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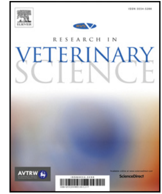

# Toll-like receptors, IFN-γ and IL-12 expression in bovine leukemia virus-infected animals with low or high proviral load

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ABSTRACT

Bovine leukemia virus (BLV) infection is widespread mainly in dairy cattle and 5–10% of infected animals will die due to lymphosarcoma; most cattle remain asymptomatic but 30% develop persistent lymphocytosis (PL). BLV transmission depends on infected cell exchange and thus, proviral load is determinant. Understanding the mechanisms which govern the control of viral dissemination will be desirable for the design of 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. We aimed to characterize cytokines and toll-like receptors (TLR) expression related to the proviral load profiles. IFN-γ and IL-12 mRNA expression level was significantly higher in PBMC from infected cattle (LPL $n = 6$ and HPL $n = 7$) compared to uninfected animals ($n = 5$). While no significant differences were observed in IL-12 expression between LPL and HPL group, IFN-γ expression was significantly higher in LPL animals. Infected cattle exhibited higher expression levels of TLR3, 7–9. Animals with HPL had significantly higher expression of TLR7/8 than uninfected cattle. TLR8 and TLR9 were up-regulated in HPL group, and TLR3 was up-regulated in LPL group. This is the first report related to TLR gene expression in BLV infected cattle and represents evidence of the involvement of these receptors in BLV recognition. Further studies on different subpopulations of immune cells may help clarify their role in response to BLV and its consequences on viral dissemination.

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## 1. Introduction

Bovine leukemia virus (BLV) is an exogenous retrovirus, which causes a lymphoproliferative disease, affecting mainly B-lymphocytes. Approximately 60% of the infected animals remain asymptomatic, 30% develop persistent lymphocytosis (PL), a frequent pre-tumoral state, and the remaining 10% usually develop lymphosarcoma. PL is characterized by a polyclonal expansion of B cells, mostly infected with the virus (Burny et al., 1978; Ferrer, 1980). Since BLV transmission depends on infected cell exchange (Hopkins and DiGiacomo, 1997), the concentration of BLV-infected cells in blood should play a major role in the success of BLV transmission. Development of PL has been considered an important risk factor for transmission. However, BLV infected non-lymphocytotic (non-PL) cattle may also play a role depending on their proviral load. Indeed, Juliarena et al. showed that approximately 60% of non-PL cattle have low proviral load (LPL), while about 40% developed high proviral load (HPL) (Juliarena et al., 2007). Apparently, the capability of controlling viral dissemination in BLV-infected animals may be associated, at least in part, with a difference in the humoral immune response to the virus (Juliarena et al., 2007) and with host genetic factors as the expression of certain alleles of the bovine leukocyte antigen (*BoLA*) *DRB3.2** gene (Juliarena et al., 2008). However, these factors would not be absolutely responsible for the LPL profile. Furthermore, the cellular response against the virus has not been well characterized in animals that develop LPL.

Cytokine expression profiles of BLV-infected animal differ depending on the stage of disease. Type 1 cytokines, interleukin-2 (IL-2) and gamma interferon (IFN-γ), are expressed in high amounts in peripheral blood mononuclear cells (PBMC) from non-PL animals. Something similar happens with IL-12, a key cytokine that induces Th1 response. However, increased expression of the type 2 cytokine IL-10 was detected in PBMC from PL or tumor-bearing animals. These findings suggest that an imbalance in the expression of cytokines may contribute to disease progression (Pyeon et al., 1996; Pyeon and Splitter, 1998). Until now, the profile of cytokines expression related to the proviral load has not been described.

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IFNs and other pro-inflammatory cytokines are rapidly induced by the innate immune system in response to pathogen invasion. These mechanisms are activated following the sensing of infection through the evolutionary conserved pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs). Viruses possess several structurally diverse PAPMs (surface glycoproteins, DNA and RNA species), present in the infecting virion or produced during viral replication; and the host cell possesses a broad range of viral nucleotide sensors (Mogensen, 2009). Toll-like receptors (TLRs) are the best PRRs characterized. Upon ligand recognition, TLRs simulate the strong production of a wide variety of cytokines, depending on the recognized PAMP and the participating TLR. TLRs 1, 2, 4, 5, 6 and 11 are expressed on the cell surface, and mainly recognize microbial membrane components, such as lipids, lipoproteins and proteins. TLRs 3, 7, 8 and 9 are expressed in intracellular vesicles, such as endosomes, lysosomes and the endoplasmic reticulum. This latter group of TLRs recognizes microbial nucleic acids, particularly of viral origin, either present in the viral genome or generated during replication. Double-stranded RNA (dsRNA) is recognized by TLR 3, single-stranded RNA (ssRNA) is detected by TLR 7/8, and TLR 9 recognizes the unmethylated CpG dinucleotides in DNA molecules (Borrow et al., 2010). Thus, the binding of viral ligands to TLRs activates transcription factors and induces the expression of antiviral factors, such as cytokines (Boo and Yang, 2010). Abundant information about the participation of these mechanisms in human viral diseases is available (Boo and Yang, 2010; Mogensen, 2009). Moreover, interference of viral proteins with TLR-induced signaling, thus preventing the antiviral host response to infection, has been demonstrated (Mogensen, 2009). Even though TLRs expression profiles in several bovine tissues and TLR polymorphisms were described (Novak, 2014; Turin and Riva, 2008), only a few studies addressed their role in the development of viral diseases in cattle (Marin et al., 2014a; Marin et al., 2014b; Zhang et al., 2006). This is the first report concerning the patterns of TLRs 3, 7–9 expression on PBMC from BLV infected animals with HPL and LPL.

## 2. Materials and methods

### 2.1. Sample collection

Blood samples were obtained from adult Holstein (Holando-Argentino) dairy cows belonging to two herds from the Tandil region (Provincia de Buenos Aires, Argentina): 5 animals were classified as BLV-free (aged 2–6 years old, average: 4 years old) and 13 animals were classified as BLV-positive (aged 2–7 years old, average: 4 years old). All animals were healthy and milking (between second and third lactation period). Serological BLV status was previously determined by testing plasma for anti-BLV antibodies by ELISA 108 (Gutierrez et al., 2001). For PBMCs separation, 10 ml blood samples were collected in heparinized syringes by jugular venipuncture. Blood samples were transferred to 15 ml tubes and centrifuged for 15 min at 3800 rpm at 4 °C. 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 blood cells. PBMCs were obtained by centrifugation at 3000 rpm during 10 min at 4 °C. Cells were washed with PBS and centrifuged at 2500 rpm for 7 min at 4 °C. PBMCs pellets were divided in 3 parts: one third was stored at −20 °C for DNA extraction; two thirds were resuspended in RNA*later* RNA Stabilization Solution (Thermo Fisher Scientific) overnight at 4 °C and stored at −80 °C until RNA extraction.

### 2.2. Proviral load determination

#### 2.2.1. DNA extraction

DNA from PBMCs was extracted using Qiagen columns (QIAamp DNA Mini Kit) according to the manufacturer's protocol. DNA was eluted in 50 μl of water. The concentration and purity of DNA were determined by the OD value at 260 nm and 260/280 coefficient respectively in a NanoDrop 2000 Spectrophotometer (Thermo Scientific). The purified DNAs were stored at −20 °C until use, no >3 months.

#### 2.2.2. Construction of a standard curve

For proviral load determination, an absolute quantification method by real-time PCR (qPCR) was set up. For the construction of a standard curve, a plasmid carrying one copy of the entire BLV genome under control of its own promoter, the long terminal repeat region (kindly supplied by Dr G.C. Buehring, University of California, Berkeley School of Public Health, California, USA) was used. TOPO10 cells were transformed with the plasmid, and DNA was purified using a commercial kit (Axygen, Biosciences). The plasmidic DNA concentration was quantified with a NanoDrop 2000 Spectrophotometer. A standard curve of six 10-fold serial dilutions of pBLV, containing $10^6$ to 10 BLV copies, was built and run 3 times for validation of the method. According to the standard curve slope (typical slope − 3.376 and R2 0.974), efficiency of reactions was calculated between 99.1 and 99.8%.

#### 2.2.3. qPCR conditions for proviral load determination.

Primers for BLV *pol* gene amplification were designed on the basis of published sequence data (Sagata et al., 1985), using Primer Express® software v. 3.0 (Applied Biosystems): forward 3′ CACCATTCACCCCCACTTG 5′ and reverse 3′ TCAGAGCCCTTGGGTGTTTC5′. The PCR reaction mix was prepared containing 0.3 μM specific forward and reverse primers, 1 x PCR Master Mix with SYBR Green (FastStart Universal SYBR Green Master Rox, Roche) and 30 ng of DNA from BLV infected animals, in a final volume of 20 μl. The amplification and detection of the specific products were carried out using an Applied Biosystems 7500 cycler, under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The standard curve and non-template control were included in each run. Experiments were always performed in triplicate.

BLV-positive animals were classified as high proviral load (HPL) (>1000 BLV copies/reaction) and low proviral load (LPL) (<100 BLV copies/reaction). Before classifying the animals into each group, the proviral load was evaluated two times at 6-month intervals.

### 2.3. mRNA gene expression

#### 2.3.1. RNA extraction and DNase I treatment

Total RNA extraction was performed from PBMC using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA was eluted in 50 μl of RNase-free water. The concentration and purity of RNA were determined by the OD value at 260 nm and 260/280 ratio respectively in a NanoDrop 2000 Spectrophotometer (Thermo Scientific). The extracted RNAs were kept at −80 °C until use. To remove any possible contaminating genomic DNA, 1 μg of each RNA was treated with RNase-free DNase I (Pierce) according to the manufacturer's instructions.

#### 2.3.2. Reverse transcription

The reverse transcription (RT) reaction was carried out with 0.5 μg of total DNase-treated RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's instructions. Negative control for cDNA synthesis (all the reagents except reverse transcriptase) were prepared for each sample. The cDNA was immediately used or stored at −20 °C until use, no >1 month.

#### 2.3.3. Primers

Primers for amplification of cytokines mRNA are listed in Table 1. For TLRs mRNA amplification, the primer sequences were described previously (Marin et al., 2016).

#### 2.3.4. qPCR conditions for mRNA gene expression.

The PCR reaction mix contained 0.5 μM specific forward and reverse primers for IFN-γ and IL-12 respectively, 1 x PCR Master Mix with SYBR

**Table 1**
Primers used for the amplification of cytokines mRNA. (*)Primers designed using Primer Express® software v. 3.0. (Applied Biosystems).

| Gene | Primer sense | 5′–3′ sequences | Melting temperature | Size of amplicon | Reference |
|---|---|---|---|---|---|
| GADPH | Fwd | CACCCTCAAGATTGTCAGCA | 51,78 °C | 142 bp | Okuda et al. (2010) |
| | Rev | GGTCATAAGTCCCTCCACGA | 53,83 °C | | |
| IFN-γ | Fwd | CAGCTCTGAGAAACTGGAGGACTT | 63 °C | 77 bp | Waldvogel et al. (2000) |
| | Rev | TGGCTTTGCGCTGGATCT | 63 °C | | |
| IL-12 | Fwd | AAACCAGACCCACCCAAGAAC | 59 °C | 61 bp | Primer Express(*) |
| | Rev | CCTCCACCTGCCGAGAATT | 59 °C | | |

Green (FastStart Universal SYBR Green Master Rox, Roche) and 2 μl of cDNA, in a final volume of 20 μl. The amplification and detection of the specific products were carried out using an Applied Biosystems 7500 cycler, under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Experiments were always performed in triplicate. Negative controls for cDNA synthesis and non-template control were included in all cases.

The qPCR for TLRs was carried out as previously described (Marin et al., 2016).

### 2.3.5. Determination of amplification efficiency

A standard curve method was employed to determine the amplification efficiency for all genes. Briefly, 5 serial dilutions (4-fold for cytokines and 10-fold for TLRs) of a reference cDNA calibrator were amplified in triplicate and the amplification efficiency was determined by a linear regression model according to the equation: $E = 10[-1/\text{slope}]$ (Pfaffl, 2001; Pfaffl et al., 2002).

### 2.3.6. Statistics analysis

Gene expression was determined by a relative quantification method. The expression of a selected gene of interest was normalized to that of GAPDH as endogenous gene. The statistical significance of the differences ($p < 0.05$) in target mRNA expression level were analyzed with the Relative Expression Software Tool (REST®, Qiagen Inc., Valencia, CA, USA), which calculates the group-wise comparisons and the statistical analyses of relative expression results for qPCR (Pfaffl et al., 2002).

## 3. Results

### 3.1. Analysis of cytokines and TLRs mRNA expression

Relative expression of IL-12, IFN-γ and major antiviral TLRs was measured using qPCR in PBMC isolated from BLV-uninfected ($n = 5$) and BLV-infected ($n = 6$ with LPL and $n = 7$ with HPL) animals. The expression of selected genes was normalized to the reference gene, bovine GAPDH. Statistical analysis were performed using REST® software (Qiagen Inc., Valencia, CA, USA), which allows comparison between two groups, with up to 16 data points in each group (Pfaffl et al., 2002). In our study, results from BLV-uninfected cattle were compared to those from animals with LPL or those with HPL. Another statistical analysis was performed comparing mean expression levels in samples from either LPL or HPL animals.

### 3.1.1. Analysis of cytokine mRNA expression

The mean IFN-γ mRNA expression level was significantly higher in PBMC from both LPL ($p = 0.000$) and HPL group ($p = 0.001$), compared to uninfected cattle (Fig. 1). This cytokine is up-regulated 1864.445 times (range 565.926–4046.680) in animals with LPL and 447.659 times (range 91.036–1276.086) in those with HPL. This expression was also significantly higher in LPL group, compared to HPL group, 4.165 times ($p = 0.007$ range 2.535–6.636).

Relative expression of IL-12 was 24.198 times ($p = 0.000$; range 4.940–267.492) in LPL group and 34.069 times ($p = 0.000$; range 3.197–404.003) up-regulated than in BLV-negative animals (Fig. 2). No significant differences were observed in IL-12 mRNA expression among both LPL and HPL groups.

### 3.1.2. Analysis of TLRs mRNA expression

The mean mRNA expression level of TLRs was always higher in PBMC from both LPL and HPL group, compared to uninfected cattle (Fig. 3). After statistical analysis, significance was found for TLR7 ($p = 0.000$) and TLR8 ($p = 0.007$) expression between uninfected cattle and those who develop HPL, up-regulated 2.107 and 2.308 times, respectively, than the control. No significant differences were observed in TLR mRNA expressions between LPL group and uninfected cattle (Fig. 3).

On the other hand, with the exception for TLR7, significant differences were observed when comparisons between LPL and HPL were done. An up-regulation of TLR8 ($p = 0.028$) and TLR9 ($p = 0.025$) were found in HPL group, while LPL group shown an up-regulation of TLR3 ($p = 0.047$) (Fig. 3).

## 4. Discussion

BLV is a major animal health problem worldwide causing important economic losses, due to virus-induced leukemia/lymphoma mortality and reduction in milk production (Erskine et al., 2012; Tiwari et al., 2007). Seroprevalence in herds from the most important dairy regions of Argentina are at or >90% (Barrios et al., 2012; Giraudo et al., 2010) and control plans involving elimination of BLV-infected animals from the herd is not an economically feasible option. Preventive management, segregation of BLV-free animals, selection of BLV-resistant cattle, therapy by epigenetic modulation of viral expression, and vaccination are other attempts developed to decrease seroprevalence of BLV (Rodriguez et al., 2011). Success of these approaches depend on feasibility/limitation of each method and, mainly, on the need for further

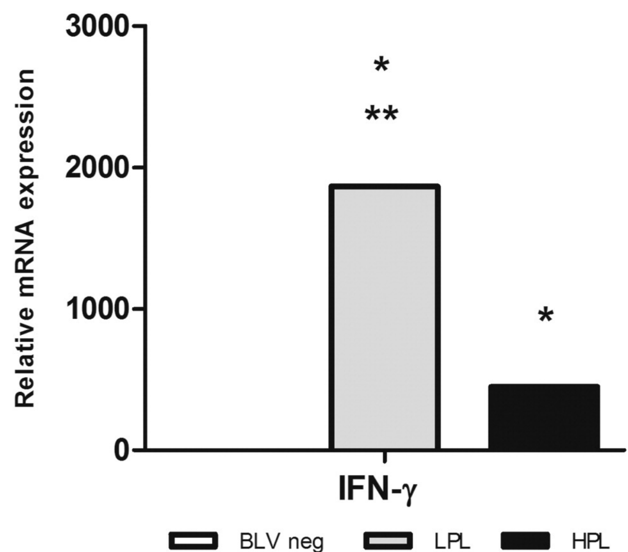


**Fig. 1.** Relative IFN-γ expression levels in PBMC from BLV-uninfected (BLV neg.) and BLV-infected animals with load proviral load (LPL) and high proviral load (HPL). One asterisk (*) indicates statistically significant differences ($p < 0.05$) in relation to uninfected cattle; two asterisks (**) indicate statistically significant differences ($p < 0.05$) between both LPL and HPL groups.

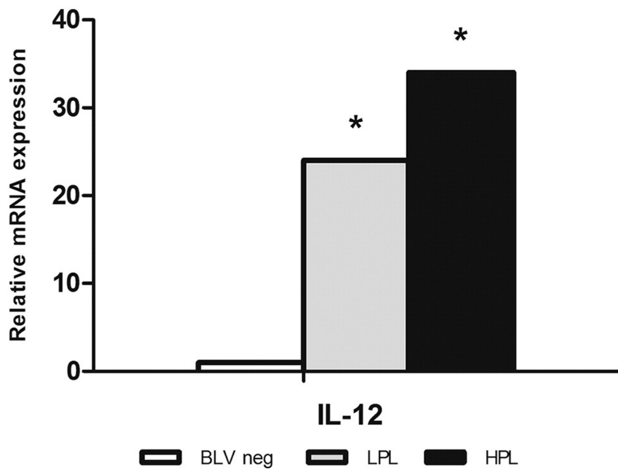


**Fig. 2.** Relative IL-12 expression levels in PBMC from BLV-uninfected (BLV neg.) and BLV-infected animals with load proviral load (LPL) and high proviral load (HPL). One asterisk (*) indicates statistically significant differences ($p < 0.05$) in relation to uninfected cattle.

fundamental research on viral dynamics in its natural host. Development of a proviral load profile is a key factor for BLV transmission. Even though certain genetic polymorphisms and humoral responses can be associated with the LPL profile (Juliarena et al., 2007; Juliarena et al., 2008; Lendez et al., 2015), they cannot fully explain this state. It might be possible that the cellular immune response in these animals could help to maintain this state, but still, it has not been fully characterized. Our study attempts to characterize the LPL profile, particularly the expression of Th1 cytokines and the main antiviral TLRs. This is the first report related to TLR gene expression in BLV infected cattle.

One characteristic of BLV infection is the lack of viral particle detection in peripheral blood concomitantly with strong specific immune responses. Certainly, viral expression occurs permanently in a subpopulation of infected cells, which are very efficiently eliminated by the immune system. However, a large proportion of infected cells, where the virus is completely silenced, escape from clearance by the immune system and accounts for proviral load (Florins et al., 2007; Gillet et al., 2007). It seems clear that a proper and strong specific cell-mediated immune response contributes to the suppression of BLV replication and thus delays disease progression (Kabeya et al., 2001). As it happens

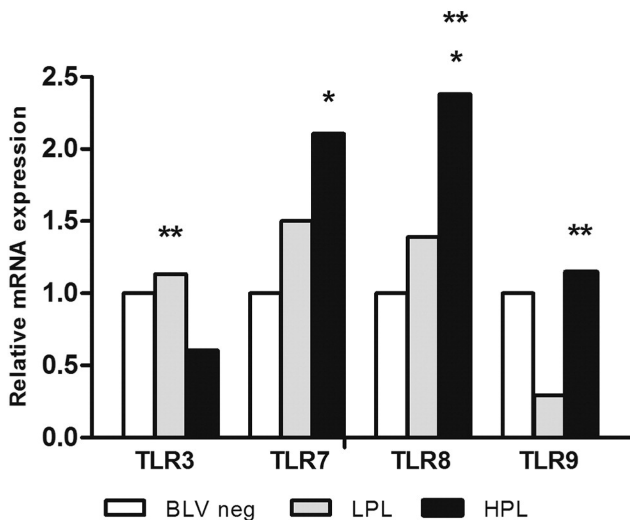


**Fig. 3.** Relative expression levels of TLRs in PBMC from BLV-uninfected (BLV neg.) and BLV-infected animals with load proviral load (LPL) and high proviral load (HPL). One asterisk (*) indicates statistically significant differences ($p < 0.05$) in relation to uninfected cattle; two asterisks (**) indicate statistically significant differences ($p < 0.05$) between both LPL and HPL groups.

in HTLV-infected patients (Bangham et al., 2009; Vine et al., 2004), this cell-mediated immune response could be determinant for the development of LPL or HPL. In response to BLV infection, several cytokines are deregulated, in particular IL-2, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α, with a prevalence of a Th1 profile (Amills et al., 2004; Amills et al., 2002; Kabeya et al., 2001; Keefe et al., 1997; Meirom et al., 1997; Pyeon et al., 1996; Pyeon and Splitter, 1998, 1999; Yakobson et al., 2000). In all these studies, authors compared cytokine expression in PL or non-PL infected cattle. In our work, we made an attempt to correlate the differences in the expression of Th1 cytokines IL-2 or IFN-γ with the development and maintenance of a particular proviral load profile. As it was described previously (Keefe et al., 1997; Pyeon and Splitter, 1998; Yakobson et al., 2000), BLV-infected animals had a strong response associated to the expression of these cytokines. This finding was not unexpected, due to the stimulation of the immune system by an intracellular pathogen like BLV. In fact, IL-12 is an immune key factor produced by activated macrophages and dendritic cells, which promotes the polarization of CD4+ T cells towards Th1 and its proliferation, as well as NK cells proliferation, and the IFN-γ production by activated CD8+ T cells and NK cells (Hamza et al., 2010). While a slight increase in IL-12 expression was detected in HPL animals, we could not find statistical differences between HPL and LPL groups. Based on the model that viral replication permanently occurs in few infected cells (Florins et al., 2008), in BLV established infection antigen-presenting cells would be continuously activated and would secrete IL-12. On the other hand, IFN-γ expression, a strong indicator of cellular immunity, was significantly higher in animals with LPL. This finding reinforces the idea of the protective role of IFN-γ in the pathogenesis of BLV infection. Indeed, it has been shown an inverse correlation between transcripts of this cytokine and BLV proviral load in experimentally inoculated sheep (Usui et al., 2007). This suggest that an efficient induction of cell-mediated immunity against the virus may be necessary to suppress viral propagation.

The main source of IFN-γ are activated T cells, including $\gamma\delta^+$ T cells, and NK cells. $\gamma\delta^+$ T cells are found in large proportions in cattle; they may comprise up to 60% of circulating lymphocytes in calves and decrease in adults up to 8–18% (Baldwin and Telfer, 2015). They can recognize antigens in their native form without MHC class I or II presentation (Baldwin and Telfer, 2015) and they have been shown to mount cytotoxic, cytokines and proliferative responses in several viral infections in cattle (Amadori et al., 1995; Bukowski et al., 1994; Silflow et al., 2005; Toka et al., 2011). $\gamma\delta^+$ T lymphocytes play a crucial role against intracellular pathogens, linking innate and specific immune responses (Baldwin and Telfer, 2015; Pollock and Welsh, 2002), and even a subpopulation of them can act as immune regulatory cells (Hoek et al., 2009). Concerning BLV, Lundberg et al. reported that the non-PL state in most infected cattle correlates with larger proportion of $\gamma\delta^+$ T cells exhibiting more efficient cytotoxic activity against antigens of the viral envelope, without interaction with molecules of the MHC (Lundberg and Splitter, 2000). The increased levels of IFN-γ in LPL cattle detected in our research, together with other findings from the literature (Usui et al., 2007), might suggest that $\gamma\delta^+$ T lymphocytes are involved in the cellular immune response against replication of BLV. In fact, administration of recombinant IFN-γ in BLV-infected animals increased the number of $\gamma\delta^+$ T cells and suppressed viral growth (Murakami et al., 2004).

The outcome of virus-mediated PRR activation can range from an antiviral response that efficiently clears the infection, to the establishment of a cellular environment that favors viral replication and spread. Viruses possess diverse PAMPs acting as immunostimulatory nucleotides, and host cells have multiple viral nucleotide sensors (Mogensen, 2009). Expression of TLRs in several cell types were mainly described in human and mice (Chang, 2010; Zarember and Godowski, 2002), as well as their modulation in response against pathogens (Boo and Yang, 2010; Chang, 2010). Even though distribution of TLR expression has been described in different tissues of domestic animal species

(Menzies and Ingham, 2006; Turin and Riva, 2008), little is known about ex vivo expression of viral TLRs in bovine cells or tissues during infection. The results presented here demonstrate for the first time the mRNA expression of viral TLRs in freshly PBMC isolated from BLV-infected and BLV-uninfected cattle. An increase in the expression of TLR3 and TLR 7–9 was detected in BLV infected cells. Similar results were obtained during infection by bovine herpesvirus type 1 and 5 (BoHV-1 and -5) in the bovine nervous system (Marin et al., 2014b), and particularly an up-regulation of these TLRs in respiratory tissues during herpesvirus reactivation (Marin et al., 2016). Zhang et al. demonstrated increased expression of TLR3 and TLR4 in nasal-associated lymphoid tissue during the acute stage of infection with foot-and-mouth disease virus (FMDV) (Zhang et al., 2006). In our work, TLR7 had the highest expression in infected animals, showing statistical significance in cattle with HPL. TLR8 expression was also significantly elevated in response to BLV infection. Given that ssRNA present inside the virion can be sensed by TLR7/8, our results might represent an evidence of involvement of these receptors in recognition of BLV genome inside PBMC cytoplasm. Moreover, other authors demonstrated that agonists of TLR7/8 impaired retrovirus (Mogensen et al., 2010; Schlaepfer et al., 2006) and herpesvirus (Marin et al., 2014a) replication.

Higher expression of TLR3 was observed in cattle with LPL than cattle with HPL, whereas PBMC from animals with HPL expressed higher levels of TLR8 and TLR9. However, significant variation in TLR7 expression was not found in PBMC from animals with HPL and LPL. Differential TLRs expression in PBMC related to viral load in retroviral infection was slightly addressed. Lester et al. reported that TLR expression levels correlated with the plasma HIV-RNA load in chronic viremic untreated HIV-1 individuals. Augmented mRNA expression of TLR 2, 3, 4, 6, 7 and 8 was observed in their PBMC, as well as inflammatory responsiveness to TLR ligands, denoting exacerbated immune activation (Lester et al., 2008). Substantial changes in TLRs expression in chronic HIV-1 patients who failed to respond to antiretroviral therapy, when compared with responder patients or healthy donors, were also reported, but with lower expression of TLR3 and TLR7 in patients with higher viral load (Scagnolari et al., 2009). Such discrepancy between studies could be related to the time after HIV-1 infection or antiretroviral treatment, but the reasons remain unclear. Continuous retroviral replication may constantly provide ligands for TLR that stimulates immune responses. The scenario in BLV infection might be similar in BLV-infected animals with HPL.

Different recognition mechanisms by PRR and immune strategies may exist depending on the involved cell type. Indeed, among human leucocytes, professional phagocytes express the greater variety of TLRs mRNAs (Zarember and Godowski, 2002). Our study was performed in total PMBC. Few studies addressed the profile of TLRs expression in different subsets of bovine PMBC. Presence of TLR1-10 (with the exception of TLR9) was demonstrated in monocytes and monocyte-derived macrophages (Guo et al., 2009); while Hedges et al. showed that bovine $\gamma\delta^{+}$ T cells express TLR1, 2, 3, 4, 5, 8 and 9 (Hedges et al., 2005). Moreover, after in vitro culture and viral infection, several authors demonstrated changes in TLRs expression in whole PBMC (Gaikwad et al., 2012; Lee et al., 2008; Marin et al., 2014a), macrophages (Franchini et al., 2006) or $\gamma\delta^{+}$ T cells (McGill et al., 2013). Our results on PMBC from animals with LPL or with HPL could have a "dilution effect" and further information could be derived by analyzing how different subpopulations of immune cells express specific transcripts for TLRs. This may help to clarify their role in response to BLV and consequences on viral dissemination. We could hypothesize that better stimulation of IFN-γ-producer cells (like activated macrophages, $\gamma\delta^{+}$ T cells, $CD8^{+}$ lymphocytes and/or NK cells) might occur in animals developing LPL, via recognition of viral components by proper TLRs.

Understanding the mechanisms which govern the control of viral dissemination will be desirable for the design of effective therapeutic or preventive strategies for BLV. It seems essential to unravel the cellular immune response triggered in animals that develop and maintain LPL. Future use of TLR agonists in the control of viral dissemination or as vaccine adjuvants must be considered.

## 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.

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