to FITC-labeled annexin V but negative for 7-AAD were classified as apoptotic. Finally, the cells that were reactive for both FITC-labeled annexin V and 7-AAD were classified as late apoptotic or necrotic.

### 2.3.3. Statistics analysis

For statistics analysis of results, the INFOSTAT (Córdoba, Argentina) software was used. In cell proliferation assays, stimulation index was calculated as the relative absorbance of mitogen-stimulated cells divided by the absorbance of unstimulated (control) cells within each group. For flow cytometry results, the normal distribution of data was evaluated with the Kolmogorov test, and differences between groups were evaluated using ANOVA followed by Tukey test. Results are reported as means  $\pm$  SD. A value of  $P \leq 0.05$  was considered significant.

## 3. Results

### 3.1. HPL animals proliferate more than LPL and BLV-uninfected animals

When comparing infected versus with uninfected animals, the first group showed statistically significant differences with all the tested stimuli: ConA ( $P = 0.002$ ), PWM ( $P = 0.007$ ), LPS ( $P = 0.0008$ ) (Fig. 1A). A higher proliferative response was observed in HPL animals compared to LPL, when stimulated with the mitogens ConA ( $P = 0.01$ ), PWM ( $P = 0.03$ ) and LPS ( $P = 0.01$ ). When BLV-uninfected and HPL animals were compared, the BLV-uninfected dairy cows showed lower lymphocyte proliferation stimulation index with all stimuli (ConA,  $P = 0.0007$ ; PWM,  $P = 0.004$ ; LPS,  $P = 0.0007$ ). However, when BLV-uninfected animals were compared with the LPL group, the proliferation response was more evident in the latter group. Proliferation rates in LPL group were higher when stimulating with ConA ( $P = 0.02$ ), PWM ( $P = 0.045$ ) and LPS ( $P = 0.002$ ) (Fig. 1B).

### 3.2. Percentages of total B- and T-lymphocytes and their subpopulations in BLV infected animals with HPL and LPL, and BLV-uninfected animals

The percentages of B-lymphocytes CD21<sup>+</sup>, and its subpopulations CD11b<sup>-</sup>/CD5<sup>+</sup>, CD11b<sup>+</sup>/CD5<sup>+</sup>, CD11b<sup>+</sup>/CD5<sup>-</sup>, CD11b<sup>-</sup>/CD5<sup>-</sup> were evaluated by flow cytometry. The analysis of flow cytometry in PBMCs from HPL, LPL and uninfected animals showed that HPL animals had a higher percentage of B cells ( $P = 0.01$ ) and CD11b<sup>-</sup>/CD5<sup>+</sup> B-lymphocytes ( $P = 0.005$ ), compared to LPL and uninfected cattle. Also, a larger population of CD11b<sup>-</sup>/CD5<sup>-</sup> B-lymphocytes in BLV-uninfected animals compared to HPL animals ( $P = 0.003$ ) (Fig. 2A) was found.

When a similar analysis is performed with T-lymphocytes both the BLV-uninfected cattle and the LPL group presented a higher percentage of CD3<sup>+</sup> lymphocytes than those of HPL group ( $P = 0.01$ ). Similar results were observed in CD4<sup>+</sup> T-cells ( $P = 0.01$ ). However, concerning the CD8<sup>+</sup> T-lymphocytes, this greater difference was only observed in LPL animals compared to HPL ( $P = 0.01$ ). The percentages of T CD4<sup>-</sup>/CD8<sup>-</sup> lymphocytes had no significant differences between the groups studied (Fig. 2B).

### 3.3. B-lymphocytes present greater early apoptosis rates in BLV-uninfected dairy cows than HPL animals

As expected, the B-lymphocyte subpopulations of PBMCs from HPL animals had a much lower percentage of cells exhibiting early apoptosis, which is related to the viability results detailed below. As shown in Fig. 3A, B-lymphocytes ( $P = 0.009$ ) and its subpopulations CD11b<sup>-</sup>/CD5<sup>+</sup> ( $P = 0.007$ ) and CD11b<sup>+</sup>/CD5<sup>+</sup> ( $P = 0.005$ ) presented significant lower early apoptosis rates than BLV-uninfected dairy cows (Fig. 3A). Concerning early apoptosis in T-cell subpopulations, no

significant differences between groups were found (Fig. 3B).

### 3.4. Increased late apoptosis in CD11b<sup>+</sup>/CD5<sup>+</sup> B-lymphocytes in LPL animals

Coincidentally, B CD11b<sup>+</sup>/CD5<sup>+</sup> lymphocytes from LPL animals had both higher early and late apoptosis ( $P = 0.03$ ) than HPL animals. In the case of BLV-uninfected animals, they had higher late apoptosis compared to HPL animals in B cells ( $P = 0.03$ ) (Fig. 4A). Related to the cellular subpopulations of T-lymphocytes which are mainly in late apoptosis, we did not find statistically significant differences between the groups (Fig. 4B).

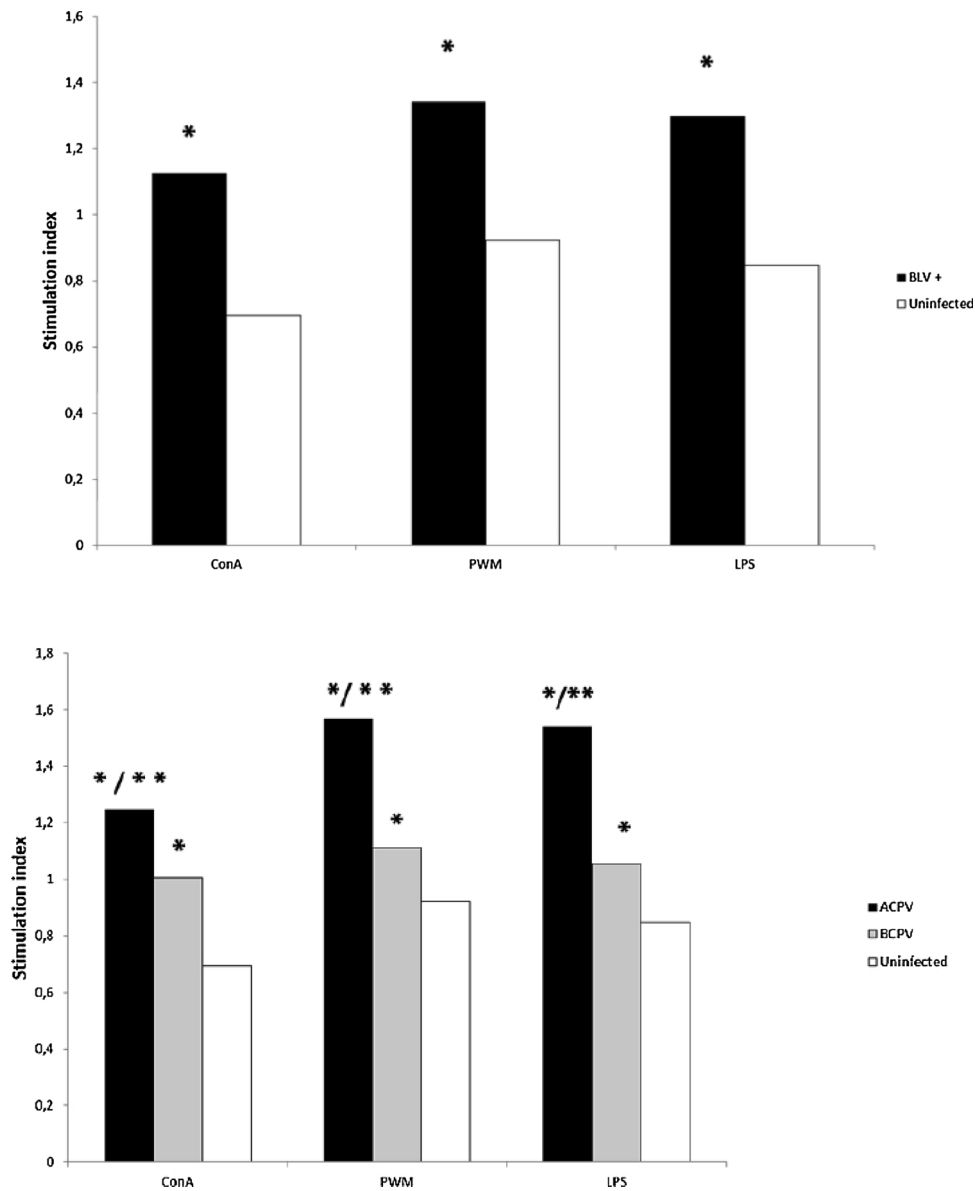
### 3.5. Increased viability in B-lymphocyte subpopulations in HPL animals compared to LPL and BLV-uninfected dairy cows

Differences were found between HPL and LPL animals: the cells of the former are more viable than the latter in the following lymphocyte populations: B lymphocytes ( $P = 0.003$ ) and B CD11b<sup>+</sup>/CD5<sup>+</sup> ( $P = 0.004$ ) cells. Also, differences were found between HPL and BLV-uninfected animals: B-lymphocytes ( $P = 0.003$ ) and its subpopulations CD11b<sup>-</sup>/CD5<sup>+</sup> ( $P = 0.0001$ ), B CD11b<sup>+</sup>/CD5<sup>+</sup> ( $P = 0.004$ ) and B CD11b<sup>-</sup>/CD5<sup>-</sup> ( $P = 0.04$ ). In the case of the LPL animals, the B CD11b<sup>-</sup>/CD5<sup>+</sup> cells were negative for Annexin-V FITC and 7-AAD (non-apoptotic) compared to BLV-uninfected cattle ( $P = 0.0001$ ) (Fig. 5A). Regarding the viability of T-lymphocytes and its subpopulations, there were no significant differences between groups (Fig. 5B).

## 4. Discussion

Although BLV infection causes EBL only in less than 10% of infected animals, recent studies suggest that it causes more negative effects on the herd than previously believed. BLV interferes with the production and performance of infected animals, and there are abnormalities in the immunity of these animals (Frie and Coussens, 2015). Several studies have focused on studying the abnormalities in cell proliferation, which would lead to PL, finding differences in the ovine experimental model related to the natural BLV host (Florins et al., 2008). Currently all the studies were studied in lymphocyte proliferation in naturally BLV-infected dairy cows, PL and non-PL animals (Erskine et al., 2011; Souza et al., 2011). No study has regarded the different BLV proviral load that may bias the previous findings, as a significant proportion of AL dairy cows showed HPL as animals with PL (Juliarena et al., 2007). As far as we know, the present study is the first one concerning cell proliferation and apoptosis developed in naturally infected cattle, considering their proviral load.

*In vitro* studies using mitotic stimuli on lymphocytes are aimed to evaluate their functionality, and cells usually respond by producing cytokines, expressing cytokine receptors, and ultimately, proliferating. In our assays, mitogens were selected based on their proliferation effect on different lymphocyte subpopulation: LPS and PWM mitogens both stimulate B-lymphocytes, while ConA stimulates T-cells. Stimulated PBMCs from HPL animals proliferate more than those from LPL and BLV-uninfected cattle; and in turn, cells from infected animals proliferate more than those from uninfected. When LPS and PWM mitogens were used, the same groups had the same behavior as when ConA was used. We could conclude that PBMCs from HPL animals are more sensitive to cell proliferation when stimulated *in vitro* with different mitogens. Sordillo et al. showed previously that lymphocytes from PL cattle had lower proliferative responses to ConA or PWM, compared with cells from AL or BLV-uninfected animals (Sordillo et al., 1994). Other data from the literature describe that, when stimulating with ConA, cells from negative animals have greater proliferation when compared with those from PL, and concerning stimulation with PWM, cells from AL animals proliferate more than those from PL and uninfected animals (Erskine et al., 2011). Differences in results might come



**Fig. 1.** Mean  $\pm$  SEM lymphoproliferative response to mitogens from PBMCs isolated from: (A) BLV-infected animals (BLV+) and BLV-uninfected animals (BLV-); (B) in HPL, LPL and BLV-. One asterisk (\*) indicates significant statistical differences ( $P < 0.05$ ) related to the uninfected control; two asterisks (\*\*) indicate statistically significant differences ( $P < 0.05$ ) related to LPL.

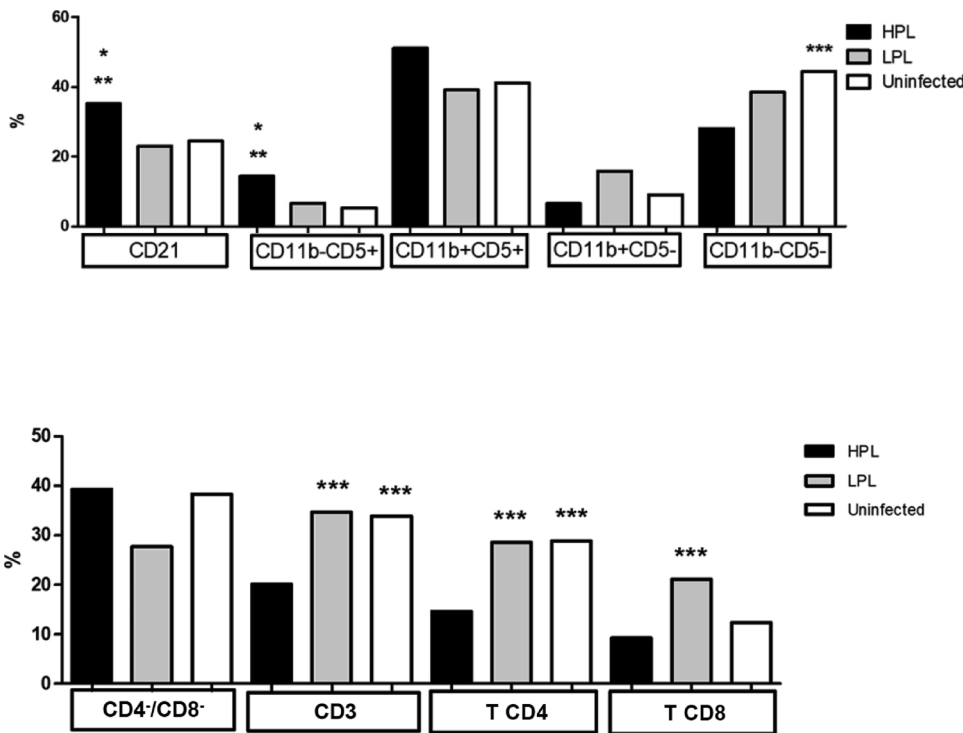
from different concentration of mitogens used in the *in vitro* studies. Besides, we classify animals in terms of their proviral load, and the group with HPL may include AL animals.

In our work, HPL animals have greater proliferation with and without stimuli in culture than the others groups. Our results might agree with those from Konnai et al., who reported spontaneous cell proliferation in PBMCs from BLV-infected cattle with high percentage of BLV-infected cell and with lymphosarcoma, and non-spontaneous cell proliferation in PBMCs derived from cattle with low percentage of BLV-infected cells or BLV-uninfected cattle (Konnai et al., 2006).

BLV-infected animals often show abnormal ratios of cell populations in comparison to healthy animals: significantly higher relative B-cell percentages and significantly lower relative T-cell percentages, including lower percentages of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells (Erskine et al., 2011; Sordillo et al., 1994; Van den Broeke et al., 2010) and a higher ratio of CD4<sup>+</sup>:CD8<sup>+</sup> T cells (Stone et al., 1995) than uninfected controls. Based on proviral load, we found that HPL animals have a significantly lower percentage of T-cells, both CD4<sup>+</sup> and CD8<sup>+</sup> cells. Therefore, when stimulated with ConA, PBMCs from these animals tend

to proliferate less than those from LPL or negative animals. As we observed that HPL animals have a higher percentage of B-cells, this could explain why PBMCs proliferate more with LPS and PWM, compared to the other two groups. Indeed, BLV primarily infects B-cells (Meiron et al., 1997) and 66% of CD5<sup>+</sup> B-cells carried the provirus (Mirsky et al., 1996). So, as others authors suggested (Frie et al., 2017), our results could indicate that infected B-cells proliferate in response to immune stimulation. A key factor to be taken into account is BLV reactivation when infected cell are *in vitro* cultured (Florins et al., 2007). Even though whole PBMCs were evaluated, the high rates of proliferation seen in our study could come from B-lymphocytes, which may also proliferate due to virus reactivation in culture.

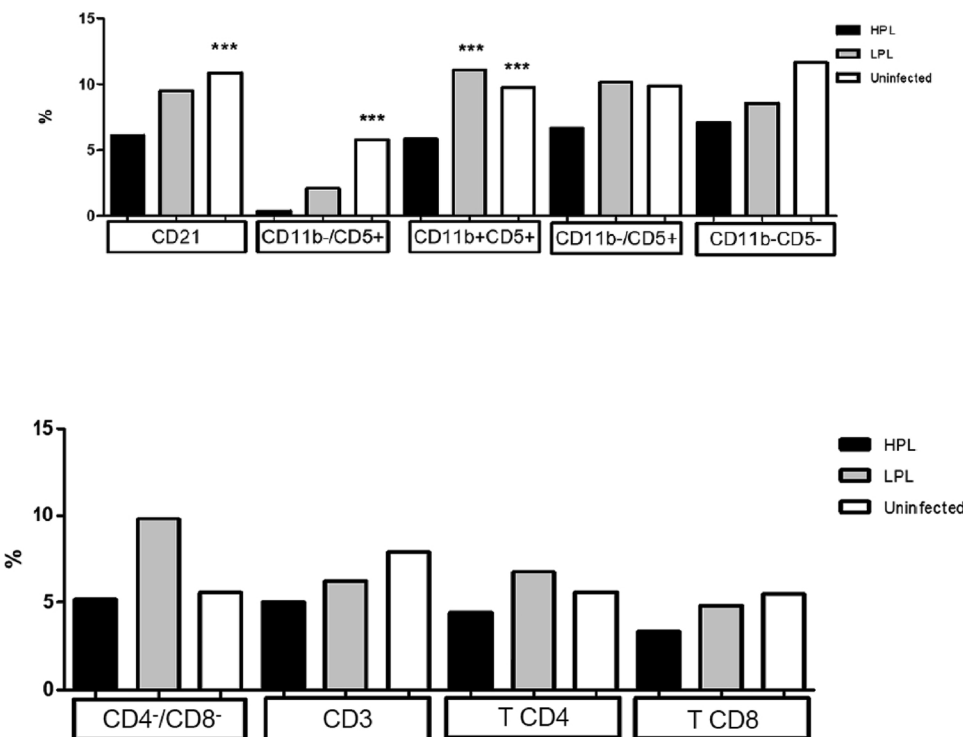
Programmed cell death (apoptosis) is a biological process for the normal functioning of the immune system. When this mechanism is altered due to BLV infection, the homeostasis of lymphocyte populations is altered (Debacq et al., 2003; Florins et al., 2008). Several studies on cell dynamics and apoptosis were carried out in experimentally BLV-infected sheep (Chevallier et al., 1998; Debacq et al., 2002; Florins et al., 2009). Moreover, concerning the natural host for the virus, most



**Fig. 2.** Percentage of B-lymphocytes (A) or T-lymphocytes (B) and its subpopulations from BLV-uninfected and BLV-infected animals with low proviral load (LPL) and high proviral load (HPL). One asterisk (\*) indicates significant difference to BLV-uninfected animals ( $P < 0.05$ ); two asterisks (\*\*) indicate statistically significant differences to LPL animals ( $P < 0.05$ ); three asterisks (\*\*\*) indicate statistically significant differences to HPL animals ( $P < 0.05$ ).

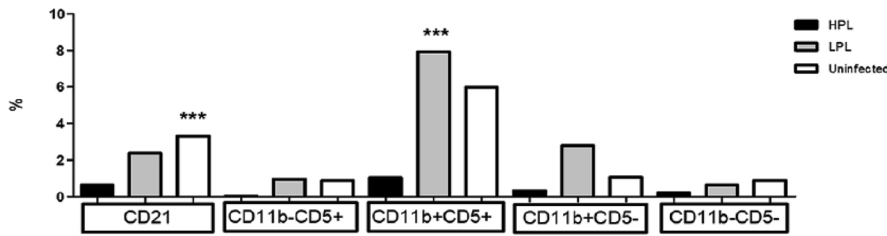
studies were done *in vitro*, on cultured cells, and showed an increased apoptosis rates in B-cells from AL cows (Cantor et al., 2001; Debacq et al., 2003). On studies demonstrated that freshly isolated B-cells do not have significantly different rates of apoptosis between uninfected and BLV-infected cattle (Erskine et al., 2011). On the contrary, Souza observed a lower lymphocyte proliferation in the animals of the LP group associated with the reduction of CD5<sup>+</sup> cell apoptosis (Souza et al., 2011). We evaluated, for the first time, B- and T-lymphocytes subpopulations, from uninfected and BLV-infected animals classified according to their proviral load, in terms of their percentage in

peripheral blood and its stage of apoptosis and viability. Data from the literature suggest that the target cell for BLV is MHCII<sup>+</sup>, IgM<sup>+</sup>, CD5<sup>+</sup> and CD11b<sup>+</sup> with some fluctuations for the three latter markers at late stages of the disease (Gillet et al., 2007). We show that the percentage of CD11b<sup>+</sup>/CD5<sup>+</sup> cells is increased in HPL animals compared to LPL and no infected animals, although without significant differences. Della Libera et al. indicate that BLV infected animals have increased values for CD21, CD5 and CD11b markers (Della Libera et al., 2012), phenomena that might be related to the modulation of apoptosis in these cells (Souza et al., 2011). As in humans and mice, bovine CD5 molecule

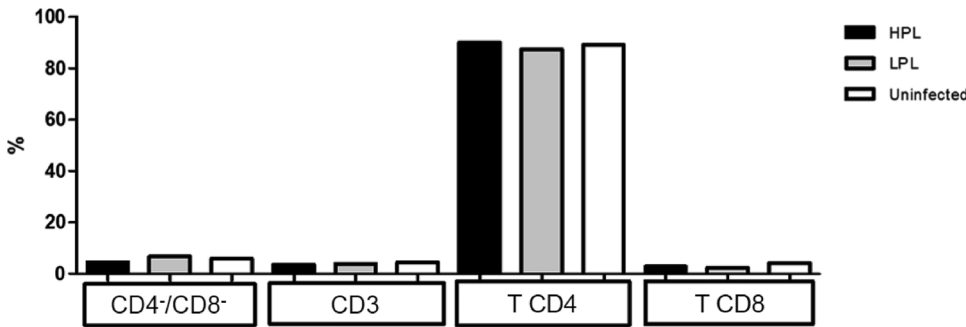


**Fig. 3.** Percentage of early apoptosis in B-lymphocytes (A) or in T-lymphocytes (B) and their subpopulations from BLV-uninfected and BLV-infected animals with low proviral load (LPL) and high proviral load (HPL). One asterisk (\*) indicates significant difference compared to not infected animals ( $P < 0.05$ ); two asterisks (\*\*) indicate statistically significant differences to LPL animals ( $P < 0.05$ ); three asterisks (\*\*\*) indicate statistically significant differences to HPL animals ( $P < 0.05$ ).





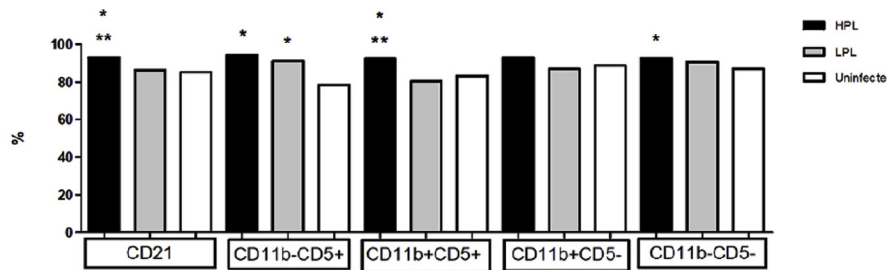
**Fig. 4.** Percentage of late apoptosis in B-lymphocytes (A) or in T-lymphocytes (B) and their subpopulations from BLV-uninfected and BLV-infected animals with low proviral load (LPL) and high proviral load (HPL). One asterisk (\*) indicates significant difference related, compared, to not infected animals ( $P < 0.05$ ); two asterisks (\*\*) indicate statistically significant differences to LPL animals ( $P < 0.05$ ); three asterisks (\*\*\*) indicate statistically significant differences to HPL animals ( $P < 0.05$ ).



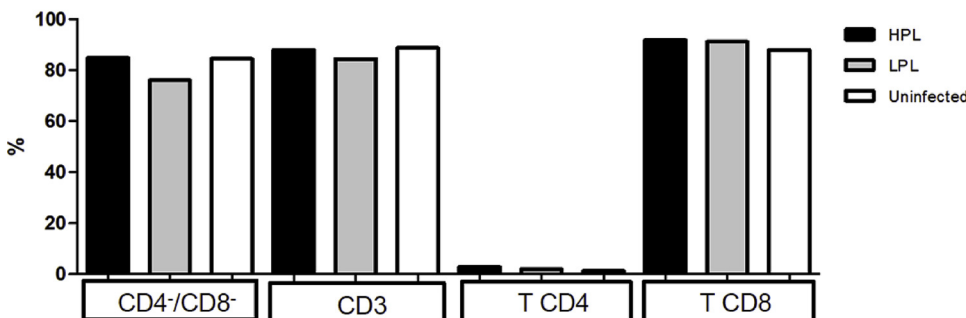
is present in most T-cells but only in a small number of normal B-cells; however, in BLV-infected PL cattle, nearly all of the B-cells are CD5<sup>+</sup> (Depelchin et al., 1989). In B-cells from uninfected animals, CD5<sup>+</sup> associates with the B-cell receptor (BCR), and this crosslinking decreases apoptosis of CD5<sup>+</sup> B-cells. However, CD5 is dissociated from de BCR in B-cells from PL cattle and it was suggested that subsequent decreased apoptosis in antigenically stimulated B-cells may thus be a mechanism of BLV induced PL (Hamilton et al., 2003; Gillet et al., 2007). Another marker, the CD11b integrin molecule, was also observed in infected animals from several studies (Gillet et al., 2007). In our study, CD11b<sup>+</sup>/CD5<sup>+</sup> cells population from HPL animals have a higher percentage of cell viability with respect to the other two groups, which is

accompanied by a decrease in early and late apoptosis. These results would be in accordance with data from the literature, where lymphocyte immune phenotyping of BLV-infected cows with PL had an increase in B-cells, mainly B CD5<sup>+</sup>/CD11b<sup>+</sup>, due to apoptosis inhibition (Blagitz et al., 2017). Moreover, Erskine et al. showed that PL animals have an increased expression of the anti-apoptotic gene *Bcl2*, and AL animals had higher expression of the pro-apoptotic gene *Bcl2L1* (Erskine et al., 2011).

When analyzing the other B-cell subpopulations, the markers that are increased in HPL animals compared to non-infected animals are CD21<sup>+</sup> and CD11b<sup>-</sup>/CD5<sup>+</sup>. In addition, these cells have a higher percentage of viability, and a decrease in early apoptosis. This could be



**Fig. 5.** Percentage of viability in B-lymphocytes (A) or in T-lymphocytes (B) and their subpopulations from BLV-uninfected and BLV-infected animals with low proviral load (LPL) and high proviral load (HPL). One asterisk (\*) indicates significant difference to not infected animals ( $P < 0.05$ ); two asterisks (\*\*) indicate statistically significant differences to LPL animals ( $P < 0.05$ ); three asterisks (\*\*\*) indicate statistically significant differences to HPL animals ( $P < 0.05$ ).



a manifestation that BLV infection also alters the balance of cell death and proliferation in other lymphocyte populations such as CD21<sup>+</sup> and CD11b<sup>-</sup>/CD5<sup>+</sup>. When uninfected and LPL animals were compared, no differences in the viability, total percentage and apoptosis were found in CD11b<sup>+</sup>/CD5<sup>+</sup> and CD21<sup>+</sup> cells. On the contrary, we observed a slight increase in the percentage of cell viability of the CD11b<sup>-</sup>/CD5<sup>-</sup> cells in LPL animals.

Our results show that HPL animals have an imbalance in terms of programmed cell death and cell viability in cells that are target of BLV. In fact, LPL animals have increased apoptosis rates in this cellular subpopulation. In turn, the main difference between BLV-uninfected and LPL animals is that the latter have a higher percentage of late apoptosis compared to HPL. Therefore, this could be specifically the way by which LPL animals control the progression of the disease.

Regarding population of T-lymphocytes, other authors showed that PL animals have a lower counting in the absolute number of T-cells, both CD4<sup>+</sup> and CD8<sup>+</sup>, in comparison with AL or uninfected animals (Gatei et al., 1989). However, Della Libera et al. described no significant changes in the absolute numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes in PL animals, but with a lower percentage of both subsets (Della Libera et al., 2012). In this way, we found that LPL animals have a higher percentage of CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T-lymphocytes than HPL animals.

The proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be related to the differential secretion of cytokines in the groups of animals. Indeed, cytokines expression in response to BLV infection is deregulated, with a prevalence of a Th1 profile observed in animals with lymphosarcoma and PL. This imbalance and suppression of T-cell proliferation in response to BLV antigens are considered to be associated with disease progression (Orlik and Splitter, 1996; Trainin et al., 1996; Pyeon and Splitter, 1998; Yakobson et al., 2000; Kabeya et al., 2001; Frie and Coussens, 2015). Our previous studies showed that expression of IFN- $\gamma$  (Th1 cytokine) is higher in LPL animals (Farias et al., 2016), indicating a protective role of IFN- $\gamma$  in the pathogenesis of BLV infection. This cytokine mediates the activation of NK cells, monocytes/macrophages and dendritic cells; it promotes the differentiation of CD4<sup>+</sup> T-cells into a Th1 profile and the activation of T CD8<sup>+</sup> cells, in favor of the development of an effective antiviral immunity. In turn, as a positive feedback, IFN- $\gamma$  is secreted by  $\gamma\delta^+$  T-cells and NK cells, CD4<sup>+</sup> Th1 cells and cytotoxic CD8<sup>+</sup> T-cells, monocytes/macrophages and dendritic cells (Schroder et al., 2004). The largest proportion of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes found in our LPL animals reinforces the concept of a better capability of these animals to eradicate/control BLV infection, may be via strong cytotoxic responses positively regulated by IFN- $\gamma$ . As mentioned,  $\gamma\delta^+$  T-cells and NK cells are major IFN- $\gamma$  sources and they have also cytotoxic functions. Clevers et al. defined the bovine T CD4<sup>+</sup>/CD8<sup>-</sup> population as  $\gamma\delta^+$  T-lymphocytes (Clevers et al., 1990). It has been described that the state of AL in BLV infection is related to a greater proportion of  $\gamma\delta^+$  T-lymphocytes (Lundberg and Splitter, 2000). However, in our study no differences were found in the percentage of T CD4<sup>+</sup>/CD8<sup>-</sup> population. Thus, the higher expression of IFN- $\gamma$  mRNA found in LPL animals (Farias et al., 2016) might not come from those cells, but it could be related to a greater cytotoxic CD8<sup>+</sup> cell, NK cell or professional antigen-presenting cell percentage in these animals.

LPL animals presented a higher percentage of apoptosis in CD11b<sup>+</sup>/CD5<sup>+</sup> B-lymphocytes, which are the primary targets for BLV integration (Meiron et al., 1997). These data, in turn, are related to the high percentage of viability of the B subpopulations of LPL animals. This reinforces the concept that LPL animals have an anti-apoptotic and proliferative status.

Our work confirmed that BLV infection alters cellular homeostasis, and it turns in a deficiency of the immune system in infected animals. Indeed, in agreement with our findings, a functional exhaustion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, associated to disease development has been demonstrated (Okagawa et al., 2018). Thus, infected animals are less competent to fight against other diseases, which generates significant

economic losses. The only way to eradicate BLV infection would be to sacrifice positive animals. As there are no economic compensation policies in our country, we suggest that one way to control the infection would be replacing HPL animals with LPL animals since they behave like “controlling animals”. At the same time, it would be interesting to know which proteins are involved in the apoptosis of these animals, in order to accurately identify how the BLV alters its target cells.

### Conflict of interest statement

The authors declared no potential conflicts of interest with respect to the research, authorship, publication of this article and/or financial and personal relationships that could inappropriately influence this work.

### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetimm.2018.10.012>.

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