Study of the proviral load levels and mRNA expression of cytokines in peripheral blood mononuclear cells and somatic milk cells in cattle with different BLV infection profiles

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Summary
The retrovirus bovine leukemia virus (BLV) might produce abnormal immune function, associated with susceptibility to developing other infectious diseases, including mastitis. This study aimed to determine the proviral load and cytokines gene expression in peripheral blood mononuclear cells (PMBC) and milk somatic cells (SC) in BLV-infected and non-infected cattle. Of 27 BLV-infected cows in PBMC, 17 (62.96%) had a high proviral load (HPL), and 10 (37.04%) had a low proviral load (LPL). All SC samples had low proviral load (LPL-SC). Higher IFN-γ and IL-10 expression, and lower IL-12 and IL-6 expression, were found in PBMC from BLV-infected compared to BLV non-infected cattle. Moreover, higher IFN-γ, IL-12, and IL-6 expression, and lower IL-10 expression were observed in cattle with LPL-PBMC compared to HPL-PBMC. In milk samples, lower IFN-γ and higher IL-12 mRNA expression were observed in LPL-SC compared to BLV non-infected cattle in SC. IL-10 and IL-6 expression mRNA was significantly lower in LPL-SC than in SC from BLV non-infected cattle. This study shows that milk SC maintains lower proviral load levels than PBMC. This first report on Th1 and Th2 cytokines expression levels in SC may be relevant to future control strategies for BLV infection, mastitis, and udder health management.

Introduction

The bovine leukemia virus (BLV) is an exogenous retrovirus belonging to the *Deltaretrovirus* genus of the *Retroviridae* family. This enveloped virus naturally infects B-lymphocytes causing enzootic bovine leukemia, a disease more common in dairy than beef herds (Giraudo *et al.*, 2010). Approximately 30% of BLV-infected cattle develop persistent lymphocytosis, with 1-10% of these cattle manifesting lymphosarcoma; the rest remain asymptomatic (Gillet *et al.*, 2007; OIE, 2021). All cattle with persistent lymphocytosis have a high proviral load (HPL). Thus, the asymptomatic can be divided into two groups: those having a low proviral load (LPL) (60%), and those having HPL (40%) (Juliarena *et al.*, 2007). Enzootic bovine leukemia has a worldwide distribution (Polat *et al.*, 2017). The latest studies in some regions of Argentina have reported an individual animal prevalence of 15-90% and a herd prevalence of 84-99% of BLV infections (Giraudo *et al.*, 2010; Gutiérrez *et al.*, 2020). Data from the USA reveal that the infection impairs the

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performance of dairy cattle, showing annual losses of up to 218 kg of milk/cow by every 10% increase in the prevalence of BLV infection, leading to annual economic losses estimated at USD525 million (Bartlett *et al.*, 2013; Erskine *et al.*, 2012; Ott *et al.*, 2003). Moreover, BLV infection could lead to cell-mediated impairment of immunity, predisposing BLV-infected cattle to develop opportunistic infections (Frie and Coussens, 2015; Ohira *et al.*, 2016). Therefore, BLV infection could be related to a higher incidence of mastitis, one of the most common diseases in dairy cattle with the most significant economic impact on the dairy industry(Frie and Coussens, 2015). These immune dysfunctions include alterations in the expression level of type 1 T helper (Th1) and type 2 T helper (Th2) cytokines (Kabeya *et al.*, 2001). Helper T cells (Th) have been defined based on their different cytokine secretion patterns and immunomodulatory effects, mainly in peripheral blood mononuclear cells (PBMC). Th1 cells produce interferon-gamma (IFN-γ), interleukin (IL)-12 primarily, and tumor necrosis factor (TNF), which are responsible for cell-mediated inflammatory reactions. Th2 cells secrete IL-4, IL-6, IL-10, and IL-13, mediating B cell activation and antibody production. The selective differentiation of naive CD4+ T cells into effector Th1 or Th2 cells defines an immune response success or disease progression (Diehl and Rincón, 2002). A Th1 response against BLV, inducing cell-mediated immunity, seems critical in preventing disease progression (Kabeya *et al.*, 2001).The somatic cells (SC) released into milk contain a mix of leukocytes and epithelial cells (Alhussien and Dang, 2018; Schultz, 1977) . Those epithelial cells come from the desquamation of the mammary epithelium of alveoli and ducts. The leukocytes in milk come directly from the blood supply to the mammary gland. After the anatomical and chemical barriers of the teat apex and canal in the mammary gland, leukocytes act as a second line of defense against any infection and assist in repairing damaged tissue (Alhussien and Dang, 2018). Therefore, a high somatic cell count (SCC) (greater than 200,000 somatic cells/mL) resulting from any intra-mammary infection would indicate poor quality and hygiene of the milk (Alhussien and Dang, 2018; SENASA, 2017). Several BLV studies have focused on viral detection in milk immune cells (della Libera *et al.*, 2015; Jaworski *et al.*, 2016; Kuckleburg *et al.*, 2003) or mammary epithelial cells (Buehring *et al.*, 1994; Motton and Buehring, 2003; Yoshikawa *et al.*, 1997). Furthermore, other studies reported that BLV infection is strongly associated with increased SCC in milk, particularly in cattle with more than four lactations (Yang *et al.*, 2016). Whether BLV infection is associated with immunological alteration in the bovine mammary gland and the development of bovine mastitis is unclear. However, previous investigations have reported a marked alteration in cytokine expression at the systemic level in BLV-infected cattle (Frie and Coussens, 2015). Moreover, other authors found alterations in the functioning of mammary gland cells from BLV-infected cattle, suggesting that this changed immune response could promote the development of other infections (della Libera *et al.*, 2015). This study aimed to compare the proviral load in PBMC and SC in BLV-infected cattle and determine mRNA expression levels for Th1 (IFN-γ, IL-12) and Th2 (IL-10, IL-6) cytokines in cells from those cell compartments.

Materials and methods

Animals and Samples

For this study, adult cattle belonging to Holstein (Holando Argentino) breed from two farms located in Tandil region (Buenos Aires Province) between 2018 and 2019, were selected. The cattle were clinically healthy; only cattle with <200,000 SCC/mL were included in the study. Fifteen BLV non-infected cattle were from 2 to 9 year old (5.10 ± 1.91) with a mean of SCC 70.90 x 10^3 cell/mL (SD, 68.02 x 10^3 cell/ml) between 1st - 6th lactation (3.44 ± 1.42), and 33 BLV-infected cows were from 2 to 8 year old (4.36 ± 1.50) with a mean of SCC 77.50 x 10^3 cell/mL (SD, 57.84 x 10^3 cell/ml) between 1st - 5th lactation (2.02 ± 1.05) (Table I). Serological BLV status had been previously determined by testing plasma for anti-BLV antibodies by ELISA 108 (Gutiérrez *et al.*, 2001). All animal experiments were conducted by applying a work protocol approved by the Animal Welfare Committee, Faculty of Veterinary Sciences, National University of the Province of Buenos Aires, UNCPBA (permit number: ResCA 087/02).

Blood samples

Blood samples from 12 BLV non-infected and 27 BLV-infected cattle were obtained. First, PBMC were separated as previously described (Farias *et al.*, 2016). Briefly, every 10 mL heparinized blood sample (5 U/ml) obtained by jugular venipuncture was transferred to a 15 mL tube and centrifuged for 15 min at 2599 g at 4 °C. Next, buffy-coat was mixed with 11 mL of cold ammonium chloride buffer (150 mM NH_4Cl, 8 mM Na_2CO_3, and 6 mM EDTA) to lyse red peripheral blood cells. Then, the cells were centrifuged at 1620 g for 10 min at 4 °C, washed with PBS, and centrifuged again at 1125 g for 7 min at 4 °C. Finally, the PBMC pellet was fractioned into 3 equal parts: one fraction was dry stored at −20 °C for DNA extraction, and two fractions were resuspended in Biozol® (PBL-Embiotec, Argentina) and stored at −80 °C until RNA extraction (described in section *RNA extraction and DNase I treatment*).

Table I. *Demographic data of groups of animals in this study.*

Cell compartment	BLV status	N	Age (mean years ± SD)	Lactation (mean ± SD)	SCC (mean 10^3/mL ± SD)	Proviral load (mean copies/30 ng DNA ± SD)
	BLV-infected	33*	4 ± 1.50	2 ± 1.05	77.5 ± 57.8	NA
SC	LPL-SC	25	4 ± 1.20	2 ± 1.02	91.8 ± 62.2	201.20 ± 240.50
	LPL-PBMC	4	3 ± 0.58	1 ± 0.50	90 ± 33.5	75.49 ± 108.53
	HPL-PBMC	21	4 ± 1.15	2 ± 0.86	92.9 ± 56.9	268.71 ± 266.99
PBMC	LPL-PBMC	10	4 ± 1.87	1 ± 1.17	71.2 ± 51.1	148.30 ± 164.48
	HPL-PBMC	17	4 ± 1.39	2 ± 1.07	69.5 ± 59.4	$6.5 \times 10^5 \pm 1.71 \times 10^6$
	BLV non-infected	15**	5 ± 1.91	3 ± 1.42	70.9 ± 68.0	NT
SC	BLV non-infected	6	4 ± 1.00	2 ± 1.53	59 ± 4	NT
PBMC	BLV non-infected	12	5 ± 2.00	3 ± 1.25	82.6 ± 73.8	NT

NT: no tested. NA: does not apply. *: 19 BLV-infected cattle paired in PBMC and SC samples plus 8 BLV-infected cattle unpaired in PBMC plus 6 BLV-infected cattle unpaired in SC.
**: 12 BLV non-infected cattle paired in PBMC and SC plus 3 BLV non-infected cattle unpaired in SC.

Milk samples

Milk samples from 6 BLV non-infected and 25 BLV-infected cows were included. For SC separation, 50 mL of pooled milk from the 4 mammary quarters were manually obtained per cow after morning milking. The samples were centrifuged for 20 min at 1620 g at 4 °C to separate and eliminate the cream coat and whey. The cellular pellet was washed twice with PBS and centrifuged at 1620 g for 10 min at 4 °C to eliminate casein and fat globules. Finally, the SC pellet was fractioned into 3 equal parts: one fraction was stored at –20 °C for DNA extraction, and two fractions were resuspended in Biozol® (PBL-Embiotec, Argentina), and stored at –80 °C until RNA extraction (described in section *Reverse transcription*).

DNA extraction

DNA from PBMC and SC was extracted using a phenol: chloroform: isoamyl protocol previously described (Farias *et al*., 2016), and DNA was eluted in 30 µL of water. To determine the concentration and purity of DNA, the OD value at 260 nm and 260/280 coefficient, respectively, in a NanoDrop 2000 Spectrophotometer (Thermo Scientific) were used. Samples with a final concentration >20 ng/µL and 260/280 values of 1.8 to 1.9 were selected. Finally, the purified DNAs were stored at –20 °C until further use, no longer than >3 months.

Proviral load determination

The proviral load determination and frequency of BLV detection were carried out in PBMC and SC. The proviral loads of all samples from BLV-infected cattle were estimated as copy numbers present in 30 ng of DNA. The absolute quantification by real-time PCR (qPCR) was done with the primers for the BLV *pol* gene as previously described (Farias *et al*., 2016) (Table II), with the slight modification of carrying out the reaction in 10 µL final volume using 2X SYBR Green Master Mix (PBL-Embiotec, Argentina).

The amplification and detection of the specific products were carried out using an Applied Biosystems 7500 cycler under the following conditions: 5 min at 94 °C, 40 cycles of 15 s at 92 °C, and 30 s at 60 °C. Both, the standard curve and non-template control were included in each run. Each curve point was amplified in triplicate, and the amplification efficiency was determined using a linear regression model according to the equation E = 10[-1/slope] (Pfaffl, 2001). The standard curve was validated by repeating the amplification at least 3 times. BLV-infected cattle were classified into two groups based on the number of proviral copies integrated into their genome (Farias *et al*., 2016). The proviral load was evaluated twice at 6-month intervals before classifying the cattle into each group.

Table II. *Sequences of the qPCR primers set used for measurement of gene expression and melt temperature.*

Gen	Forward primer (5' → 3')	Tm °C	Reverse primer (5' → 3')	Tm °C
GAPDH	CACCCTCAAGATTGTCAGCA	51.78	GGTCATAAGTCCCTCCACGA	53.83
IFN-γ	CAGCTCTGAGAAACTGGAGGAC	63	TT TGGCTTTGCGCTGGATCT	63
IL-6	CAGCTATGAACTCCCGCTTCA	63	AGGAGCAGCCCCAGGG	62
IL-10	CTGACAGCAGCTGTATCCACTTG	62	GTGCAGTTGGTCCTTCATTTGA	62
IL-12	AAACCAGACCCACCCAAGAAC	63	CCTCCACCTGCCGAGAATT	59.6
POL	CACCATTCACCCCCACTTG	60.2	TCAGAGCCCTTGGGTGTTTC	61,8

Gene expression of mRNA

RNA extraction and DNase I treatment

Total RNA extraction from PBMC and SC was performed using Biozol® (PBL-Embiotec, Argentina) according to the manufacturer's instructions. RNA was eluted in 30 µL of RNase-free water. To determine the concentration and purity of RNA, we use the OD value at 260 nm and 260/280 coefficient, respectively, in a NanoDrop 2000 Spectrophotometer (Thermo Scientific).

Samples with a final concentration >70 ng/µL and 260/280 values of 1.9 to 2 were selected. The extracted RNA was kept at −80 °C until use. To remove any possible contaminating genomic DNA, 0.1-1 µg of total RNA was treated with RNase-free DNase I (PBL-Embiotec, Argentina) according to the manufacturer's instructions.

Reverse transcription

First-strand cDNA was synthesized in 20 µl reaction volume, which contained 1 µg total RNA, 1 µl M-MLV reverse transcriptase (200 U/µl) (PBL-Embiotec, Argentina), 4 µl 5X First-Strand Buffer (PBL-Embiotec, Argentina), 2 µl dNTPs Mix (10 mM) (PBL-Embiotec, Argentina), and 2 µl Random hexamer primers (Roche) under the following conditions: 10 min at 25 °C, 60 min at 37 °C and 5 min at 95 °C.

Negative controls (all the reagents except reverse transcriptase) for each sample to cDNA synthesis were included.

The cDNA was either immediately used or stored at −20 °C until use, no longer than one month.

Real-time PCR (qPCR) conditions for mRNA gene expression

The qPCR reaction mix contained specific forward and reverse primers for the different cytokines, 2X Master Mix qPCR with SYBR Green (PBL-Embiotec, Argentina), and 2 µL of cDNA (previously diluted 1:2), in a final volume of 10 µL.

The specific forward and reverse primers concentrations were 0.15 µM for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Okuda *et al*., 2010), 0.3 µM for IFN-γ (Waldvogel *et al*., 2000), 0.3 µM for IL-12 (Farias *et al*., 2016), and 0.5 µM for IL-10 (Almeida *et al*., 2007) and IL-6 (Werling *et al*., 2002).

The amplification and detection of the specific products were carried out using an ABI 7500 cycler under the following conditions: 5 min at 94 °C, 40 cycles of 15 s at 92 °C, and 30 s at 60 °C.

All experiments were carried out in triplicate. Negative controls for cDNA synthesis and non-template control were included in all cases.

Data analysis

In a previous study, three endogenous genes (*GAPDH*, *β-actin*, and *RN18S*) in the PBMC of BLV-negative cattle were tested. Target gene expression was normalized to that of the endogenous gene (bovine *GAPDH*) because it was validated *GAPDH* as the most stable reference gene. GAPDH amplified with a similar dynamic range as that of our target genes. Standard curves were performed with eight serial dilutions (8-fold) of a reference cDNA calibrator in triplicate to determine the PCR efficiency of each gene (efficiencies *GAPDH* 1.813, *IFN*-γ 1.945, *IL-12* 1.969, *IL-10* 1.991, and *IL-6* 2.004).

Amplification efficiency was calculated using the equation: $E = 10^{(-1/slope)}$, and a relative expression software tool (*fgStatistics* software, version 2009) was used to get the relative expression, statistical analysis, and SE for the results (di Rienzo *et al*., 2009). This method uses the following calculations with efficiency correction and normalization by a reference gene to determine the relative expression ratios:

$$\text{Ratio} = \frac{E\ target^{\Delta Cq\ Target(control\text{-}sample)}}{E\ reference^{\Delta Cq\ reference(control\text{-}sample)}}$$

E is the efficiency of the qPCR reaction, and Cq is the crossing point above background fluorescence (Pfaffl, 2001; Pfaffl *et al*., 2002).

Statistical analysis

Pearson's test assessed the correlation between levels of the proviral load in PBMC vs. SC of milk samples, and proviral load vs. SCC, using InfoStat software. P-values <0.05 were considered significant.

P-values for expression analysis were calculated in the fgStatistics software by 5000 randomly selecting combinations of biological and technical replicates following the permutation test paradigm. Statistically significant differences were determined at $p < 0.05$ (Pfaffl et al., 2002).

Results

Determination of BLV proviral loads in SC and PBMC

BLV-infected cattle were classified into two groups according to their proviral load in PBMC: high proviral load when having >1000 BLV copies/30 ng of DNA reaction (HPL-PBMC) or low proviral load when having <100 BLV copies/30 ng of DNA reaction (LPL-PBMC).

From 27 BLV-infected cattle in PBMC, 17 (62.96%)

were HPL-PBMC (2.7 x 10^3- 6.10 x 10^6 copies/30 ng of DNA reaction; mean, 6.5 x 10^5 ± 1.71 x 10^6 copies/30 ng of DNA reaction), and 10 (37.04%) were LPL-PBMC (54 - 608 copies/30 ng of DNA reaction; mean, 148.30 ± 164.48 copies/30 ng of DNA reaction).

When the qPCR reaction was applied to SC samples from 25 cattle serological BLV positive, all samples resulted positive (100%). All SC samples had less than 1000 BLV copies/reaction (2.96 – 1000 copies/30 ng reactions; mean, 201.20 ± 240.50 copies/30 ng of DNA reaction), even retesting samples.

Correlation of BLV proviral load between SC and PBMC

The proviral load in PBMC has been associated with BLV infection and disease progression (Farias *et al.*, 2016; Juliarena *et al*., 2007).

We constructed a scatter graph using sixteen paired infected cattle (excluding samples with a proviral load 10-fold higher than measured in other samples) to assess whether the proviral load in milk could be associated with the proviral load in PBMC, a correlation analysis between the number of copies of the provirus per reaction in PBMC and SC, and a linear regression analysis was performed.

The correlation coefficient (r) was 0.78 (p-value = 0 .00), indicating an intermediate to strong correlation, which would suggest that when the proviral load in PBMC is high, the proviral load in SC is also increasing (Figure 1).

In addition, we found that for every unit change in proviral load in SC, it is expected that the proviral load in PBMC will increase by 1.89 (p-value = 0.00181).

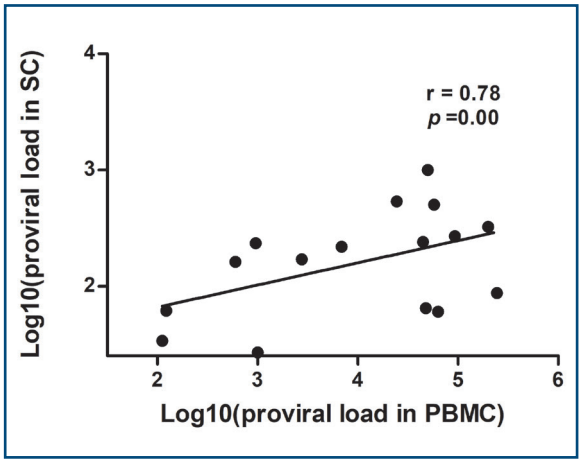

Figure 1. Correlation between the BLV proviral loads (copies/30 ng of DNA) in PBMC and SC (X, Y pairs = 16). *The bold line represents the approximate curve (r=correlation coefficient), and the p-value is indicated.*

Correlation of BLV proviral load between SC and somatic cells count

To investigate whether somatic cell count (SCC) was associated with proviral load levels in SC, a correlation analysis of variations in the proviral load in SC of BLV-infected cattle was performed. No significant correlation between proviral loads of SC and the SSC of BLV-infected cattle was found (r = 0.22, p-value = 0.42).

Comparison of cytokine mRNA expression levels in PBMC and SC between BLV-positive and BLV-negative cattle

Relative quantification of cytokine expression in PBMC and SC was performed to verify the effect of BLV infection on the cellular immune response. Five genes in PBMC and SC samples were analyzed: GAPDH, IFN-γ, IL-12, IL-10, and IL-6, using GAPDH as a normalizer. Even though results in PBMC show no significant differences, ratios of mRNA expression were higher for IFN-γ (ratio = 1.56, p-value = 0. 2444) and IL-10 (ratio = 1.18, p-value = 0. 4256), and lower for IL-12 (ratio = 0.31, p-value = 0. 2568) and IL-6 (ratio = 0.24, p-value = 0. 1828) in BLV-infected compared to BLV non-infected cattle (Figure 2.a). Regarding infection profiles, in cattle with LPL-PBMC the ratios of expressions were higher for IFN-γ (ratio = 2.71, p-value = 0. 1057), IL-12 (ratio = 2.42, p-value = 0. 2092) and IL-6 (ratio = 1.56, p-value = 0. 0961), and lower for IL-10 (ratio = 0.19, p-value = 0. 0856) compared to HPL-PBMC cattle (Figure 2.b). The gene expression level in LPL-SC (or BLV-infected) cattle was lower for IFN-γ (ratio = 0.250, p-value = 0. 0774), and higher in IL-12 (ratio = 1.49, p-value = 0. 4160) than BLV non-infected cattle. In contrast, the gene expression level of IL-10 was significantly 0.09-fold (p-value = 0.0113) and IL-6 was significantly 0.004-fold (p-value = 0.0124) lower in LPL-SC compared to BLV non-infected cattle (Figure 3.a). All SC samples were classified according to the infection profile in PBMC (LPL-PBMC or HPL-PBMC), and the cytokines expression levels were compared. No significant differences were found between both infection profiles; however, ratios of expression were higher for IFN-γ (ratio = 2.91, p-value = 0.1838), IL-12 (ratio = 1.14, p-value = 0. 4663) and IL-10 (ratio = 1.20, p-value = 0. 4305), and lower for IL-6 (ratio = 0.01, p-value = 0. 0978) in SC samples from cattle having LPL-PBMC compared to HPL-PBMC. When LPL-PBMC and HPL-PBMC cattle were compared with BLV non-infected group, significantly lower expression levels were found in both profiles for IL-6 (LPL-PBMC: ratio = 0.00024-fold, p = 0.0306 and HPL-PBMC: ratio = 0.02, p = 0.024) and IL-10 (LPL-PBMC: ratio = 0.10-fold, p = 0.0132 and HPL-PBMC: ratio = 0.09, p = 0.0227) than in BLV non-infected cattle (Figure 3.b).

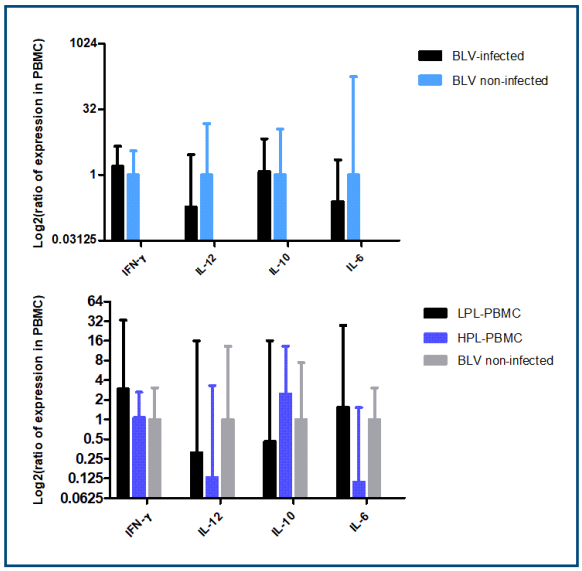

Figure 2. Bar charts represent the ratio of cytokine gene expression in PBMC. *2a: Represents log$_2$ ratio of cytokines gene expression of IFN-γ, IL-12, IL-10, and IL-6 in BLV -infected and BLV non-infected cattle. 2b: Represents log$_2$ ratio of cytokines gene expression of IFN-γ, IL-12, IL-10, and IL-6 in low proviral load, high proviral load, and BLV non-infected cattle. ±S.E. based upon permuted expression data in* fgStatistics.

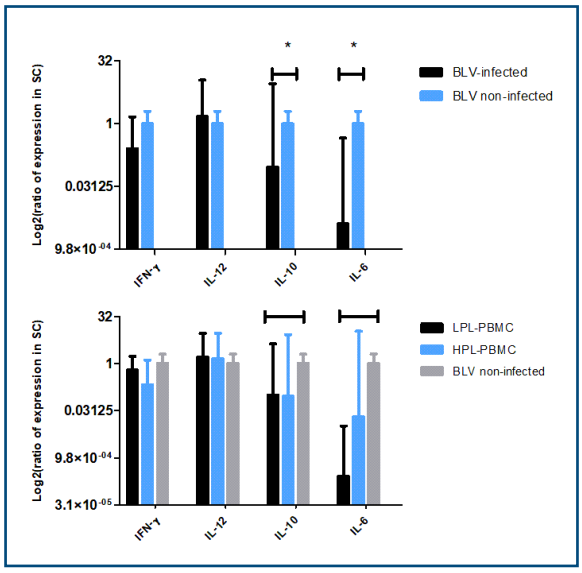

Figure 3. Bar charts representing the ratio of cytokine gene expression in SC. *3a: Represents log$_2$ ratio of cytokines gene expression of IFN-γ, IL-12, IL-10, and IL-6 in BLV -infected and BLV non-infected cattle. 3b: Represents log$_2$ ratio of cytokines gene expression of IFN-γ, IL-12, IL-10, and IL-6 in low proviral load, high proviral load, and BLV non-infected cattle. ±S.E. based upon permuted expression data in* fgStatistics. *LPL-PBMC, cattle harbor low proviral load in peripheral blood mononuclear cells. Stars indicate significant differences (*fgStatistics *statistical randomization test; p-value <0.05).*

Discussion

The success of natural BLV infection, especially in dairy cattle, depends on the number of cells with the BLV provirus transferred to healthy cattle through iatrogenic way, i.e., during dehorning, use of needles contaminated with BLV-infected blood, transrectal palpation (Hopkins and DiGiacomo, 1997), and during routine milking. Most cattle naturally BLV-infected are asymptomatic, and 30% develop persistent lymphocytosis. This classification (asymptomatic and persistent lymphocytotic) has been used in several studies that evaluate the alterations produced by BLV infection for many years. Nevertheless, this classification does not represent BLV infected cattle total. Studies reported that asymptomatic cattle comprise two groups differentiated in terms of their proviral load in PBMC: the HPL group, which is not statistically different from persistent lymphocytosis cattle in terms of the proviral load in PBMC or humoral immune response to BLV, and the LPL group which develops very low proviral load in PBMC after BLV infection (Farias *et al.*, 2016; Juliarena *et al.*, 2007).

For this reason, cattle were defined in two groups regarding their proviral load in our study. Moreover, the risk of transmitting the infection to other cattle could be classified according to the proviral load. It has been suggested that HPL cattle are the most efficient transmitters because most harbor a high percentage of infected lymphocytes. In contrast, LPL cattle could be inefficient transmitters of the infection under standard husbandry conditions (Juliarena *et al.*, 2016).

So far, few studies consider the proviral load of BLV in milk as a key risk factor for transmitting the virus. Therefore, the proviral load in SC and PBMC was compared in the present study. The proviral load was always low in SC with a significant positive association compared to PBMC proviral load, and a progressive increase of 1.89 copies of BLV in PBMC for each copy of provirus in SC was found. This fact suggests that milk could be a suitable and more feasible compartment for sampling to predict systemic proviral load in infected herds. On the other hand, the intrinsic capability in BLV-infected cattle to maintain a low proviral load could be associated with a difference in the immune response to the virus.

The evaluation of cell count in milk (SCC) is a simple and useful indicator of the health of the mammary gland in dairy farms. Values higher 200,000 SCC/mL are a predictor of subclinical mastitis (Hernández and Bedolla, 2008); an influx of immune cells might take place and thus might affect the number of BLV-infected cells in the mammary gland in infected cattle. Only cattle with <200,000 SCC/mL were included in our study. Probably for this reason, even with variations in SC counts in milk, we found no correlation between proviral loads of SC and the SSC of BLV-infected cattle, as other authors reported (Watanabe *et al.*, 2019).

We found that all SC samples were positive by qPCR from the BLV-positive cattle, unlike other studies. Watanuki *et al*. (2019) reported 45.8% of positivity in SC samples by CoCoMo-qPCR detecting BLV *LTR* gen (Watanuki *et al.*, 2019). Using a qPCR similar to our detecting *pol* gen with SybrGreen described by Farias *et al*. (2016), Jaworski *et al*. (2016) reported 59% of positivity in SC samples from healthy cattle, and Watanabe et al. (2019) used the TaqMan method reported 84% of positivity in milk samples from dairy cattle with clinical mastitis (Farias *et al.*, 2016; Jaworski *et al.*, 2016; Watanabe *et al.*, 2019). However, the limit of BLV copy detection reported by those authors was lower than our method, which could explain the differences in our results. It has been reported that lymphocytotic BLV-infected cattle have an increased incidence of mastitis (Sandev *et al.*, 2004). Moreover, a high proviral load in milk has been associated with the severity of clinical mastitis (Watanabe *et al.*, 2019). In our study, BLV-infected cattle showed an LPL profile in milk. In SC extracted from the milk provirus copies were fewer than in PBMC from the same BLV-infected cattle. Thus, all SC samples enter a new classification as LPL-SC. The upper limit determined for the LPL-SC cattle was 1000 copies/30 ng of DNA reaction, while an upper limit of 100 copies/30 ng of DNA reaction was established for LPL-PBMC cattle (Farias *et al.*, 2016). These LPL levels remained constant in all milk samples in our study, and there is a significant association with the proviral load levels found in PBMC. Other reports (Jaworski *et al.*, 2016) found similar LPL levels in milk. The LPL levels are probably related to the proportion of B-lymphocytes in samples, which are the primary target for BLV infection. B-lymphocytes comprise the most negligible percentage of leukocyte components in milk (3,2%), in contrast to a mean of 21% in the blood compartment (Butler *et al.*, 2015; Duhamel *et al.*, 1987). Therefore, fewer B-lymphocytes containing integrated BLV may be present in milk, thus making less provirus quantified (Jaworski *et al.*, 2016; Kuckleburg *et al.*, 2003).

Several studies demonstrated that BLV infection causes an alteration in cytokine production, immune cell proliferation, and apoptosis, evidenced in lymphocytotic animals; this abnormal immune function has been associated with susceptibility to the development of other infectious diseases (Frie and Coussens, 2015). Moreover, infected cattle with higher BLV titers in PBMC and T-cell unbalance could be prone to clinical mastitis (Kakinuma *et al.*, 2014). In PBMC, we found that expression levels of IFN-γ, a cytokine associated with a Th1-type response, tend to be higher in infected cattle than in BLV non-infected cattle, and this trend is more evident in LPL-PBMC compared to HPL-PBMC cattle. While, expression levels of IL-10, a cytokine associated with a Th2-type response, tend to be lower in LPL-PBMC than in HPL-PBMC cattle. Limited evidence exists in the literature about *ex vivo* cytokines expression in PBMC from BLV-infected cattle, and most studies classify them as lymphocytotic or aleukemic. Previous reports found that IFN-γ is up-regulated in PBMC from BLV-infected cattle than in non-infected ones (Konnai *et al.*, 2003; Pyeon *et al.*, 1996). Authors from the present study have previously characterized the LPL profile in PBMC from BLV-infected cattle, particularly concerning the expression of Th1 cytokines (IFN-γ, IL-12) (Farias *et al.*, 2016). IFN-γ mRNA expression levels were significantly higher in PBMC than in BLV-infected cattle with LPL, which supports our results with increased levels of Th1 cytokines in LPL cattle, suggesting that IFN-γ has a protective role in the pathogenesis of BLV infection. These findings indicate that LPL cattle might express a profile that prevents disease progression due to the protective effect of IFN-γ and less induction of proliferation and differentiation because of less production of Th2-type cytokines, such as IL-10.

Little information is available on the behavior of BLV in the mammary gland. A recent study reported the low phagocytic function of milk macrophages in cattle infected with BLV compared to those not infected (Lima *et al.*, 2021). In turn, another study showed that infection by BLV inhibits neutrophil migration, and its effect is closely related to the low provirus load of BLV (Lv *et al.*, 2021). Thus, it was interesting to evaluate the cellular immune response concerning cytokine expression in the SC of the milk from BLV-infected cattle. We found that IFN-γ expression levels tended to be lower, and IL-12 tended to be higher in the LPL-SC cattle compared to the BLV non-infected group. Both IL-10 and IL-6 mRNA expressions were significantly down-regulated, suggesting a reduced Th2 activity response to viral infection. Regarding the mRNA expression levels in LPL-SC cattle based on the infection profile of each animal in peripheral blood, those with LPL-PBMC expressed more Th-1 cytokines, mainly IFN-γ, and fewer Th-2 cytokines, particularly IL-6, in the mammary gland. It is the first report to evaluate cytokine expression levels in the mammary gland compared to PBMC classified according to proviral load.

Cytokine expression patterns in peripheral blood differed from mammary gland in infected and non-infected cattle, except for the similar IL-6 mRNA expression pattern observed in both compartments. However, interestingly, when the cattle were classified by PBMC infection profile, a similar pattern of higher IFN-γ and IL-12 expression was observed in LPL-PBMC compared to HPL-PBMC, both in the blood and mammary gland. These reinforce the concept that cattle with LPL could control viral dissemination, also in the mammary gland, through a high Th-1. Although we have found a trend toward higher IL-6 and lower IL-10 expression in LPL-PBMC in

blood, cattle with this profile expressed significantly less mRNA expression level of these cytokines in SC when compared to BLV non-infected. This is an important point for mammary gland health as IL-6 is involved in both innate and adaptative immunity and participates in the inflammatory and mucosal responses; and IL-10 is a Th2 key cytokine for humoral responses and exerts anti-inflammatory effects (Bannerman, 2009; Butler *et al.*, 2015; Riollet *et al.*, 2000).

IL-12 is an important cytokine involved in the cellular differentiation towards Th-1 cells (Frie and Coussens, 2015). mRNA expression levels of IL-12 p40 have been found in healthy mammary glands, showing significant IL-12 p40 at late lactation compared with mid-lactation (Alluwaimi and Cullor, 2002; Frie and Coussens, 2015). Concerning IL-12 mRNA expression levels in bacterial infection, there is controversy. Nevertheless, the studies assert the crucial role of IL-12 in mammary gland immunity. On the other hand, no *ex vivo* studies show IFN-γ biological activity in the bovine mammary gland. A study of adjuvant activity of recombinant bovine IFN-γ was performed in normal and infected mammary glands showing activation of T cells in the normal mammary gland (Alluwaimi, 2004), being this cytokine an essential modulator of lymphocyte trafficking. In our study, the variations in the expression levels of IFN-γ and IL-12 in SC between cattle infected and not infected with BLV show this cytokine's central role in the adaptive immunity of the bovine mammary gland. Furthermore, mRNA expression towards Th-1 cytokines in SC of LPL-PBMC cattle mirrors the crucial role of IL-12 in the polarization of CD4 T cells towards the type 1 T cell phenotype in the bovine mammary gland in infections. IL-6 is a pleiotropic and proinflammatory cytokine implicated as a significant mediator of the pathology accompanying infections (Riollet *et al.*, 2000). However, the gene expression level of IL- 6 in some mammary gland infections is contradictory: studies have reported high-level expression of IL-6 with coliform mastitis, whereas other studies have found low levels of IL-6 in *S. aureus* infection (Alluwaimi, 2004). In the same way, we found low levels of IL-6 expression in SC from cattle infected with BLV and lower IL-10 expression. It is necessary to expand studies of these cytokines in cattle infected with BLV to know their possible contributions as indicators of the prognosis of this infection in the mammary gland.

Even though BLV infection has a significant economic impact on the dairy industry worldwide, viral effects on the immune system of the mammary gland are just beginning to be elucidated. The present study shows that the BLV-infected cattle maintain a low proviral load profile in SC of milk correlated positively with the proviral load in PBMC. And the BLV infection could alter the immune response in both blood and mammary gland.

Classifying SC samples according to proviral load in PBMC, similar expression patterns of Th1-type cytokines in both compartments were observed. Results suggest a Th1-type response in cattle that maintain low proviral load compared to high proviral load cattle in blood. While the expression levels of most cytokines are kept low in BLV-infected cattle, it might suggest a reduced immune response and susceptibility to other infectious diseases. This study is the first to report the differences in the expression of Th1 and Th2 cytokines levels in PBMC from LPL-PBMC and HPL-PBMC cattle and in SC from BLV-infected cattle with LPL in the mammary gland. These findings may be relevant to future control strategies for BLV infection, mastitis, and udder health management.

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