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ALICE MARIA MELO DO NASCIMENTO

A INFECÇÃO DO VÍRUS DA LEUCEMIA BOVINA PROLONGA A IMUNOSSUPRESSÃO EM VACAS LEITEIRAS DURANTE O PERÍODO PERIPARTURIENTE PELA PERSISTÊNCIA DA ALTA EXPRESSÃO DE PONTOS DE CONTROLE IMUNOLÓGICOS EM CÉLULAS T

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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Federal da Paraíba, como requisito parcial à obtenção do título de Mestre em Ciência Animal

Orientador: Prof. Dr. Fernando Nogueira Souza.

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“Rivers do not drink their own water; trees do not eat their own fruit; the sun doesn’t shine on itself and flowers do not spread their fragrance for themselves. Living for others is a rule of nature. We are all born to help each other. No matter how difficult it is… Life is good when you are happy; but much better when others are happy because of you.”

Pope Francis

RESUMO

O vírus da leucemia bovina (BLV) é causado por um deltaretrovírus e tem sido associado à imunossupressão, bem como a comorbidades como a mastite bovina, a doença mais onerosa no setor de laticínios. No entanto, nenhum estudo anterior explorou o efeito imunossupressor sinérgico do período periparturiente com uma doença viral imunossupressora como o BLV. Assim, nosso estudo explorou o efeito da infecção pelo BLV no período periparturiente na expressão de PD-1 e CTLA-4 nos linfócitos T sanguíneos, e o impacto da infecção pelo BLV na taxa de novas infecções intramamárias durante o início da lactação. Aqui, descobrimos que vacas leiteiras infectadas com BLV sempre tiveram uma expressão estatisticamente significativa de CTLA-4 e PD-1 nas células T do sangue. Além disso, a infecção por BLV prolonga a imunossupressão em vacas leiteiras durante o período periparturiente, sustentando maior expressão de pontos de controle imunológicos nas células T. Além disso, vacas leiteiras infectadas pelo BLV têm uma taxa mais alta de novas infecções intramamárias durante o início da lactação. Assim, nosso estudo fornece novas informações sobre o efeito imunossupressor do BLV no período mais crítico da vida das vacas, com efeito prejudicial marcante na imunidade protetora das células T e comorbidades, como a mastite bovina.

Palavras-Chave: PD-1; CTLA-4; deltaretovírus; período de transição; mastite; gado de leite.

ABSTRACT

Bovine leukemia virus (BLV) is caused by a deltaretrovirus and has been associated with immunosuppression as well as comorbidities such as bovine mastitis, the costliest disease in the dairy sector. However, no previous study has explored at the synergistic immunosuppressive effect of the peripartum period with an immunosuppressive viral disease such as BLV. Thus, our study explored the effect of BLV infection in the periparturient period on the expression of PD-1 and CTLA-4 in blood T lymphocytes, and the impact of BLV infection on the rate of new intramammary infections during the early lactation. Here, we found that BLV-infected dairy cows always had a statistically significant higher expression of CTLA-4 and PD-1 in blood T cells. Furthermore, BLV infection prolongs immunosuppression in dairy cows during the periparturient period by sustaining higher expression of immunological checkpoints in T cells. In addition, BLV-infected dairy cows have a higher rate of new intramammary infections during early lactation. Thus, our study provides new insights of the immunosuppressive effect of BLV on the most critical period of the cows' life with marked detrimental effect on protective T-cell immunity and comorbidities, such as bovine mastitis.

Keywords: PD-1; CTLA-4; deltaretovirus; transition period; mastitis; cattle.

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1 INITIAL CONSIDERATIONS

The three weeks prior to calving through the next three weeks after parturition are widely known as the periparturient period. According to Bell (1995), this period is characterized by several orchestrated metabolic and endocrine changes due to the increased nutrient demands that aim to support milk production.

Dairy cows' immune systems are weakened during the parturition period, making them more vulnerable to diseases. This period is crucial for health, productivity, and profitability, but due to hormonal changes brought on by pregnancy and the significant increase in milk production, the animal is under stress at this time, such as immunity depression, negative energy balance, and low dry matter intake due to pregnancy, and to maintain the normal metabolism and productive efficiency of dairy cows. Additionally, a negative energy balance typically develops because the demand for nutrients to support milk production rises throughout the periparturient period by a greater amount than the supply (Khan *et al.*, 2020). The negative energy balance is indicated by high levels of non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHBA), which impair immune cell functions (Lacetera et al., 2014).

It is widely accepted that the periparturient period is when dairy cattle are most susceptible to a significant increase in metabolic and production-related disorders like mastitis, metritis, milk fever, and ketosis (Bertoni *et al.*, 2008; Itle *et al.*, 2015; Khan *et al.*, 2020). These disorders can also occur secondary to other immunosuppressive diseases in this critical period, such as Bovine Leukemia Virus (BLV).

The ramifications of BLV infections for mammary gland immunity, primarily supported by the malfunctioning of the milk neutrophils, are discussed by Della Libera *et al.* (2015). However, due to their indirect impacts, such as the already mentioned secondary diseases, it is essential to emphasize the significance of controlling BLV infections.

Inhibitory receptors known as immune checkpoint molecules are expressed on immune cells and start immunosuppressive signaling cascades. Effector immune cells, particularly T cells, can experience a condition described as "exhaustion" as a result of signaling from these molecules. Diminished effector function, persistent expression of immunological checkpoint molecules [such as programmed death-1 (PD-1) and

cytotoxic T-lymphocyte antigen (CTLA-4)], and diminished T cell activity are the characteristics of T cell exhaustion (Wykes *et al.*, 2018). The receptors PD-1 and CTLA-4 contribute to T-cell exhaustion caused by BLV infection in cattle (Okagawa *et al.*, 2018; Suzuki et al., 2015).

In their study, Souza *et al.* (2022) revealed greater levels of CTLA-4 and PD-1 expression in T cells at parturition and during the prepartum period, which may suggest a connection between these immune checkpoints and immunological tolerance in dairy cows during gestation. Furthermore, a higher expression of these immune checkpoint molecules was associated with reproductive health during the postpartum period in dairy cows. Therefore, in this work, we proceeded with the hypothesis that by analyzing these immunological checkpoints and linking the data with the periparturient period, we could set the aim of establishing a relationship between their measurement and the rate of BLV in two herds from Paraíba, Brazil.

2 CHAPTER I – LITERATURE REVIEW

2.1 PERIPARTURIENT PERIOD

The transition period is the most challenging time a dairy cow faces in her lifetime because of the changes in physiological conditions that occur as they move from the dry and pregnant to the nonpregnant and lactating stage. Compared to earlier lactation cycle stages, this is when animals experience most of their health problem episodes. Hence, animals are more vulnerable to various metabolic and infectious disorders when these nutrients and metabolites are in insufficient levels (Keshri et al., 2019). In addition to having a negative influence on the health of the animals, diseases at this stage have a significant financial impact on dairy farms since, aside from the cost of treatment, sick cows won't produce their maximum amount of milk (Abuelo et al., 2014).

It consequently is not surprising that in herds, about 75% of disease incidence (mastitis, metritis, ketosis, displacements of the abomasum, etc.) occurs within the first month of lactation (LeBlanc et al., 2006), with the time frame with the highest risk of infectious and metabolic disorders being the first 10 days following calving, which also coincides with the milk yield's most extraordinary increasing (Ingvartsen et al., 2003; Abuelo et al., 2014).

In this regard, the cumulative effects of numerous stressors resulting from nutritional deficiency, parturition, moving to the milking herd, and herd management practices during the periparturient period can both increase and prolong the magnitude of immunosuppression, increasing susceptibility to diseases and negatively affecting the animal's capacity to overcome illness and recover (Aleri et al., 2016). Due to the fact that the effects of production diseases on dairy cow health and productivity persist thoroughly into the subsequent lactation, this period is linked to an increased incidence of metabolic and infectious diseases (Abuelo et al., 2014).

2.2 BOVINE LEUKEMIA VIRUS

The primate T-lymphotropic virus types 1–5 (HTLV-1 to HTLV-4) are genetically and structurally related to the bovine leukemia virus (BLV), a member of the Retroviridae family and the Deltaretrovirus genus. BLV is one of the most common livestock infections in many countries, especially in dairy herds. (Della Libera et al., 2015; Blagitz et al., 2017; Gillet et al., 2007). Both viral and non-viral causes can contribute to bovine leukemia. Calf type, thymic type, and cutaneous type of non-viral bovine leukemia are three subtypes with unknown etiology. However, most instances of bovine leukemia are caused by enzootic bovine leukemia, which BLV spreads, and its frequency is rising (Konnai et al., 2017).

Hematogenous transfer of infected B cells appears to be the primary method of BLV transmission in cattle, allowing the virus to infect B cells in the new host. The newly infected animal generates a robust immune response within a few weeks, preventing infection of other B cells and leading the virus to become latent and only replicate through B cell mitosis (Norby et al., 2015). BLV infections are known as aleukemic (AL) and typically show no symptoms. On the other hand, B cell lymphoma occurs less frequently in persistent lymphocytosis (PL), which occurs in 20–30% of infected cattle and is characterized by non-malignant polyclonal proliferation of B cells (Gillet et al., 2007). After a long latent period, less than 5% of infected cattle develop malignant B-cell lymphoma in various lymph nodes, so-called enzootic bovine leukosis (EBL) (Okagawa et al., 2018; Gillet et al., 2007).

Ott et al. (2003) assessed the direct losses caused by clinical BLV infections to the dairy sector and consumers to be more than $500 million annually, and Rhodes et al. (2003) estimated the economic losses caused by lymphoma to be $412 per case. However, the overall impact of some chronic diseases with low lethality rates, such as BLV, is usually neglected, as the association of BLV infection with comorbidities are underestimated (Della Libera et al., 2015). This BLV-related phenomenon can make animals more susceptible to different coinfections and make the disease more severe, reducing cow lifetime and raising the culling rate (Blagitz et al., 2017).

2.3 MASTITIS AND THEIR ASSOCIATION WITH BLV

The most prevalent condition affecting dairy cattle is bovine mastitis, an mammary gland infection that results in decreased milk production and poor milk

quality. A wide range of Gram-positive and Gram-negative bacteria, including contagious bacteria like Staphylococcus aureus, Streptococcus agalactiae, and Mycoplasma spp., as well as environmental bacteria like Escherichia coli, Enterococcus spp., non-aureus Staphylococcus, and Streptococcus uberis, are among the most common etiological agents (Cheng and Han, 2020). This intramammary inflammation is still a devastating condition in dairy animals, negatively affecting both animal welfare and the dairy industry's economy due to poor milk production performance and higher culling rates (Sharun et al., 2020).

We can additionally highlight Norby et al. (2015) findings alongside the previously mentioned causes that the BLV infection can be associated with decreased milk production in dairy cows. Della Libera et al. (2015) have also emphasized the higher susceptibility of BLV-infected cows to secondary diseases, such as mastitis, the most expensive disease afflicting cattle, which is one example of how important it is to control BLV infections. According to the lactation stage and parity, a BLV infection may lower milk production while raising somatic cell counts (Yang et al., 2016). Thus, it is evident that BLV infection can affect macrophages' ability to phagocytose mastitis-causing organisms, such as Staphylococcus aureus, and may increase an infection's susceptibility or lessen its ability to remove an intramammary infection (spontaneous cure) (Souza Lima et al., 2021). Given that macrophages are the major population seen in milk from healthy mammary glands and are the first defense cells to come into touch with the virus that causes mastitis, these findings prompted concerns regarding the impact of BLV on mammary gland immunity (Miller et al., 1991; Souza Lima et al., 2021.

These results are also shown by Watanabe et al. (2019), whose findings indicated that proviral load (PVL) and season are related to the severity of clinical mastitis and that cows with high BLV proviral load (H-PVL) had less immunological function in their mammary glands than cows with low BLV proviral load (L-PVL). Cuesta et al. (2019) also showed that mammary epithelial cells infected with BLV had altered the apoptotic and immune pathways, probably affecting their response to bacteria and favoring the development of mastitis.

2.4 T LYMPHOCYTES

A major issue in immunology that has consequences for autoimmune disease, pathogen immunity, and immunotherapy is how an effector T cell response's intensity is regulated. The sum of the independent signals from the antigen, co-stimulation, and cytokines determines the initial amplitude of the T-cell response (Dowling *et al.*, 2018).

T cells, which include $CD8^+$ and $CD4^+$ (T helper, Th) T cells, are lymphocyte subsets. Th cells can also be divided into Th1, Th2, Th17, and T regulatory cells (Treg). The effectiveness of immune responses essential for maintaining homeostasis and an organism's health is determined by the balance between pro-inflammatory Th17 cells and anti-inflammatory Treg cells (Li *et al.*, 2022). Therefore, while creating age- and herd-specific preventative and therapeutic methods, it is important to consider a combination of common and distinctive factors that drive the bovine adaptive immune responses (Vlasova and Saif, 2021).

The mammary gland's cellular and humoral immunity is primarily mediated by T and B lymphocytes, which can eliminate infections by direct cytotoxicity, produce antibodies and cytokines, activate macrophages, and recruit neutrophils into the body. In addition, the cow mammary gland's defense is aided by the production of vital cytokines by lymphocytes, including IL-17A and IFN-γ, for instance (Souza *et al.*, 2023).

2.5 IMMUNE CHECKPOINTS

Recent research has established that antigen-specific T-cell dysfunction, also known as T-cell exhaustion, is characterized by the loss of effector capabilities during persistent infections and malignancies in humans. The phenotypic characteristics of exhausted T cells include the surface expression of immunoinhibitory receptors such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1) (Konnai *et al.*, 2017).

Functional inhibition of immunosuppressive factors is a promising method to reactivate immune cells in a state of tolerance and is anticipated to be useful as a new therapeutic strategy for persistent infections or tumor disorders or as a method to

improve the efficiency of vaccinations, as evidenced by the recent success of immune checkpoint inhibition in cancer therapy (Wykes and Lewin, 2018; Konnai *et al.*, 2017). In cattle infected with BLV, for instance, CTLA-4 was once linked to the progression of the disease, suggesting that an anti-bovine CTLA-4 antibody can reawaken lymphocyte functions and be used as a new treatment for chronic illnesses that have failed to respond to other treatments (Watari *et al.*, 2019).

Moreover, PD-1 is a well-known immunoinhibitory receptor that supports infections like the bovine leukemia virus (BLV) infection and different tumor cells to evade the immune system (Ikebuchi *et al.*, 2013). Therefore, it is crucial to suppress persistently activated T cells specific for pathogens in various forms of chronic infections and malignancies, such as BLV infection in cattle, leading to an accumulation of "exhausted" T cells (Okagawa *et al.*, 2018). Furthermore, inhibiting the PD-1 pathway with a PD-L1 antibody improves immune responses and cytokine production, which lowers viral load (Konnai *et al.*, 2017).

It is anticipated that PD-1 will be a promising target for reviving the function of exhausted T cells in veterinary medicine since nowadays, for various human cancers, antibodies that target PD-1 (Pembrolizumab; Nivolumab), CTLA4 (ipilimumab), and PD-L1 (atezolizumab; avelumab) are now approved as monotherapies (Wykes and Lewin, 2018). Furthermore, in their research, Ikebuchi *et al.* (2013) show that anti-PD-1 monoclonal antibodies could be applied to new therapy targets for many types of infection in cattle via upregulation of immune responses.

As significantly shown in Souza Lima *et al.* (2021) study, prepartum T cells from dairy cows expressed greater levels of CTLA-4 and PD-1, suggesting that these molecules play a significant role in maintaining the maternal immunological tolerance that develops throughout pregnancy, as has been observed previously in humans (Andrikopoulou *et al.*, 2021) and rats (Tiam *et al.*, 2016). In addition, anti-programmed death ligand 1 (PD-L1), anti-PD-1, or anti-CTLA-4 monoclonal antibodies can reverse the suppression of T cells caused by these immunological checkpoints, offering fresh possibilities for the treatment and prevention of disease. Therefore, functional inhibition of immunosuppressive factors constitutes a workable instrument to reactivate immune cells in a state of tolerance, offering a new treatment strategy for persistent infections and tumor illnesses or as a method to boost the effectiveness of vaccinations.

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2 CAPÍTULO II – Short communication[1]: THE BOVINE LEUKEMIA VIRUS INFECTION PROLONGS IMMUNOSUPPRESSION IN DAIRY COWS DURING THE PERIPARTURIENT PERIOD BY SUSTAINING HIGHER EXPRESSION OF IMMUNOLOGICAL CHECKPOINTS IN T CELLS[1]

3.1 ABSTRACT

Bovine leukemia virus (BLV) is caused by a deltaretrovirus and has been associated with immunosuppression as well as comorbidities such as bovine mastitis, the costliest disease in the dairy sector. However, no previous study has explored at the synergistic immunosuppressive effect of the peripartum period with an immunosuppressive viral disease such as BLV. Thus, our study explored the effect of BLV infection in the periparturient period on the expression of PD-1 and CTLA-4 in blood T lymphocytes, and the impact of BLV infection on the rate of new intramammary infections during the early lactation. Here, we found that BLV-infected dairy cows always had a statistically significant higher expression of CTLA-4 and PD-1 in blood T cells. Furthermore, BLV infection prolongs immunosuppression in dairy cows during the periparturient period by sustaining higher expression of immunological checkpoints in T cells. In addition, BLV-infected dairy cows have a higher rate of new intramammary infections during early lactation. Thus, our study provides new insights of the immunosuppressive effect of BLV on the most critical period of the cows' life with marked detrimental effect on protective T-cell immunity and comorbidities, such as bovine mastitis.

Key-words: PD-1; CTLA-4; deltaretovirus; transition period; mastitis; cattle.

3.2 INTRODUCTION

[1] Artigo submetido à revista Veterinary Immunology and Immunopathology

The bovine leukemia virus (BLV) infection is caused by a deltaretrovirus, and it is one of the most widespread bovine diseases in several countries. It has been associated with immunosuppression and comorbidities in cattle (Della Libera et al., 2015; Blagitz et al., 2017; Souza Lima et al., 2021), resulting in a significant economic impact on the cattle industry (Nakada et al., 2023). For instance, BLV has a negative impact on udder health (Yoshikawa et al., 1997; Della Libera et al., 2015; Norby et al., 2016; Watanabe et al., 2019; Souza Lima et al., 2021).

In the last decade, the expression of immune checkpoints molecules, such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), has been clearly associated with immunosuppression during BLV infection (Okagawa et al., 2017; Okagawa et al., 2018; Sajiki et al., 2019; Ikebuchi et al., 2013; Nakamura et al., 2023). Indeed, T-cell exhaustion, which is marked by the expression of the immune checkpoint molecules, is an important hallmark of chronic infections, as it hampers cell-mediated protective immunity (Dyck et al., 2017). Thus, a promising therapeutic method for BLV infection has been emerged by the blockade of the immune checkpoints, which has a substantial positive effect on immunity restoration (Nakamura et al., 2023).

In dairy cows, the periparturient period is also defined by immunosuppression, corresponding to the most critical period of the cow's life (Aleri et al., 2016). Nonetheless, no previous study has investigated the synergistic immunosuppressive effect of the periparturient period and an immunosuppressive viral disease, such as BLV. Thus, our study explored: 1) the effect of BLV infection in the periparturient period on the expression of PD-1 and CTLA-4 in blood T lymphocytes, and 2) the impact of BLV infection on the rate of new intramammary infections during the early lactation.

3.3 MATERIALS AND METHODS

The present study was approved by the Animal Research Ethics Committee of the Federal University of Paraíba, Brazil (protocol number: 4595011020).

3.3.1 Herds, Animals and Experimental Design

In this study, we used 10 Guzerá (Herd A) and 10 Girolando (Herd B) clinically healthy dairy cows, including 4 primiparous and 16 pluriparous dairy cows. The Guzerá cows were kept under Massai grass pastures a natural hybrid between Panicum maximum and Panicum infestum, and were supplemented with vitamins and minerals as well as soybean meal, corn meal, and cottonseed meal and cake as concentrates based on their milk production. The calves were kept half the day with their dams since the high weight of the calves was prioritized due to their high economic value. These zebu dairy cows were milked once daily by manual milking with the calf at foot and produced an estimated average of 20 kg milk/day. In this herd, both natural and artificial insemination were used.

The Girolando dairy cows were kept on Mombaça grass (Panicum maximum) pasture for a portion of the day (in the morning), and they also received vitamins and minerals, as well as palm roughage and soybean, corn, and cottonseed meal concentrates based on their milk production. All of the dairy cows in this herd were artificially inseminated and were milked twice daily using a machine. They produced an average of 27 kg milk/day.

Peripheral blood samples were aseptically collected into vacutainer® tubes containing sodium heparin (cat. n. 367871, BD Biosciences, New Jersey, USA) from the jugular vein at 14 days prior to conception (T-14), at calving (T0), and 30 days postpartum (T30) to determine the expression of PD-1 and CTLA-4 in blood T lymphocytes, and in tubes without anticoagulant for the serological diagnosis of BLV. Furthermore, udder quarter milk samples were collected from all dairy cows at parturition, and 3, 7, 15, and 30 days after parturition for microbiological analysis to diagnose intramammary infections.

3.3.2 Serological diagnosis of BLV

The serum blood samples were obtained by centrifugation at 2,500 x g for 10 min at room temperature. The serological diagnosis of BLV were assessed by IDEXX®

Leukosis serum X2 Ab test using the glycoprotein gp51 as the antigen (IDEXX®, Hoofddorp, Netherlands).

3.2.3 Milk sampling

First, the strip cup test was performed to determine the presence of clinical mastitis. After discarding the first three milk streams, the teats apices were scrubbed with 70% ethanol using cotton balls, and milk samples from the individual udder quarters were aseptically collected for microbiological analysis.

3.2.4 Microbiological analyses

The bacteriological analysis of the milk sample was performed by cultivating 10 µL on 5% defibrinated sheep blood agar plates, which were incubated at 37 °C for 24 to 72 h (Oliver et al., 2004). The bacterial identification was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, as previously described (Braga et al., 2018).

3.2.5 Definition of new intramammary infections (IMIs)

A new IMI was defined as a quarter without an IMI initially but had an IMI in the subsequent milk sampling (Reyher et al., 2013) or a distinct pathogen was detected in an udder quarter.

3.2.6 Expression of PD-1 and CTLA-4 in the T lymphocytes

The expression of PD-1 and CTLA-4 in T lymphocytes were performed as previously described by our research group (Souza et al., 2022). Briefly, peripheral venous blood mononuclear cells (PBMCs) were isolated using Ficoll-PaqueTM PLUS density gradient (GE Healthcare, Darmstadt, Germany). To identify T lymphocytes (CD3+) and the immune checkpoints PD-1 and CTLA-4, the following monoclonal antibodies (mAbs) were used: tube A) 1 µL of the primary mouse anti-bovine IgG1 antibody CD3 (clone MM1A, cat. n. BOV 2009, Washington State University Monoclonal Antibody Center, USA), and 1 µL of the goat anti-human PD-1 with cross-reactivity to cattle (diluted 1/10 in PBS with 1% heat-sterilized inactivated fetal bovine serum and 0.09% sodium azide; cat. n. LS-C55247-100, LSBIO, USA), and tube B) 1 µL of the primary mouse anti-bovine IgG1 CD3 (clone MM1A, cat. n. BOV 2009, Washington State University Monoclonal Antibody Center, USA) and 1 µL of the goat anti-human CTLA-4 crossreactive with cattle (diluted 1/10 in PBS with 1% heat-inactivated sterile-filtered fetal bovine serum and 0.09% sodium azide; cat. n. AF-386-PB, R&D Systems, USA). The PBMCs were incubated with these mAbs for 30 min at room temperature in two 5-mL round-bottom polypropylene tubes (A and B), suitable for flow cytometry. Following the incubation time, the cells were washed with 1 mL PBS, and centrifuged at 250 x g for 8 min at 4 °C, the supernatant was removed, and the cells were resuspended in 100 µL of PBS. Afterward, the 0.5 µL of the secondary goat anti-mouse IgG1-conjugated secondary antibodies conjugated with PE-Texas Red and 1 µL of the crossadsorbed donkey IgG conjugated with Alexa Fluor 488 (cat. No. A11055, Thermo Fisher, Carlsbad, USA) were added. Following the incubation time, the cells were washed with 1 mL PBS, and centrifuged at 250 x g for 8 min at 4 °C, the supernatant was removed, and the cells were resuspended in 300 µL of PBS containing 1% heat-inactivated fetal bovine serum. The samples were analyzed by BD FACSCantoTM II flow cytometer (BD Biosciences, New Jersey, USA). In this study, 10,000 cells from each sample were analyzed. As compensation controls, single-stained samples, isotype controls, secondary antibody controls, and unstained controls were also prepared and used.

The data were analyzed using Flow Jo Tree Star software (FlowJo - Treestar 10.5.3 for Windows, Tree Star Inc., Ashland, USA). In contrast to the percentage of positive cells, the relative geometric mean fluorescence intensity (GMFI) was chosen since it was much more selective. The number of receptors per cell is determined by

the GMFI, which precisely quantifies the brightness of the stained cells (Della Libera et al., 2015).

3.2.7 Statistical analysis

Initially, data distribution was initially determined using the Shapiro-Wilk test. The three-way repeated measures ANOVA followed by the Student-Newman-Keuls test was used to assessed the effect of sampling time, parity and farm/breed. The unpaired-T test was used to compare BLV-infected and BLV-uninfected dairy cows. The Kaplan-Meier survival curve was used to distinguish the rate of new IMIs in BLV-infected and -uninfected dairy cows. Statistical analysis was performed using the GraphPad Prism 9.4.1 (GraphPad Software, Inc., USA). The P value was set as $P < 0.05$.

4 RESULTS AND DISCUSSION

Here, we identified 7 BLV serologically positive dairy cows and 13 BLV serologically negative dairy cows. No effect of herd and parity (primiparous vs. pluriparous) were observed. In the present study, BLV-infected dairy cows always had a statistically significant higher expression of CTLA-4 (Fig. 1) and PD-1 (Fig. 2) in T cells. In agreement with our findings, Suzuki et al. (2015) and Okagawa et al. (2017) who found that in BLV infection led to T-cell exhaustion mediated by CTLA-4 and PD-1/PD-L1, respectively, which probably promotes BLV persistent infection and disease development.

Furthermore, the expression of CTLA-4 markedly decreased from the prepartum period (T-14) compared to partum (T0) and 30 days after parturition (T30) (Fig. 1) in BLV-uninfected dairy cows, but this phenomenon was not observed in BLV-infected dairy cows ($P = 0.12$). Besides that, the expression of PD-1 significantly decreased from T-14 to T30 (Fig. 1) in BLV-uninfected dairy cows, but this phenomenon was also not found in BLV-infected dairy cows ($P = 0.27$). Thus, our data indicated that BLV

infection causes a profound T cell exhaustion during the periparturient period and prolongs immunosuppression in dairy cows during the periparturient period by sustaining higher expression of immunological checkpoints in T cells beyond this critical period of cows' life. In this scenario, T cell exhaustion associated with BLV evasion mechanisms (Okagawa et al., 2018) resulted in poor effector functions and recall responses that could evidently translate to increased susceptibility to infectious diseases (Wykes and Lewin, 2018). Therefore, the use of combined immune checkpoint pathway blocking will contribute to not only the antiviral action against BLV, but also the restoration of T cell-mediated immunity (Nakamura et al., 2023), minimizing the rate of new infectious infections.

Indeed, BLV infection negatively impact the udder health in our study, as BLV-infected dairy cows has a higher rate of new intramammary infections during the first month of lactation ($P = 0.047$; Fig. 3). In this context, the higher expression of the immune checkpoints associated with BLV infection could clearly impact udder health, as T cells are essential for protective mammary gland immunity (Souza et al., 2020; Souza et al., 2023). The bacteriological outcomes of the udder quarter milk samples were showed in Supplemental Table 1.

Thus, our study provides further insights of the immunosuppressive effect of BLV on the most critical period of the cows' life with marked detrimental effect on protective T-cell immunity and comorbidities, such as bovine mastitis. Therefore, immune checkpoints blockade appears as a promising strategy to be used in this period, particularly in BLV-infected dairy cows.

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APÊNDICE A – FIGURES AND SUPPLEMENTAL TABLE

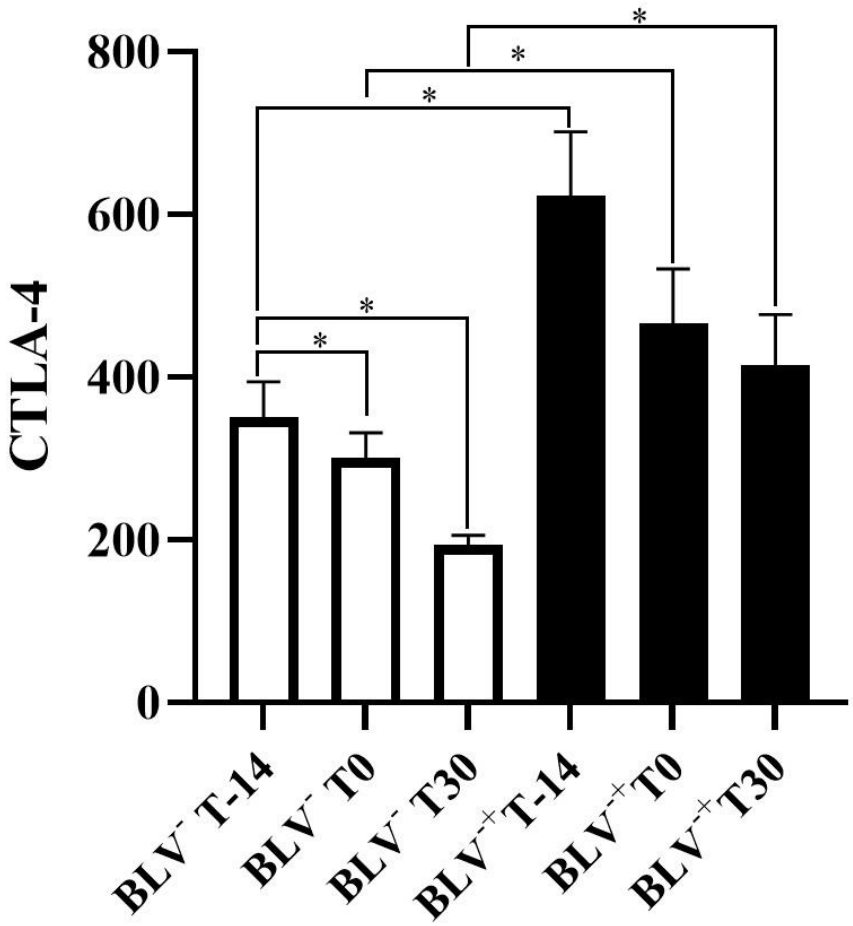

Fig. 1. Expression of CTLA-4 in T lymphocytes in bovine leukemia virus (BLV)-uninfected (BLV^-) and BLV-infected (BLV^+) dairy cows during the periparturient period. Results are expressed as mean ± SEM. CTLA-4: cytotoxic T lymphocyte-associated antigen-4; PD-1: programmed cell death protein 1; T-14 = 14 days before parturition; T0 = day of parturition; T30 = 30 days postpartum. $^*P \leq 0.05$.

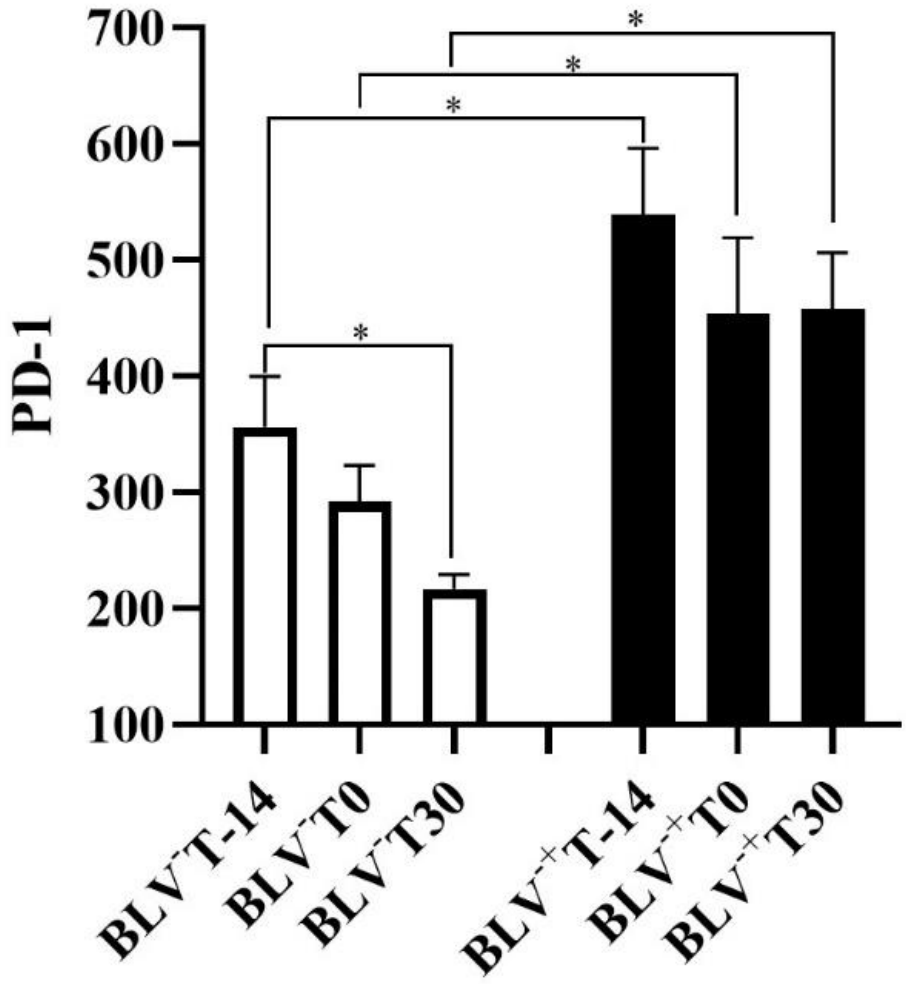

Fig. 2. Expression of PD-1 in T lymphocytes in bovine leukemia virus (BLV)-uninfected (BLV^-) and BLV-infected (BLV^+) dairy cows during the periparturient period. Results are expressed as mean ± SEM. CTLA-4: cytotoxic T lymphocyte-associated antigen-4; PD-1: programmed cell death protein 1; T-14 = 14 days before parturition; T0 = day of parturition; T30 = 30 days postpartum. $^*P \leq 0.05$.

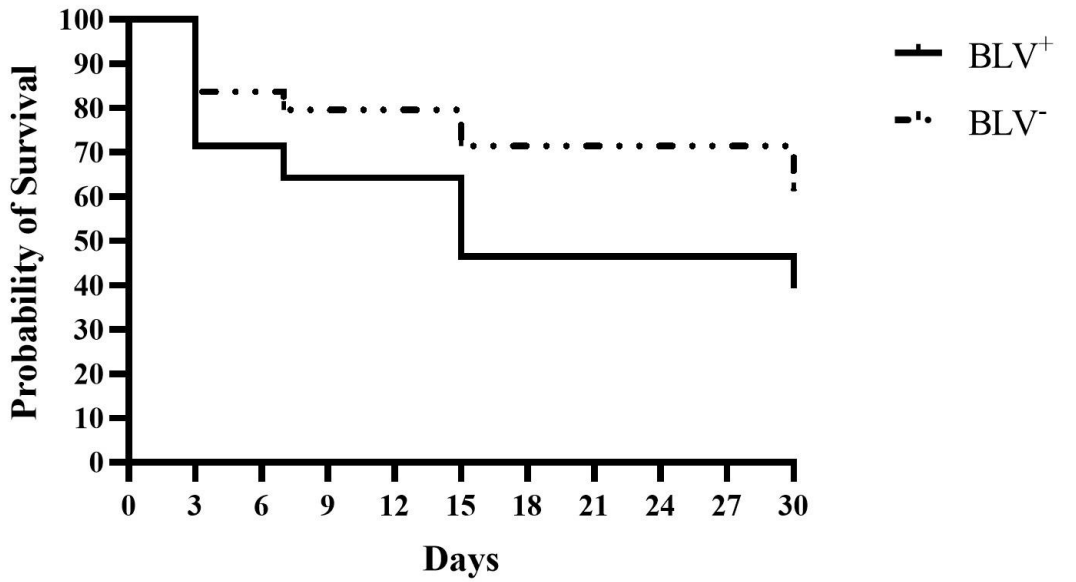

Fig. 3. Survival curve showing the rate of new intramammary infections in udder quarters from bovine leukemia virus (BLV)-uninfected (BLV^-) and BLV-infected (BLV^+) dairy cows during the first month of lactation.

Supplemental Table 1. The bacteriological results of the 520 udder quarter milk samples at distinct times throughout the first month of lactation.

Pathogens isolated	At parturition	3 days after calving	7 days after calving	14 days after calving	30 days after calving
Negative	55 (52.88%)	62 (59.62%)	70 (67.31%)	55 (52.88%)	57 (54.81%)
Staphylococcus chromogenes	22 (21.15%)	23 (22.12%)	20 (19.23%)	26 (25.00%)	20 (19.23%)
Staphylococcus aureus	10 (9.62%)	7 (6.73%)	4 (3.85%)	7 (6.73%)	9 (8.65%)
Staphylococcus hyicus	8 (7.69%)	0 (0.00%)	5 (4.81%)	5 (4.81%)	5 (4.81%)
Corynebacterium ulcerans	4 (3.85%)	5 (4.81%)	1 (0.96%)	4 (3.85%)	6 (5.77%)
Staphylococcus cohnii	2 (1.92%)	1 (0.96%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Streptococcus dysgalactiae	2 (1.93%)	1 (0.96%)	0 (0.00%)	1 (0.96%)	2 (1.92%)
Rothia endophytica	1 (0.96%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Staphylococcus xylosus	0 (0.00%)	3 (2.88%)	3 (2.88%)	2 (1.92%)	0 (0.00%)
Acinetobacter nosocomialis	0 (0.00%)	1 (0.96%)	0 (0.00%)	0 (0.00%)	0 (0.00%)
Staphylococcus capitis	0 (0.00%)	1 (0.96%)	0 (0.00%)	0 (0.00%)	0 (0.00%)

Staphylococcus epidermidis	0 (0.00%)	0 (0.00%)	1 (0.96%)	0 (0.00%)	0 (0.00%)
Pseudomonas stutzeri	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (0.96%)	0 (0.00%)
Streptococcus pluranimalium	0 (0.00%)	0 (0.00%)	0 (0.00%)	2 (1.92%)	3 (2.88%)
Escherichia coli	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (0.96%)	1 (0.96%)
Staphylococcus warneri	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (0.96%)
Trueperella pyogenes	0 (0.00%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	1 (0.96%)