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Expression-based analysis of genes related to single nucleotide polymorphism hits associated with bovine leukemia virus proviral load in Argentinean dairy cattle

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ABSTRACT

In dairy cattle infected with bovine leukemia virus (BLV), the proviral load (PVL) level is directly related to the viral transmission from infected animals to their healthy herdmates. Two contrasting phenotypic groups can be identified when assessing PVL in peripheral blood of infected cows. A large number of reports point to bovine genetic variants (single nucleotide polymorphisms) as one of the key determinants underlying PVL level. However, biological mechanisms driving BLV PVL profiles and infection progression in cattle have not yet been elucidated. In this study, we evaluated whether a set of candidate genes affecting BLV PVL level according to whole genome association studies are differentially expressed in peripheral blood mononuclear cells derived from phenotypically contrasting groups of BLV-infected cows. During a 10-mo-long sampling scheme, 129 Holstein cows were phenotyped measuring anti-BLV antibody levels, PVL quantification, and white blood cell subpopulation counts. Finally, the expression of 8 genes (*BOLA-DRB3*, *PRRC2A*, *ABT1*, *TNF*, *BAG6*, *BOLA-A*, *LY6G5B*, and *IER3*) located within the bovine major histocompatibility complex region harboring whole genome association SNP hits was evaluated in 2 phenotypic groups: high PVL (n = 7) and low PVL (n = 8). The $\log_2$ initial fluorescence value (N_0) transformed mean expression values for the *ABT1* transcription factor were statistically different in high- and low-PVL groups, showing a higher expression of the *ABT1* gene in low-PVL cows. The *PRRC2A* and *IER3* genes had a significant positive (correlation coefficient = 0.61) and negative (correlation coefficient = −0.45) correlation with the lymphocyte counts, respectively. Additionally, the relationships between gene expression values and lymphocyte counts were modeled using linear regressions. Lymphocyte levels in infected cows were better explained (coefficient of determination = 0.56) when fitted a multiple linear regression model using both *PRRC2A* and *IER3* expression values as independent variables. The present study showed evidence of differential gene expression between contrasting BLV infection phenotypes. These genes have not been previously related to BLV pathobiology. This valuable information represents a step forward in understanding the BLV biology and the immune response of naturally infected cows under a commercial milk production system. Efforts to elucidate biological mechanisms leading to BLV infection progression in cows are valuable for BLV control programs. Further studies integrating genotypic data, global transcriptome analysis, and BLV progression phenotypes are needed to better understand the BLV–host interaction.
Key words: bovine leukemia virus, proviral load level, differential gene expression

INTRODUCTION

Bovine leukemia virus (**BLV**) is an oncogenic retrovirus (subfamily *Orthoretroviridae*, genus *Deltavirus*) that infects cattle (Kettmann et al., 1994). Bovine leukemia virus infection spreads in dairy herds worldwide, causing important economic losses owing to milk production decline, lymphosarcoma-associated deaths, and international trade restrictions imposed on livestock products derived from infected animals (Erskine

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et al., 2012; Bartlett et al., 2014; Castellano and Goizueta, 2014). In Argentina, more than 80% of lactating cows in the main dairy production area are infected (Gutiérrez et al., 2012), and in surveys conducted in the United States and Canada, the prevalence of BLV reached 70 to 85% at the farm level (VanLeeuwen et al., 2005, 2006; NAHMS, 2007). Bovine leukemia virus shares its genomic organization (structural and functional) with the T-cell leukemia virus of humans (**HTLV**) and simians (Poiesz et al., 1980; Sagata et al., 1985; Coffin, 1996). Conversely, B-lymphocytes are the main cellular target for BLV DNA integration into the bovine genome (provirus; Schwartz et al., 1994; Mirsky et al., 1996). The proviral BLV is maintained through the mitotic division of infected B-lymphocytes undergoing BLV mRNA transcriptional silencing. Essentially in infected cows, BLV is responsible for a lymphoproliferative disorder (leukosis) and fatal neoplastic transformation (leukemia/lymphosarcoma; Burny et al., 1988; Kettmann et al., 1994). However, biological mechanisms modulating BLV leukemogenesis are largely unknown (Gillet et al., 2007). It is worth noting that an increased BLV proviral load (**PVL**) in peripheral blood of infected individuals has been associated with infection progression (Mirsky et al., 1996; Jimba et al., 2010). Also, high-PVL cows have an enhanced ability to transmit the virus to healthy mates, boosting the BLV dissemination within herds (Mammerickx et al., 1987; Mirsky et al., 1996; Juliarena et al., 2007; Jimba et al., 2010). A recurrent observation in bovine dairy farms is the interindividual variation in the peripheral blood BLV PVL level of infected cows (Gutiérrez et al., 2012). Early candidate gene population genetics studies pointed to polymorphisms in the bovine major histocompatibility complex (**MHC**) class II *DRB3* gene as a key modulator of the in vivo PVL level (Mirsky et al., 1998; Juliarena et al., 2008; Jimba et al., 2012; Miyasaka et al., 2013; Carignano et al., 2017; Takeshima et al., 2019). However, *BOLA-DRB3* allele associations were not validated among studies or failed to explain variations observed in BLV PVL. Furthermore, the genetic basis of resistance or susceptibility to diseases in cattle is usually polygenic—that is, involving many loci of minor effects (Minozzi et al., 2012; Sahana et al., 2013; Neibergs et al., 2014; Brym and Kamiński, 2017). With the advent of high-throughput SNP genotyping technologies, GWAS aimed to dissect additional loci underlying PVL variation. A series of BLV PVL GWAS identified a genomic region within the bovine MHC, approximately from the beginning of bovine leukocyte antigen class IIa region (BTA23 ~20.6 Mb) extending to the histone cluster I at the end of the complex (BTA23 ~33.3 Mb; Takeshima et al., 2017; Carignano et al., 2018). This region is well known for harboring genes related to innate and adaptive immune response. Accordingly, the effect of the MHC locus in the susceptibility or resistance to diseases is the most preponderant one, greater than any other genomic region (Lechler and Warrens, 2000; Blackwell et al., 2009; Howell, 2014). However, the highly gene-dense MHC region holds an intricate structure of linkage disequilibrium. Thus, GWAS hits in this region could represent the effect of multiple correlated SNP spanning over several closely located genes (Stewart et al., 2004; Miretti et al., 2005; de Bakker et al., 2006; Traherne et al., 2006). Therefore, identifying genes potentially relevant to phenotypic variation is a challenging task considering association *P*-values alone. In an effort to link GWAS results to biological processes, large-scale functional genomic data showed that most disease-associated SNP are located in regulatory regions influencing gene expression (Schaub et al., 2012). In this study we evaluated the expression of genes in which we have previously mapped SNP (within the bovine MHC region) significantly associated with BLV PVL level in Argentinean Holsteins cows (Carignano et al., 2018).

MATERIALS AND METHODS

Selection of Animals

A total of 129 Holstein cows were screened using anti-BLV ELISA (Supplemental Table S1, https://doi.org/10.3168/jds.2020-18924) and divided into 2 contrasting groups on the basis of their antibody level. Then, selected animals were subjected to PVL determination as previously described (Gutiérrez et al., 2012). Adult cows belonged to the main dairy farm region in Argentina (31°16′S, 61°29′W), were over age 3 yr, and shared the same lactation period (>1 lactation). This geographic area presented a BLV individual mean prevalence higher than 80% (Gutiérrez et al., 2012). We arbitrarily chose a 10-mo interval of positive anti-BLV antibodies to avoid BLV neoinfections in selected cows. The percentage of reactivity (**PR**) obtained in the ELISA assay was used as a cost-effective indirect measure of PVL as previously reported (Gutiérrez et al., 2012). All cows were evaluated using anti-BLV ELISA twice, at −10 mo (**T1**) and −5 mo (**T2**) from the final sampling time; the mean PR was 122.7 ± 34.8 for T1 and 146.3 ± 55.6 for T2. Then, animals that consistently presented the highest and the lowest antibody reactivity in both sampling times were selected (10 cows for each group) as follows. The low-PR group included cows in the lowest PR quartile (**Q1**; Q1 T1: 25.0–102.8%; Q1 T2: 25.0–118.2%), and the high-PR group included cows in the highest PR quartile (**Q4**; Q4 T1: 148.6–178.6%; Q4 T2: 194.4–239.6%; Supplemental

Table 1. Percentage of reactivity (PR) and proviral load (PVL) values for all sampling times[1]

	T1	T2	T3		T4		
Sample ID	PR (%)	PR (%)	PR (%)	PVL (copies/μg)	PR (%)	PVL (copies/μg)	Cow phenotype[2]
5570	149.3	232.0	182.9	30,994.6	185.1	32,081.7	HPVL
5671	157.3	220.5	167.2	26,231.3	169.6	100,065.7	HPVL
5841	166.3	239.6	178.9	33,660.5	180.4	54,808.0	HPVL
6021	169.9	222.8	168.3	17,973.6	118.1	87,831.1	HPVL
6097	162.6	218.3	185.3	79,864.3	166.6	102,647.5	HPVL
6115	161.7	228.4	172.6	110,437.3	171.5	119,882.4	HPVL
6227	155.3	232.3	166.7	186,771.1	166.7	184,051.1	HPVL
5324	79.6	59.5	42.0	<10.0	49.5	295.5	LPVL
5633	66.8	52.3	57.6	173.6	78.5	166.1	LPVL
5656	81.6	37.9	89.8	0.0	78.6	41.9	LPVL
5659	51.2	28.9	31.2	0.0	57.5	166.2	LPVL
5701	64.3	56.0	61.0	0.0	63.5	192.5	LPVL
6121	77.9	52.7	81.9	0.0	69.4	31.1	LPVL
6190	81.3	59.1	41.4	86.94	86.6	79.3	LPVL
6236	47.0	44.9	58.8	0.0	79.1	0.0	LPVL

[1]T1 = −10 mo from final sampling time; T2 = −5 mo from final sampling time; T3 = −3 mo from final sampling time; T4 = 0 mo from final sampling time.

[2]HPVL = high proviral load; LPVL = low proviral load.

Table S2, https://doi.org/10.3168/jds.2020-18924). Finally, PVL was measured twice [at −3 mo (**T3**) and 0 mo (**T4**)] in 15 selected animals using quantification PCR (**qPCR**; described later), resulting in 7 cows with high PVL (**HPVL**) and 8 with low PVL (**LPVL**) with the highest and lowest antibody reactivities in each group, respectively (Table 1). In addition, MHC class II *BOLA-DRB3* alleles were genotyped for all 15 animals according to Carignano et al. (2017). Animal handling and sampling procedures followed recommendations of the Institutional Animal Care and Use Committee of the National Institute of Agricultural Technology (Buenos Aires, Argentina).

Peripheral Blood Mononuclear Cell Isolation

Animal fresh blood samples (45 mL) were collected by jugular venipuncture and supplemented with EDTA (225 μ*M*). Peripheral blood mononuclear cells (**PBMC**) were isolated immediately after blood sampling using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient centrifugation following the manufacturer's protocol. The plasma fraction was kept for anti-BLV ELISA determination. Immediately after isolation, PBMC were conserved in RNAlater solution (Ambion, Austin, TX) and stored at −80°C until use. Sample collection, processing, and PBMC isolation were done in the same working day.

Cell Counts

A hematologic analyzer (Sysmex XN-1000, Sysmex Co., Kobe, Japan) was used for absolute and relative blood cell counts and classification of immune subpopulations (i.e., lymphocytes, neutrophils, eosinophils, and monocytes).

Anti-BLV ELISA

An indirect ELISA against the whole BLV viral particle antigen was used as previously described by Trono et al. (2001). A weak positive international control standard serum was used as reference to calculate a normalized sample to positive ratio. The difference between the optical density value obtained for the weak positive control and a negative BLV control sample was set up as 100% of reactivity. All tested samples were referred against it. Samples with PR above the cut-off level (>25%) were considered positive.

BLV PVL Quantification

Genomic DNA was extracted from PBMC using the High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. Concentration and quality of DNA were measured using a nanophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA).

A validated BLV *POL* gene-based PVL qPCR assay based on the SYBR Green dye detection system was used (Petersen et al., 2018). Briefly, each qPCR reaction (final volume = 25 μL) contained Fast Start Universal SYBR Green Master Mix (2×; Roche), forward and reverse primers (800 n*M*; BLVpol_5f: 5′-CCTCAATTCCCTTTAAACTA-3′; BLVpol_3r: 5′-GTACCGGGAAGACTGGATTA-3′; Thermo Fisher

Scientific), and genomic DNA template (200 ng). The amplification and detection reaction was performed using Step One Plus equipment (Applied Biosystems, Foster City, CA). The specificity of each BLV-positive reaction was confirmed using melting temperature dissociation curve (**Tm**) analysis. Assuming a very low level of natural infection as a proportion of 1% of BLV-infected cells in peripheral blood of infected cattle, PVL values <1,500 copies/μg of total DNA were considered low; otherwise, they were considered high. This proportion of BLV-infected cells is even lower than the 5% described for aleukemic animals (Hopkins and DiGiacomo, 1997).

Candidate Gene Selection

Briefly, in our previous study (Carignano et al., 2018) we identified 24 SNP significantly associated with PVL in BLV-infected cattle. These SNP (located within the bovine MHC region) were assigned to putative biological function if they fell within gene coding, intronic, or regulatory regions. For those SNP mapping outside genic regions, we scrutinized the closest gene considering the linkage disequilibrium extent. Therefore, we reduced the landscape of genes potentially related to BLV PVL to a list of specific candidate genes. In this study, we chose 8 relevant genes potentially related to the pathogenesis of viral infections (Supplemental Table S3, https://doi.org/10.3168/jds.2020-18924): antigenic peptide presentation by class I (*BOLA-A*) and II (*BOLA-DRB3*) MHC molecules, regulation of apoptosis and proteasome degradation (*TNF*, *BAG6*, *IER3*), T-cell response to interferon and viral shedding prevention during innate response (*LY6G5B*), and host transcription factors (**TF**; *ABT1*, *PRRC2A*). The TF genes were selected as candidates due to the tight mechanism of transcriptional regulation exerted by the transactivating viral protein TAX, which modulates several host-signaling pathways [activator protein 1, nuclear factor (**NF**)-κB, cAMP response element-binding protein] to induce oncogenic transformation (Grassmann et al., 2005). Particularly, the *PRRC2A* TF was selected because it is exclusively expressed in lymphocytes (the main cell target of BLV), and the SNP linked to this gene are located in an exonic region provoking a synonymous amino acidic change.

Candidate Gene Real-Time qPCR

The assay was conducted following the recommendations proposed by the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). Total RNA was extracted from PBMC with the High Pure RNA Isolation Kit (Roche) using a slightly modified in situ protocol. A DNase treatment was modified from 15 min at 25°C to 20 min at 37°C, allowing us to prevent DNA contamination. Concentration and quality were measured using a nanophotometer (Nanodrop, Thermo Fisher Scientific). The RNA integrity was evaluated by running between 0.5 and 1 μg of total RNA in 1% agarose bleach gel (Aranda et al., 2012; Supplemental Figure S1, https://doi.org/10.3168/jds.2020-18924). A quantity of 300 ng of RNA was used for cDNA synthesis using the MultiScribe High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. In detail, 300 ng of RNA diluted in 10 μL of ultrapure water (Invitrogen, Carlsbad, CA) was added to a master mix (final volume = 20 μL) containing 2 μL of 10× reverse transcription buffer, 2 μL of 10× reverse transcription random primers, 4 m*M* dNTP, 2.5 U/μL MultiScribe reverse transcriptase, and RNasin ribonuclease inhibitors (1 U/μL; Promega, Madison, WI). Then, cDNA was diluted 1:10 in ultrapure water (Invitrogen) and stored at −80°C until use.

Primers for each candidate target and reference genes (Table 2) were designed using the Primer-BLAST tool (Ye et al., 2012). Self-dimer and heterodimer tendencies, GC content, Tm, and potential secondary structure for each primer pair were evaluated using OligoAnalyzer 3.1v software (Owczarzy et al., 2008). For real-time (**RT**) qPCR experiments, 2 different commercial mixes were used. Mix 1 reactions were performed using the Fast Start Universal SYBR Green Master Mix (Roche) in 5 candidate target genes (*BOLA-DRB3*, *PRRC2A*, *TNF*, *BAG6*, and *BOLA-A*); this mix was later discontinued by the vendor from the Argentinean market. Mix 2 reactions were performed using the SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA) to assay 3 target genes (*LY6G5B*, *IER3*, and *ABT1*). The final reaction volume was 10 μL in both mixes, adding specific gene primers (300 n*M*; Table 2) and 3 μL of cDNA dilution. The RT-qPCR reactions were conducted using a StepOne Plus device (Applied Biosystems) following a standard running condition: (1) activation step at 95°C, 10 min for mix 1 and 30 s for mix 2, and (2) 40 cycles of 95°C for 15 s and 60°C for 1 min. The specificity was confirmed by Tm dissociation curve analysis to verify that a single qPCR product was amplified per reaction (single peak profile). Ambiguous results (broader curve profile or a weaker extra peak or both) were confirmed by agarose gel electrophoresis. Technical duplicates were assayed for all RT-qPCR

reactions. For each evaluated gene, no retrotranscribed pooled sample RNA template and no template controls were included. The reaction efficiencies were calculated using LinRegPCR software (Ramakers et al., 2003). Finally, the between-plate measurement variation was removed using Factor-qPCR software (Ruijter et al., 2006). Relative expression values were normalized against previously described bovine reference genes [ribosomal protein large P0 (*RPLP0*) and β-2-microglobulin (*B2M*); Brym et al., 2013] with both mix 1 and mix 2.

Statistical Analyses

The differential gene expression between HPVL and LPVL phenotypic groups was measured using the N_0 values (Equation 1; Ruijter et al., 2009):

$$N_0 = \frac{N_t}{E^{C_q}}, \quad [1]$$

where N_0 = initial concentration measured in fluorescence values; N_t = user-defined fluorescence threshold; E = reaction efficiency; and C_q = fractional number of cycles needed to reach the established threshold. The N_0 values for the candidate genes were normalized against the geometric mean of N_0 values for 2 reference genes as proposed by Vandesompele et al. (2002). This normalization process was done for all candidate genes in each animal cDNA.

The normalized N_0 values and the cells count data were transformed to logarithmic scale to approximate a normal distribution. The normality of the transformed data was evaluated with a Shapiro-Wilk test (Supplemental Table S4, https://doi.org/10.3168/jds.2020-18924).

Relevant hypothesis tests to evaluate differential gene expression were performed with the Student test when normality assumptions were met; otherwise the Mann-Whitney-Wilcoxon test was applied. Correlations among lymphocyte counts and target gene expression levels were assessed using the Pearson correlation coefficient or the Spearman's rank correlation as appropriate. Linear regression models were adjusted for variables showing significant correlations. All statistical tests were performed using the free software R (R Core Team, 2014) with a 95% confidence interval.

To contrast the results based on the N_0 value analysis, we also performed RT-qPCR curve analysis and ratio calculation using REST 2009 V2.0.13 software (Pfaffl, 2002). Briefly, the relative expression ratio (**R**; Equation 2) was used as defined by Hellemans et al. (2007) for multiple reference genes:

Table 2. Candidate (target) and reference gene amplification details

Gene name and symbol	Primer sequence[1] (5′→3′)	Product size (bp)	GenBank accession no. and reference	Gene type
Ribosomal protein large P0 (*RPLP0*)	F: CAACCCTGAAGTGCTTGACAT	227	NM_001012682	Reference
	R: AGGCAGATGGATCAGCCA		(Donaldson et al., 2005)	
β-2-Microglobulin (*B2M*)	F: AGCAAGGATCAGTACAGCTGCCG	105	NM_173893	Reference
	R: ATGTTCAAATCTCGATGGTGCTGCT		(Brym et al., 2013)	
MHC class II DRB3 (*BOLA-DRB3*)	F: GGAATCAGAAAGGACGCCCTAC	112	NM_001012680.2	Target
	R: CCTTTCCATGCTGTGAAGAAGC			
Tumor necrosis factor (*TNF*)	F: CTTGTGCCTCAGCCTCTTCT	107	NM_173966.3	Target
	R: CTGGGGACTGCTCTTCCCTC			
Proline rich coiled-coil 2A (*PRRC2A*)	F: AGCCTGAAAGCCGAGAACAA	114	NM_0012055557.1	Target
	R: TGAGGCATCGGAACTCTTGG			
BAG cochaperone 6 (*BAG6*)	F: GCCGCCAGGAACTCACTAT	178	NM_001075366.2	Target
	R: CCGACAGCTCTCGTCACTTC			
MHC class I A (*BOLA-A*)	F: GGAGACGCAGAGAACTAAGGA	194	NM_001040554.1	Target
	R: TCGTTCAGGGCGATGTAA		(Wu et al., 2018)	
Lymphocyte antigen 6 family G5B (*LY6G5B*)	F: TTAGCAGCTCATCCCCATGC	108	NM_001192202.1	Target
	R: GCTTTTCTTGGCAGCGGTAG			
Immediate early response 3 (*IER3*)	F: TCCTCTACCCACGAGTGGTC	109	NM_001075202.2	Target
	R: GCCATTAGGATCTGGCAAAA		(Villalba et al., 2017)	
Activator of basal transcription 1 (*ABT1*)	F: AACCTCAAGTACCTGCACCG	124	NM_001076152.2	Target
	R: AGAAGTCAGTCTCACGCTTGG			

[1]F = forward; R = reverse.

$$R = \frac{E_{goi}^{(ControlCqmean - SampleCqmean)_{goi}}}{\sqrt[n]{\prod_{k=1}^{n} E_{ref}^{(ControlCqmean - SampleCqmean)_{ref}}}}, \quad [2]$$

where control refers to LPVL and sample refers to HPVL; C_q = quantification cycle value; E_{goi} = reaction efficiency for the target gene of interest; E_{ref} = reaction efficiency for the reference genes; and n = number of reference genes. Differential gene expression among groups was assessed for statistical significance by permutation tests. To ensure a good estimate of the *P*-value (SE < 0.005 at $P = 0.05$), 2,000 resamples were made (Pfaffl, 2002).

RESULTS

In this work, we studied the expression of candidate genes linked to SNP associated with BLV PVL in cattle identified in a previous GWAS (Carignano et al., 2018). We included genes previously related to susceptibility or resistance to BLV infection in cattle (*BOLA-DRB3*, *BOLA-A*, and *TNF*), immune response and regulation of cell apoptosis (*LY6G5B*, *BAG6*, and *IER3*), and host transcription factors (*ABT1* and *PRRC2A*; Table 2). From a larger set of cows screened by anti-BLV ELISA, 8 (with the lowest PR and low PVL) and 7 (with the highest PR and high PVL) cows were classified as LPVL and HPVL phenotypes, respectively (Table 1). Also, *BOLA-DRB3* genotypes were identified in the selected animals (Table 3). Six animals in the LPVL phenotypic group were heterozygous for the **0902* allele and the remaining 2 were heterozygous for the **14011* allele. Within the HPVL group, 1 cow was homozygous for the **1501* allele and 3 were heterozygous carriers of the **1001*, **1201*, and **1501* alleles.

Table 3. The *BOLA-DRB3* genotype in selected cows

Sample ID	Cow phenotype[1]	Genotype
5570	HPVL	*BOLA-DRB3*0101 + BOLA-DRB3*1501*
5671	HPVL	*BOLA-DRB3*1101 + BOLA-DRB3*2703*
5841	HPVL	*BOLA-DRB3*1001 + BOLA-DRB3*14011*
6021	HPVL	*BOLA-DRB3*0101 + BOLA-DRB3*1101*
6097	HPVL	*BOLA-DRB3*0101 + BOLA-DRB3*1101*
6115	HPVL	*BOLA-DRB3*1201 + BOLA-DRB3*1501*
6227	HPVL	*BOLA-DRB3*1501 + BOLA-DRB3*1501*
5324	LPVL	*BOLA-DRB3*14011 + BOLA-DRB3*1501*
5633	LPVL	*BOLA-DRB3*0902 + BOLA-DRB3*1101*
5656	LPVL	*BOLA-DRB3*0902 + BOLA-DRB3*2703*
5659	LPVL	*BOLA-DRB3*0902 + BOLA-DRB3*14011*
5701	LPVL	*BOLA-DRB3*0902 + BOLA-DRB3*1201*
6121	LPVL	*BOLA-DRB3*0902 + BOLA-DRB3*1501*
6190	LPVL	*BOLA-DRB3*1101 + BOLA-DRB3*14011*
6236	LPVL	*BOLA-DRB3*0902 + BOLA-DRB3*1201*

[1]HPVL = high proviral load; LPVL = low proviral load.

Normalized N_0 values were transformed using logarithm base 2 ($\log_2 N_0$) to approximate a normal distribution of the N_0 expression data. At the 5% level of significance, no significant differences were observed in the distribution of the expression values in 5 genes (mix 1) between HPVL and LPVL cows. In the case of the normalized N_0 values for the 3 target genes assayed with mix 2 (*LY6G5B*, *IER3*, and *ABT1*), 1 of them, *ABT1*, showed a statistically significant difference in the expression values between HPVL and LPVL groups (Mann-Whitney-Wilcoxon test $P = 0.029$; Figure 1), showing higher expression in the LPVL group.

The analysis performed considering quantification cycle values using the REST software did not show any statistically significant expression ratio between LPVL and HPVL phenotypes for any of the candidate genes tested. Nonetheless, differences in *PRRC2A* gene activity approached significance (R = 1.293, $P = 0.08$) with higher expression levels observed in the HPVL group (Table 4; Figure 2).

Lymphocyte counts (**LC**) performed at T4 and 4 mo later (T5) are shown in Supplemental Table S5 (https://doi.org/10.3168/jds.2020-18924). Only 2 animals had total LC 3 or more standard deviations from the normal lymphocyte value (≥8,200 lymphocytes/μL; Bendixen, 1963) at times T4 and T5, thus being considered to be lymphocytotic (Juliarena et al., 2007). These 2 animals belonged to the HPVL phenotypic group (Supplemental Table S5, https://doi.org/10.3168/jds.2020-18924). Then, we investigated whether blood cell counts differences existed between HPVL and LPVL groups using a statistical Student *t*-test. When white blood cell counts were considered, significant mean differences between groups were observed (*t*-test $P = 0.036$). Then, we analyzed immune cell (lymphocytes, neutrophils, eosinophils, and monocytes) subpopulations separately and found that only the mean LC was significantly higher, as expected, between the HPVL and LPVL groups (*t*-test $P = 0.003$; Figure 3).

We then evaluated the correlations among the LC and the candidate gene expression levels. Statistically significant positive and negative correlations with $\log_2$ LC were found for *PRRC2A* (r = 0.61, $P = 0.02$) and *IER3* expression level (r = −0.45, $P = 0.01$), respectively. Furthermore, we applied linear regression models to evaluate whether the $\log_2$ LC values are better fitted considering $\log_2$ *PRRC2A*, $\log_2$ *IER3*, or $\log_2$ *PRRC2A* + $\log_2$ *IER3* as independent variables. The coefficients of determination, *P*-values, and model parameters are shown. The linear regression model including $\log_2$ *PRRC2A* + $\log_2$ *IER3* resulted in a higher coefficient

Table 4. Differential gene expression analysis using relative expression ratio (R) and randomization test

Gene symbol	E[1]	R	Log_2 R	Randomization test *P*-value
BOLA-DRB3	0.778	0.806	−0.311	0.20
PRRC2A	0.847	1.293	0.255	0.08
TNF	0.839	0.700	−0.515	0.19
BAG6	0.829	0.986	−0.020	0.90
BOLA-A	0.830	1.201	0.264	0.30
LY6G5B	0.865	0.900	−0.152	0.67
IER3	0.854	0.629	−0.669	0.11
ABT1	0.869	0.803	−0.316	0.14

[1]Real-time quantification PCR reaction efficiency.

of determination ($R^2 = 0.56$, $P = 0.003$) compared with the models considering only $\log_2$ *PRRC2A* ($R^2 = 0.32$, $P = 0.02$) or $\log_2$ *IER3* ($R^2 = 0.35$, $P = 0.01$) as independent variables (Table 5).

DISCUSSION

In this work we evaluated whether several candidate genes holding or linked to genetic variants associated with BLV infection levels were differentially expressed between HPVL and LPVL groups of Argentinean Holstein cows. The selected cows were, on average, in their second lactation (≥3 yr old); at this stage, the PVL level in peripheral blood is apparently stable for extended periods of time (Florins et al., 2007). Previous studies have reported that the anti-BLV antibody level positively reflects the in vivo PVL in BLV-infected animals (Juliarena et al., 2007; Gutiérrez et al., 2012). Applying this rationale, we preselected animals positive for BLV antibodies (10-mo interval). Then, we selected those cows with contrasting levels of serological reactivity to later determine their PVL level by qPCR. This strategy has been implemented in previous studies and allowed us to establish contrasting phenotypic groups based on BLV PVL (Gutiérrez et al., 2012; Carignano et al., 2017, 2018). Although we had 19 animals (10 LPVL and 9 HPVL) at the T3 sampling, 4 of them were not in lactation at the T4 sampling; therefore, they were removed from further analysis (Supplemental Table S6, https://doi.org/10.3168/jds.2020-18924).

Of the 8 genes studied, only the *ABT1* gene presented a statistically higher expression in the LPVL group (Mann-Whitney-Wilcoxon test $P = 0.029$; Figure

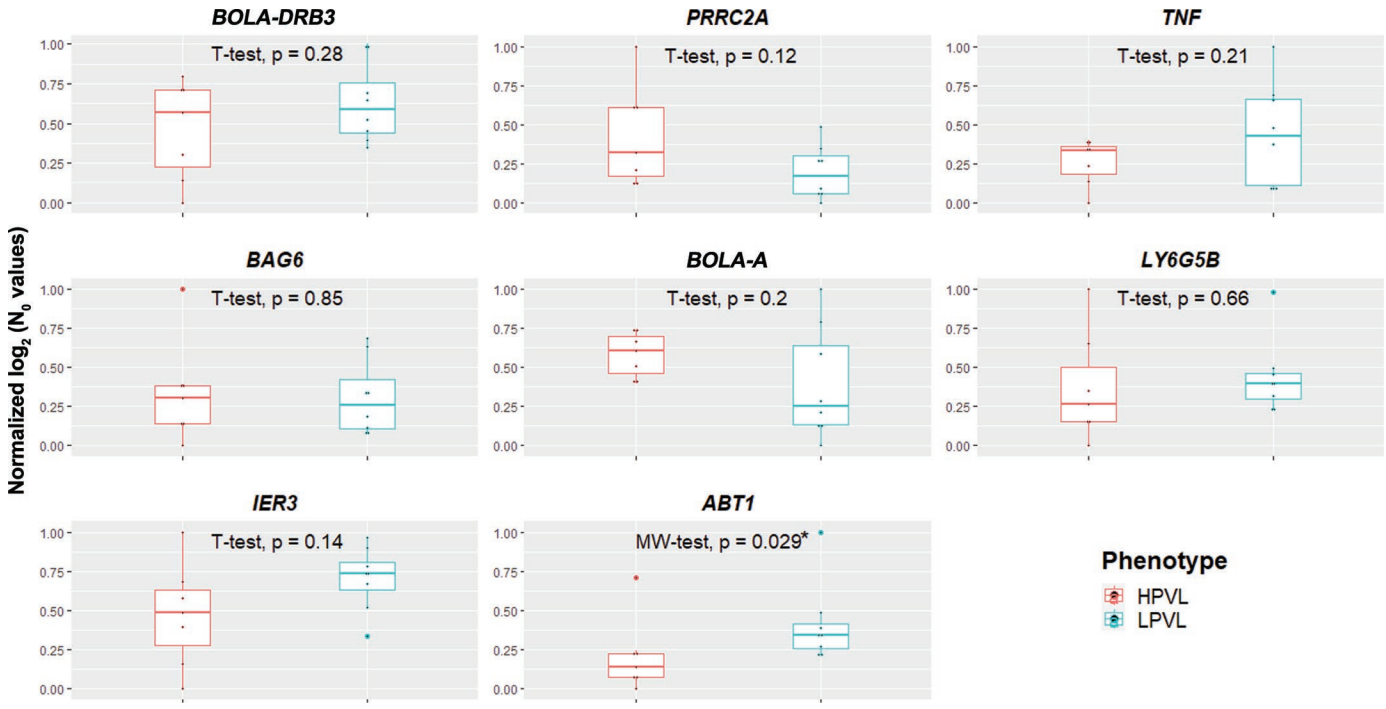

Figure 1. Differential gene expression ($\log_2$ initial fluorescence value; N_0) in high proviral load (HPVL) and low proviral load (LPVL) phenotypic groups. Boxplots of gene expression quantification for 8 genes in HPVL (red) and LPVL (green) cows. Box = interquartile range (IQR); midline = median value; dots = initial fluorescence values of each sample; whiskers = minimum and maximum values excluding outliers (>1.5 × IQR). *P*-value estimated with Student test (*t*-test) or Mann-Whitney-Wilcoxon test (MW-test) are indicated below each gene name. *The expression of the *ABT1* gene is significantly higher in LPVL animals (MW-test $P = 0.029$). Y-axis values were normalized [0,1]|$(Y - Y_{minimum}/Y_{maximum} - Y_{minimum})$.

1). Additionally, we performed a different approach using the C_q expression values obtained for the candidate gene and permutation tests for significance calculation as implemented in the REST software (Pfaffl, 2002). In this case, none of the analyzed genes showed significant expression differences between LPVL and HPVL phenotypes. Only *PRRC2A* gene expression had higher values in HPVL cows than in LPVL cows approaching significance ($P = 0.08$; Table 4).

Both methodologies resulted in similar patterns adding consistency to the differential gene expression analyses, as can be seen in Figures 1 and 2 and in Table 4. For example, even though the *ABT1* gene quantification cycle expression value did not show significant differences between the contrasting phenotypic groups, a trend could be observed in Figure 2 where 75% of the measured *ABT1* R values (Equation 2) were below the unit, meaning a higher expression in LPVL cows than in HPVL cows. These differences would be reflected in a permutation test *P*-value close to 0.1 (Table 4). The same trend was observed in *ABT1* when using N_0 values (Figure 1). In turn, the presence of 2 outliers in the *ABT1* box plots, 1 in each group of animals (Figure 1), could be interfering with the permutation test sensitivity.

Associations of ubiquitous genes in the bovine MHC region with resistance or susceptibility to BLV have been reported for more than 30 yr (Lewin and Bernoco, 1986; Lewin et al., 1988; Xu et al., 1993; Mirsky et al., 1998; Konnai et al., 2006a; Juliarena et al., 2008; Jimba et al., 2012; Miyasaka et al., 2013; Carignano et al., 2017, 2018; Takeshima et al., 2019). Such is the case of MHC class I genes, which encode glycoproteins expressed on the surface of most somatic cells. These glycoproteins are involved in the activation of CD8+ T lymphocytes through the presentation of antigens derived from processed intracellular proteins (Germain, 1994). Previous studies reported a correlation between BLV infection progression and *BOLA-A* genotypes (Lewin and Bernoco, 1986; Lewin et al., 1988). At the *BOLA-A* gene expression level, we did not find significant differences between HPVL and LPVL phenotypes (Figure 1; Table 4). No differences were described by Teutsch and Lewin (1996) for bovine MHC class I and

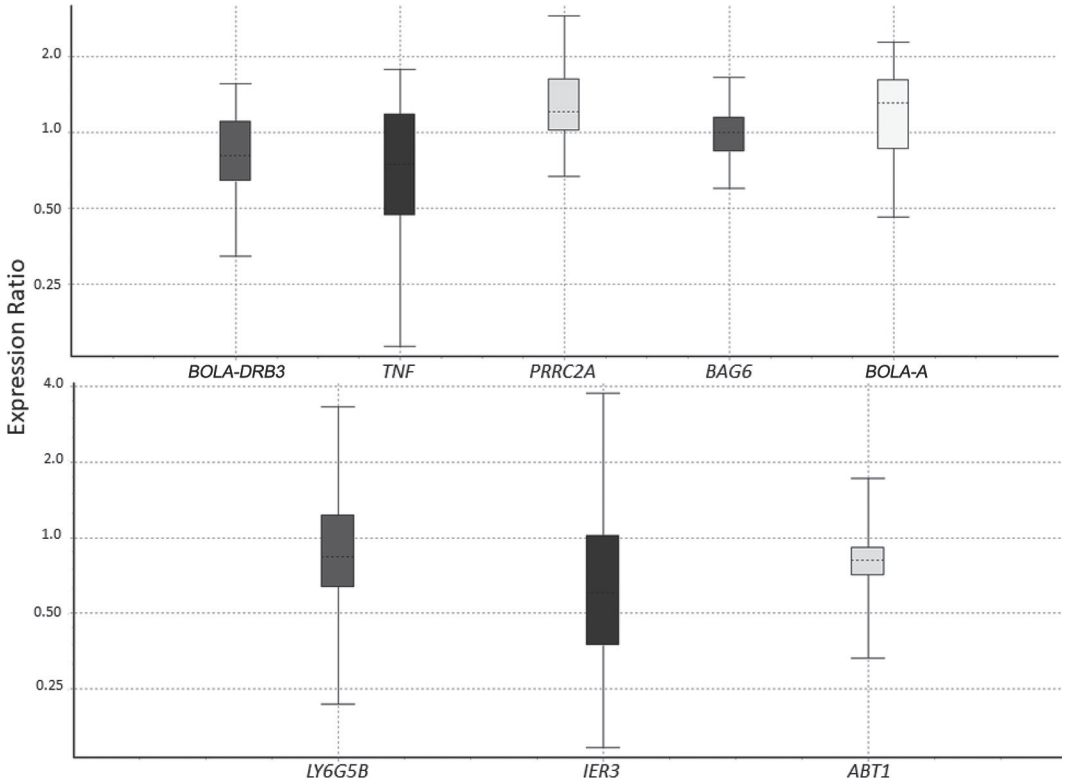

Figure 2. Gene expression ratios (R) calculated using REST version 2009 (Pfaffl, 2002). Gene expression ratios were measured based on quantification cycle and efficiency values and then normalized using geometric means of the reference genes. R = 1 indicates no differences, R >1 indicates higher gene expression in the high proviral load group, and R < 1 indicates higher gene expression in the low proviral load group. Box = interquartile range (IQR); midline = median value; whiskers = minimum and maximum values excluding outliers (>1.5 × IQR).

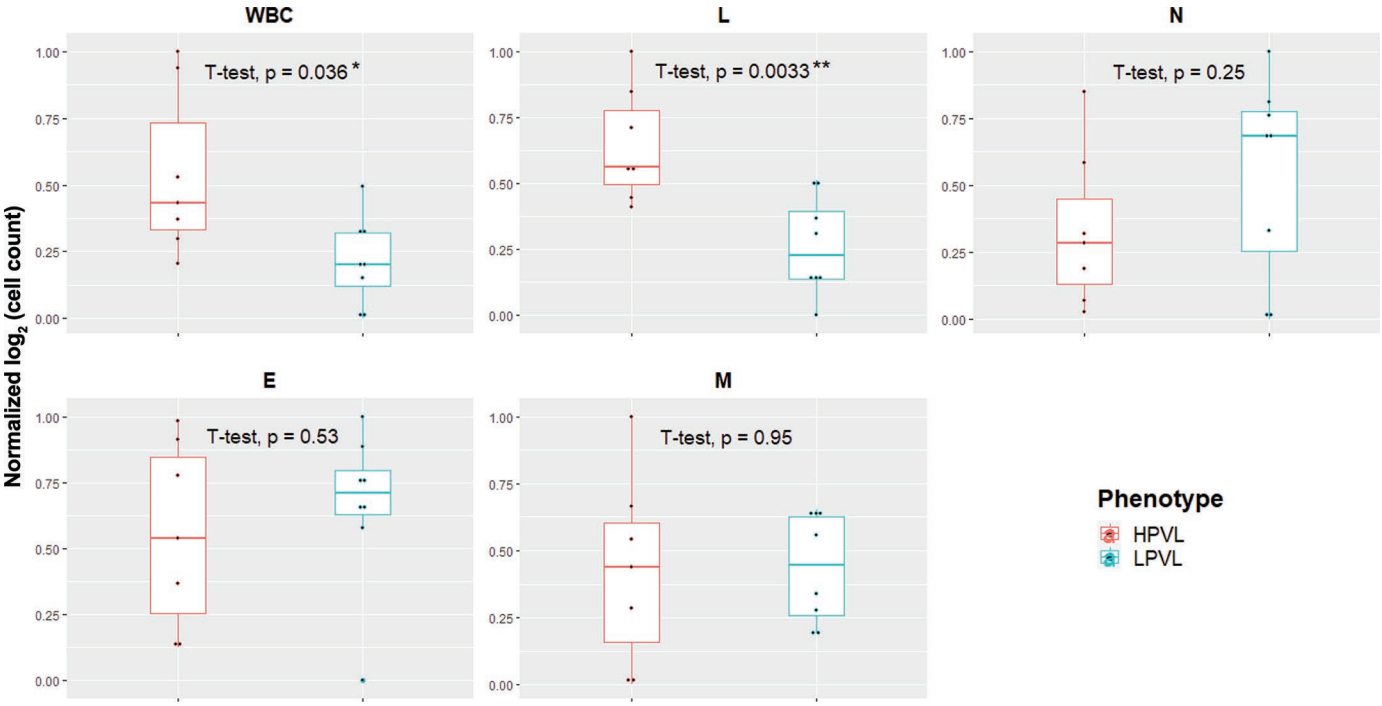

Figure 3. Cell count distributions in high proviral load (HPVL) and low proviral load (LPVL) phenotypic groups. Boxplots show white blood cell (WBC) and individual cell population (L = lymphocytes; N = neutrophils; E = eosinophils; M = monocytes) count distributions in HPVL (red) and LPVL (green) cows. Box = interquartile range (IQR); midline = median value; dots = log cell count values of each sample; whiskers = minimum and maximum values excluding outliers (>1.5 × IQR). *P*-values estimated with Student test (*t*-test) are indicated under each cell name. Statistically significant differences were observed in WBC count (* = *t*-test $P = 0.036$) and L count (** = *t*-test $P = 0.003$). Y-axis values were normalized $[0,1]|(Y - Y_{minimum}/Y_{maximum} - Y_{minimum})$.

II gene expression when comparing animals infected with BLV, with or without persistent lymphocytosis, and negative animals.

The MHC class II genes are expressed in antigen-presenting cells, such as dendritic cells and macrophages. Specific motifs present in the peptide-binding cleft of the MHC class II BoLA-DRB molecule have been associated with resistance to the progression of BLV infection to persistent lymphocytosis in cattle (Xu et al., 1993). However, significantly associated alleles did not always coincide among the cattle populations studied by different authors. In HPVL animals, several alleles associated with *BOLA-DRB3* have been identified (e.g., *BOLA-DRB3*1001*, **1201*, **1501*, and **1503*; Juliarena et al., 2008; Carignano et al., 2017; Takeshima et al., 2019). On the other hand, several alleles were described as being associated with an LPVL phenotype (e.g., *BOLA-DRB3*14011*, **0201*, and **1701*). Particularly, the *BOLA-DRB3*0902* allele was consistently associated with an LPVL phenotype in several studies involving different cattle breeds (Juliarena et al., 2008; Carignano et al., 2017; Hayashi et al., 2017;

Table 5. Linear regression model analysis considering lymphocyte counts ($\log_2$ LC) as dependent variable and *PRRC2A* and *IER3* gene expression values ($\log_2$ initial fluorescence value) as independent variables

	Linear model		
Item	$\log_2$ LC = a + m x		$\log_2$ LC = a + b x_1 + c x_2
Independent variable	x = $\log_2$ *PRRC2A*	x = $\log_2$ *IER3*	x_1 = $\log_2$ *PRRC2A* + x_2 = $\log_2$ *IER3*
F-test *P*-value	0.02	0.01	0.003
R^2	0.32	0.35	0.56
Parameters[1]	a = 16.17 ± 1.33	a = 9.93 ± 0.89	a = 13.34 ± 1.46
	m = 0.90 ± 0.32	m = −0.55 ± 0.19	b = 0.72 ± 0.27
			c = −0.45 ± 0.16

[1]m, b, and c = slope (± SE); a = intercept (± SE).

Takeshima et al., 2019). Specifically, an amino acidic motif in the peptide antigen binding domain present in the **0902* allele was associated with an LPVL phenotype (Carignano et al., 2017). However, the biological mechanism leading to an LPVL phenotype has not yet been elucidated. We evaluated the differential expression of the *BOLA-DRB3* gene without finding statistically significant differences between HPVL and LPVL phenotypes (Figure 1; Table 4). Table 3 shows that all cows carrying the **0902* allele were LPVL; however, differential expression of the *BOLA-DRB3* gene in these cows did not appear to be responsible for the PVL phenotypic differences compared with the HPVL group. Genetic variation for resistance or susceptibility to PVL development in cattle is mainly polygenic (Brym and Kamiński, 2017; Carignano et al., 2018); hence, other candidate genes were further functionally characterized.

Tumor necrosis factor (**TNF**)-α is a proinflammatory cytokine playing a key role in the host response to various types of infection (Imanishi, 2000; Roux et al., 2006). A differential expression in this cytokine has been reported for cows infected with BLV considering HPVL and LPVL phenotypes (Konnai et al., 2006b). The differential expression (measured in ex vivo cultivated PBMC) has been attributed to polymorphisms in the *TNF* gene regulatory region (Konnai et al., 2006a; Lendez et al., 2015). The BLV transcription is mostly silent during nonacute stages of infection (Gillet et al., 2013), although some spontaneous reactivation could be observed in vivo (Alvarez et al., 2019; Jaworski et al., 2019). On the other hand, BLV transcription is easily reactivated by ex vivo whole-blood cultivation from infected cows (Tajima and Aida, 2005). In this work, we detected and quantified *TNF* mRNA from uncultured isolated PBMC. Nonetheless, there were no statistically significant differences between the 2 phenotypic groups studied (Figure 1; Table 4). Therefore, we argued that bulk TNF expression ex vivo could be promoted by the presence of BLV expression products (higher in HPVL cows). Instead, in our case, in vivo BLV transcription would be silent, so TNF mRNA levels would be similar between HPVL and LPVL animals.

The *PRRC2A*, *ABT1*, *IER3*, *LY6G5B*, and *BAG6* genes assayed in this work have not been previously studied in relation to BLV infection in cattle. The *BAG6* gene functions have been related to the apoptotic signaling pathway (Grover and Izzo, 2012). Disturbed expression of *BAG6* has been reported in hepatocellular carcinoma tumor cells (Zhang et al., 2019) and Hodgkin lymphomas (Hartmann et al., 2013). Late in the life course of an infected animal, BLV eventually causes a lymphoproliferative disorder leading to deadly lymphosarcoma (Burny et al., 1988; Kettmann et al., 1994). In this regard, previous research suggests that apoptosis and B-cell proliferation are affected in BLV-infected animals (Frie and Coussens, 2015), so we hypothesized that genes in the apoptotic pathway, such as *BAG6*, would be differentially expressed in HPVL and LPVL groups of animals. On the other hand, the superfamily of proteins lymphocyte antigen 6 (LY6) have functions related to the immune response. They have been involved in numerous cellular functions, such as leukocyte differentiation, cell adhesion and migration, and cytokine production (Lee et al., 2013). Susceptibility to human immunodeficiency virus (**HIV**) disease progression has been associated with an SNP close to the *LY6* gene family in humans (Loeuillet et al., 2008). Particularly, the *LY6G* family member is expressed in circulating blood and limited to neutrophils. However, *LY6G* expression has been reported recently in viral skin infections (Fischer et al., 2011). In this work, neither *BAG6* nor *LY6G5B* showed expression differences between BLV HPVL and LPVL contrasting phenotypes (Figure 1; Table 4).

The expression of *IER3* is induced by a variety of stimuli, and the protein product of this gene exerts its effect on signaling pathways involving cell cycle and apoptosis (Arlt and Schäfer, 2011). Notably, an increase of *IER3* expression as a response to viral infections has been reported (Villalba et al., 2017). In this study, nonsignificant *IER3* expression differences were observed between HPVL and LPVL animals, but a trend toward lower expression level could be observed in HPVL cows (Figures 1 and 2). Klase et al. (2009) found diminished expression of *IER3* in vitro due to the action of an HIV-1 viral noncoding microRNA. This HIV-1 microRNA could be favoring the survival of infected cells by decreasing the *IER3* proapoptotic function. Therefore, the tendency to a lower expression of *IER3* in HPVL animals could result from a differential host response to BLV virulence factor promoting the survival of infected cells. Also, *IER3* has been associated with the inhibition of the nuclear factor (**NF**)-κβ TF (Arlt and Schäfer, 2011). The NF-κβ TF was postulated to be a stimulator of BLV *TAX* regulatory protein expression, which is ultimately responsible for the BLV viral transcription transactivation (Brooks et al., 1998). In humans infected with HTLV-1, the Tax protein-dependent transactivation of NF-κβ is relevant for immortalization of infected cells (Robek and Ratner, 1999). Therefore, very small differences in the expression of *IER3* could be involved in the progression of BLV disease.

Transactivation of BLV viral transcription induced by NF-κβ (Hiscott et al., 1995; Brooks et al., 1998),

cAMP response element-binding protein, and activating transcription factor 1 and 2 TF has been reported (Willems et al., 1992; Adam et al., 1996). In turn, FOXP3 (Grant et al., 2006) and p53 could interfere in the TATA-binding protein/HTLV-1 LTR TATA motif interaction (Mori et al., 1997). We evaluated the expression of 2 different TF. The *PRRC2A* gene is a TF whose biological function remains poorly understood, but it is important to point out that an SNP previously associated with BLV PVL development in cattle is located in the *PRRC2A* coding region of provoking a synonymous substitution (Carignano et al., 2018). Sequence-level polymorphisms linked to *PRRC2A* have been associated with susceptibility to different diseases in humans, such as susceptibility to rheumatoid arthritis (Singal et al., 2000), insulin-dependent diabetes mellitus (Hashimoto et al., 1999), or development of non-Hodgkin's lymphoma (Nieters et al., 2012). The expression of this gene seems to be higher in HPVL compared with LPVL animals (Figures 1 and 2) but not statistically significant (Figure 1; Table 4). Increasing the number of animals per phenotype would allow us to determine whether this trend is really due to differences in the level of gene expression or the result of a limited sample size.

As previously stated, the expression of *IER3* is related to the inhibition of NF-κβ, a TF whose function is important for viral replication and immortalization of infected cells (Robek and Ratner, 1999; Arlt and Schäfer, 2011). The biological function of the TF *PRRC2A* remains unknown, but some genetic polymorphisms in this gene have been associated with the susceptibility to develop a lymphoid tissue cancer (non-Hodgkin's lymphoma) in humans (Nieters et al., 2012). In this work, correlations among the expression ($\log_2 N_0$) of both genes and lymphocytes counts ($\log_2$ LC) showed that *PRRC2A* (low) and *IER3* (high) gene expressions are related to a decreased lymphocyte count (Table 5), possibly preventing an uncontrolled replication of infected cells and limiting the spreading of BLV provirus within the host.

The other TF analyzed was *ABT1*, which is a polymerase II basal transcription activator associated with TATA-binding proteins (Oda et al., 2000). The interaction of the HTLV-1 *TAX* protein with TATA-binding proteins has been described in humans, increasing the viral transcription (Caron et al., 1993; Grassmann et al., 2005). However, the continuous expression of viral antigens activates the host immune response, stimulating the clearance of infected cells expressing viral proteins. Mori et al. (1997) argued that the p53 protein would promote the survival of infected cells carrying HTLV-1 provirus by silencing viral expression. A lower expression of the *ABT1* gene in HPVL animals would diminish the expression of BLV viral antigens compared with LPVL animals, thus allowing cells infected with BLV provirus to escape the immune response and to proliferate those provirus-carrying clones until levels of BLV circulating provirus are high.

CONCLUSIONS

The biological mechanisms underlying the determination of the level of BLV PVL and white blood cell counts in cattle have not been yet elucidated. In this study, we focused on a limited number of genes related to BLV PVL SNP hits to assess whether differences in their expression could be associated with BLV disease progression in cattle. The expression of the *ABT1* transcription factor was higher in LPVL cows at a 95% significance level. On the other hand, the LC showed a statistically significant correlation with the expression of *PRRC2A* and *IER3* genes. We argued that *ABT1* TF promoted BLV antigen expressions in LPVL cows. This viral expression in BLV-infected cells could favor viral clearance by the host immune response, thus limiting provirus clonal expansion. Similarly, *PRRC2A* and *IER3* expression patterns in LPVL animals would be contributing to limit the number of infected cells in peripheral blood. Although the sample size was limited, evidence of differential expression and correlations with lymphocytes in contrasting BLV phenotypes were reported for genes not previously involved in the progression of BLV infection. This information is valuable in understanding BLV biology and the immune response in naturally infected cows under real milk production conditions. Deciphering the mechanisms leading to an HPVL phenotype in cows is relevant for the development of BLV infection control programs within herds. Overall, these results encourage further studies integrating genome-wide genotypic data, global transcriptome analysis, and characterized BLV progression phenotypes to understand the BLV–host interaction.

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