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Research article

TNF-A GENE VARIANTS AND RECEPTOR EXPRESSION ASSOCIATED WITH BLV INFECTION IN COLOMBIAN HOLSTEIN CATTLE

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Bovine leukemia virus (BLV) is a single-stranded RNA retrovirus whose persistence in infected cattle indicates that innate immune responses, particularly antiviral cytokines, are insufficient to control infection. Tumor necrosis factor alpha (TNF-α) is a key proinflammatory cytokine that may contribute to antiviral defense, acting through two membrane-bound receptors: TNF-α receptor I (TNF-α RI) and TNF-α receptor II (TNF-α RII). Both receptors participate in apoptotic and immune-regulatory pathways. This study aimed to investigate the – 824 A/G polymorphism in the promoter region of the TNF-α gene and to quantify TNF-α RI and TNF-α RII transcript levels, assessing their association with proviral load and persistent lymphocytosis (PL) in Holstein cattle. Blood samples from 140 cows were analyzed for TNF-α genotypes and receptor mRNA expression. Data were normalized and analyzed using one-way analysis of variance (ANOVA) to assess differences in gene expression among the experimental groups. The TNF-α G/G genotype was significantly associated with increased odds of BLV infection (p=0.006). TNF-αRI mRNA expression differed significantly between BLV-positive and BLV-negative cows (p=0.0017), whereas TNF-αRII expression showed no differences according to infection status (p=0.999). In BLV-negative animals, TNF-αRI and TNF-αRII expression levels differed significantly, with lower RII expression (p<0.0001); this pattern was not observed in infected cows, regardless of aleukemic or persistent lymphocytosis status. Aleukemic cows exhibited reduced RI and RII expression compared with RI levels in uninfected animals (p<0.05). No significant correlations were detected between TNF-αRI or TNF-αRII expression and PBMC counts or proviral load in infected cattle ($\varrho \leq |0.23|$; p>0.05). The results indicate an association between TNF-α genetic variation, receptor expression patterns, and BLV infection status, suggesting that TNF-α signaling may contribute to the host–virus interaction during BLV infection. Further longitudinal and functional studies are required to clarify the biological mechanisms linking TNF-α regulatory polymorphisms with viral persistence and disease progression.

Keywords: Cytokine, dairy herds, gene expression, SNP, proviral load.

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INTRODUCTION

Bovine leukemia virus (BLV) is a single-stranded RNA (ssRNA) retrovirus that primarily affects dairy cattle. Like other ssRNA viruses, BLV is a poor inducer of interferons (IFNs), which compromises the efficiency of the innate immune response in controlling viral replication and dissemination [1,2]. Once integrated into the host genome, the BLV provirus undergoes cycles of active transcription and latency, with gene silencing mechanisms modulating its expression [3]. Due to the rapid immune-mediated clearance of cells expressing viral antigens, infected animals typically do not exhibit viremia [4].

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine involved in numerous biological processes, including inflammation, apoptosis, and the modulation of both innate and adaptive immune responses. It stimulates chemokine and cytokine release, enhances antigen presentation, and promotes the activation of leukocytes and co-stimulation of T cells. TNF-α may influence viral containment during BLV infection through apoptotic signaling pathways such as the nuclear factor kappa B (NF-ϰB) and caspase cascades. However, its antiviral efficacy can be modulated by two main factors: (1) the presence of the – 824 A/G single nucleotide polymorphism (SNP) in the TNF-α gene promoter and (2) the expression levels of TNF-α receptor I (TNF-α RI) and receptor II (TNF-α RII) [5,6].

The TNF-α gene is regulated at both the promoter and 3' untranslated regions (3'UTR). The – 824 A/G SNP, located within the promoter region, can influence gene transcription, mRNA stability, and translational efficiency of TNF-α protein [6-8]. TNF-α exerts its biological functions through two membrane-bound receptors: TNF-α RI, which is broadly expressed across various tissues, and TNF-α RII, which is more selectively expressed on immune cells [9].

Interestingly, during BLV infection, cattle with a higher resistance to disease progression tend to produce more TNF-α and exhibit lower TNF-α RII expression, thereby preserving TNF-α RI-mediated pro-apoptotic responses. In contrast, susceptible animals often show elevated TNF-α RII transcript levels, reducing the availability of TNF-α RI receptors and potentially favoring viral persistence and lymphoproliferation [5]. This receptor profile may modulate disease outcome, with high TNF-α RI expression associated with enhanced apoptosis of infected cells and reduced proviral load. Accordingly, the objective of this study was to evaluate the – 824 A/G polymorphism in the TNF-α gene promoter, quantify the transcriptional levels of TNF-α RI and TNF-α RII, and determine their relationship with proviral load and persistent lymphocytosis in a Holstein cattle population from Colombia.

MATERIALS AND METHODS

Ethical approval

All animal procedures were conducted in accordance with institutional and national guidelines for the care and use of animals in research. The study protocol was reviewed and approved by the Ethics Committee of the Universidad Nacional de Colombia, Medellín campus (Approval ID: CEMED-022; July 13, 2015).

Study area and sampling

A total of 140 Holstein cows between their first and fifth lactation, ranging in age from 3 to 7 years, were included in the study. The selected cows belong to three specialized dairy systems located in Medellín and Belmira municipalities in the state of Antioquia, Colombia. Sampling was carried out from February to June 2017. For each animal, two peripheral blood samples were collected into 15 mL conical tube three months apart. Each sample consisted of two EDTA-coated tubes and subsequently transported under refrigerated conditions (4°C). Double sampling was performed exclusively to establish persistent lymphocytosis.

DNA and RNA extraction

Blood samples were centrifuged at 3,000 rpm for 4 minutes at 4°C. The *salting-out* technique was used to obtain DNA total [10]. The DNA was suspended in buffer TE 1X pH 8.0 (Tris HCl 1 M and EDTA 0.5 M) and stored at 4°C until analysis. The quality and quantity of the DNA were evaluated in a spectrophotometer (NanoDrop2000®, Massachusetts, United States) and 1% agarose gel.

A 1 mL aliquot of whole blood was transferred to a 1.5 mL conical tube and centrifuged for 3,500 rpm for 15 minutes at 4°C. Subsequently, 100 µL of the buffy coat layer, containing peripheral blood leukocyte count (cells/µL), were collected into a pre-chilled 1.5 mL microcentrifuge tube. To this fraction, 2 µL of Ribolock RNAse Inhibitor (ThermoScientific®) and 1 mL of TRIzol Reagent (life technologies®) were added at room temperature. Total RNA was extracted following the manufacturer's protocol for TRIzol Reagent (TRIzol Reagent). Genomic DNA was removed by treating 16 µL of RNA with 1 U of DNase I (ThermoScientific®) at 37°C for 30 minutes. A total of 40 ng of DNase-treated RNA from each sample was used for reverse transcription. The cDNA synthesis was performed according to the manufacturer's instructions using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems®).

-824 A/G polymorphism in the TNF-α gene promoter

A promoter region of the TNF-α gene was amplified to obtain a 249 bp fragment using previously reported oligonucleotides [11]. The PCR reaction was

performed in a final volume of 25 μL with 140 ng of DNA, 3.0 μL (10μM) of each TNF-α FW (5´-GAGAAATGGGACAACCTCCA-3') and TNF-α RV (5' – CCAGGAACTCGCTGAAACTC-3') oligonucleotide, 0.4 mM of dNTPs, 1X of PCR buffer (ThermoScientific®), 2.5 mM of $MgCl_2$, and 1 U de Taq DNA polymerase. The PCR reaction was performed in a MultiGene™ OptiMax thermocycler (Labnet International®) with the following protocol: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, ending with a final extension at 72°C for 5 min. The reaction product was verified on a 2% agarose gel in a gel documenter (Biorad®). The PCR products were digested with 20 U of SacI (ThermoScientific®) using 1μg/μL of DNA at 37 °C for 16 hours. Digestion was verified on a 2% agarose gel in a gel documenter (Biorad®).

BLV PCR

A nested PCR was performed for all samples to determine BLV infection in the analyzed Holstein cows. A region of the viral *env* gene was amplified to obtain a 444 bp fragment using the previously reported oligonucleotides [12] and conditions [13]. Negative and positive controls were included in each PCR assay. The negative control consisted of a reaction without template DNA, while the positive control included DNA from a cow previously confirmed as BLV-positive [13].

Persistent lymphocytosis in cows

Peripheral blood smears were prepared from BLV-positive cows. Smears were prepared using the wedge-slide technique at three-month intervals. Blood smears were prepared from 1 μL of blood, air-dried, fixed in 96% methanol for 5 min, and stained with Wright's stain for 5 min, followed by dilution with deionized water and incubation for 10–15 min. Stained smears were examined under a light microscope (400X), and leukocytes were counted in 100 fields to determine total white blood cell concentration. Hematological classification followed by [14]: cows with leukocyte counts >10,000 cells/μL in two consecutive samplings were classified as persistently lymphocytic (PL), while those below this threshold were considered aleukemic (AL).

Proviral load

The qPCR reaction was carried out in a final volume of 20 μL with 50 ng of DNA, 12.5 μL of QuantiTect-SYBR (Quiagen®), and 0.75 μL (10 mM) of each BLV-LTR 256 (5'GAGCTCTCTTGCTCCCGAGAC'3) and BLV-LTR 453 (5'GAAACAAACGCGGGTGCAAGCCAG'3) oligonucleotide [5]. The qPCR reaction was completed in a RotorGene (Quiagen®) thermal cycler with the following protocol: initial denaturation at 95°C for 15 s, followed by 40 cycles at 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. A negative control was included in each run, and each sample was tested in duplicate. The standard curve was elaborated through the serial dilution method proposed by [15]. DNA copy number/ng of a BLV-positive sample

was determined by serial dilution of the sample 15-fold in 1X TE (1 mM Tris HCl pH 7.5 and 0.1 mM EDTA pH 8.0). The LTR fragment was detected in each sample using the same primers from the qPCR reaction. Ten more amplifications were performed in dilutions in which no amplification was obtained. The number of copies of the target gene (λ) was determined according to Poisson's distribution model: $-Log_e\ f(\chi = 0)$ where *f* is the frequency of success [15,16]. Animals positive for BLV infection were classified as having high proviral load (HPL) if they had more than 1,000 copies per reaction or with low proviral load (LPL) if they had less than 1,000 copies per reaction.

Expression of TNF-α RI and TNF-α RII

Quantitative PCR assays were validated prior to expression analysis. Primer annealing temperature was optimized by gradient endpoint PCR, establishing 58°C for all targets. Amplification efficiencies were calculated using five-point standard curves generated from 1:2 serial dilutions (three technical replicates per dilution), yielding efficiencies ranging from 1.95 to 1.98. Mean Ct values were approximately 22 for β-actin, 24 for TNF-α RII, and 29 for TNF-α RI. No significant variation in β-actin Ct values was observed across experimental groups. qPCR reaction was performed in a final volume of 20 μL with 40 ng of cDNA, 12.5 μL of QuantiTect-SYBR (Quiagen®), and 0.75 μL (10 mM) of the TNF-α RI F (5'CGCCTCTGTCGTCTTAGCAT3') and TNF-α RI R (5´-TGGAGACAGGACTGGAACTT-3') oligonucleotides, or TNF-α RII F (5´-CTCGACCAGCAGCACGGACA-3') and TNF-α RII R (5´-GCGTCTGTGTCCCTCGTGGA-3') oligonucleotides. As a control, the β-actin gene was used in each sample using the β-actin F (5´-CGCACCACTGGCATTGTCAT-3') and β-actin R (5'-TCCAAGGCGACGTAGCAGAG-3') primers [5,17]. The qPCR reaction was performed in a RotorGene thermocycler (Quiagen®) with the following protocol: initial denaturation at 95°C for 15 s, followed by 40 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 30 s. A negative control was included in each run, and TNF-α RI and TNF-α RII receptors and the β-actin gene were evaluated in duplicate for each sample in the same run.

Statistical analysis

A frequency table was used to establish the number of BLV-positive and negative animals. The – 824 A/G genotype of TNF-α was determined, and genotype frequencies were calculated compared with the absence or presence of the virus. A binomial logistic regression model was fitted to evaluate the association between TNF-α genotype and BLV infection status in Holstein cows. BLV status was defined as a binary outcome (1= positive, 0= negative), and TNF-α genotype (AA, AG, GG) was included as a categorical predictor using AG as the reference category. The model was estimated by maximum likelihood, and coefficients were expressed as log-odds and transformed into odds ratios (OR) with 95% confidence intervals. Statistical significance was

assessed using Wald tests (Z statistics), with $p<0.05$ considered significant. Model fit was evaluated using deviance, AIC, and McFadden's pseudo-R^2.

The relative expression analysis of TNF-α RI mRNA and TNF-α RII mRNA was carried out through the $2^{-\Delta\Delta C}{}_T$ method. A complete factorial experimental design was employed. The data obtained were normalized by applying the square root transformation. A one-way analysis of variance followed by Student's t-test was applied to evaluate the differences between the means in the relative expression level of TNF-α RI mRNA and TNF-αRII mRNA in BLV-negative cows and BLV-infected cows with AL and PL and similarly, to assess mean differences in the relative expression level of TNF-αRI mRNA and TNF-αRII mRNA in BLV-positive cows with low proviral load (LPL) and high proviral load (HPL). Pearson's correlation was used to assess the relationship between the relative expression of TNF-α RI mRNA and TNF-α RII mRNA from BLV-positive cows with total lymphocyte number and proviral load. Group differences were considered significant if a p-value <0.05 was obtained. Statistical analyses were performed in the programs Gradpad Prism ® V8 (San Diego, United States), Jamovi 2.5.3 and SAS® version 9.2 (SAS Institute Inc, Cary, NC, USA) for Windows.

RESULTS

The molecular prevalence of BLV infection among the evaluated Holstein cows was 39.2% (55/140). Among BLV-positive animals, 67.3% exhibited a low proviral load (LPL), whereas 32.7% showed a high proviral load (HPL). Based on hematological classification, 70.9% of BLV-positive cows were categorized as aleukemic (AL) and 29.1% as presenting persistent lymphocytosis (PL). Regarding the TNF-α – 824 A/G polymorphism, the A/G heterozygous genotype was the most frequent, observed in 76% of the animals. The frequencies of the A/A and G/G homozygous genotypes were 0.11 and 0.12, respectively. Logistic regression analysis showed that cows carrying the G/G genotype had significantly higher odds of BLV infection compared with the AG reference genotype (OR = 4.058; 95% CI: 1.505–10.94; $p=0.006$), indicating a significant association between this genotype and BLV infection status (Table 1)

Table 1. Binomial logistic regression analysis of the association between TNF-α genotype and BLV infection status.

Predictor	Estimate	SE	Z	p	Odds ratio	95% Confidence Interval	
						Lower	Upper
Intercept	-0.1967	0.199	-0.989	0.323	0.821	0.556	1.21
Genotype:							
GG – AG	14.007	0.506	2.767	**0.006***	4.058	1.505	10.94
AA – AG	0.0789	0.525	0.150	0.881	1.082	0.387	3.03

Note 1. Estimates represent the log odds of BLV = 1 and BLV = 0.
Note 2. OR > 1 indicates higher odds of BLV infection relative to the AG reference genotype. OR < 1 indicates lower odds of infection relative to the AG reference genotype.

When analyzing the genotype distribution between BLV-infected and non-infected cows (Figure 1), the frequency of the A/G genotype was similar in both groups. A comparable pattern was observed for the A/A genotype. In contrast, the G/G genotype was more prevalent among BLV-positive animals than among BLV-negative animals, suggesting a potential association between the G/G allele and BLV infection status.

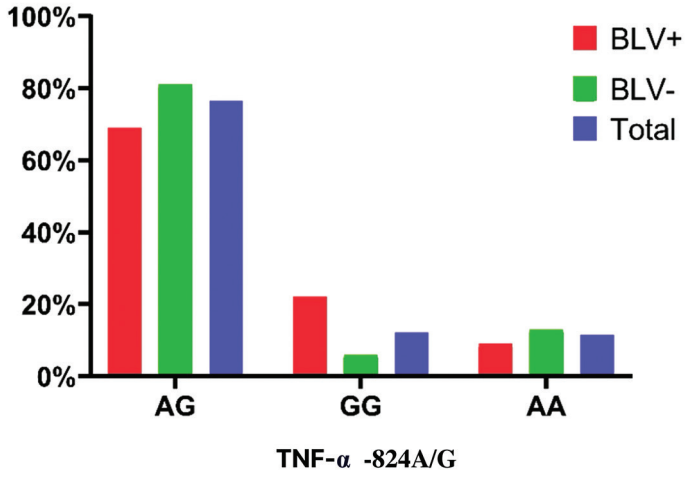

Figure 1. Bar graph for TNF-α – 824 A/G polymorphism. The bars show the percentage for the A/A, A/G, and G/G genotypes of TNF-α in BLV-positive and negative Holstein cows. The green bars show the percentages for each genotype in BLV-negative cows. The red bars present the percentages for each genotype in the BLV-positive cows. The blue bars show the percentage of each genotype for the total number of cows evaluated (n=140).

Within the BLV-positive group, the TNF-α – 824 polymorphism was associated with proviral load (Figure 2A). Cows carrying the G/G genotype exhibited a significantly higher number of viral copies compared with those carrying at least one A allele. When BLV-positive animals were classified according to hematological status (AL or PL), cows with A/G or G/G genotypes and PL showed higher proviral loads than cows with the same genotypes and AL ($p<0.05$). Notably, no animals with the A/A genotype were identified among the PL group. Furthermore, BLV-positive A/A cows presented significantly lower proviral loads compared to G/G cows ($p<0.05$). Figure 2B shows a statistically significant difference between cows with AL and those with PL with the A/G genotype. Likewise, a statistically significant difference was found between the cows with AL and PL when they had the G/G genotype.

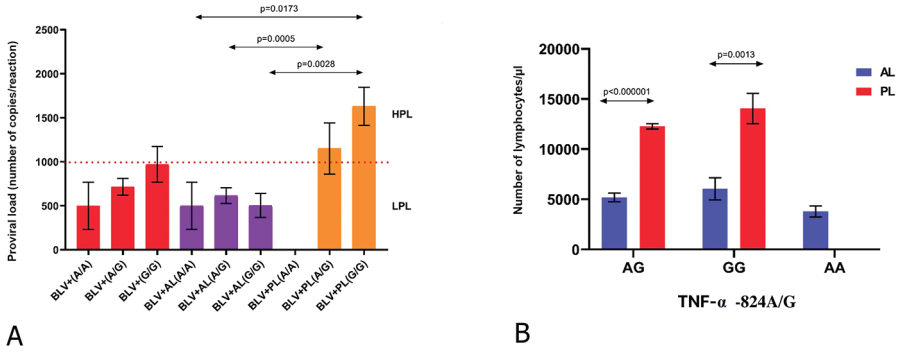

Figure 2. Association of the TNF-α – 824 A/G polymorphism with proviral load and lymphocyte count in BLV-positive Holstein cows. (A) Proviral load is shown for A/A, A/G, and G/G genotypes in BLV+, BLV+ AL, and BLV+ PL groups. Red bars represent BLV+ cows, purple bars BLV+ AL cows, and orange bars BLV+ PL cows. Data are presented as mean ± standard error of the mean (SEM). *p*-values indicating differences between BLV+ AL and BLV+ PL groups are shown above the bars. Statistical significance was set at $p<0.05$. HPL: high proviral load; LPL: low proviral load. (B) The number of lymphocytes per µL is shown for A/A, A/G, and G/G genotypes in cows classified as aleukemic (AL) or with persistent lymphocytosis (PL). Blue bars represent AL cows and red bars PL cows. Data are presented as mean ± SEM. *p*-values indicating differences between AL and PL groups are shown above the bars. Statistical significance was set at $p<0.05$.

Figure 3A shows that TNF-α RI mRNA expression levels were statistically different ($p<0.05$) between positive and negative cows. However, no difference was found between the TNF-αRII mRNA expression levels of BLV-positive and negative cows ($p>0.05$). Cows negative for BLV infection presented a statistically significant difference ($p<0.05$) between the relative expression level of TNF-α RI mRNA and the relative expression level of TNF-α RII mRNA, exhibiting a lower level of expression of RII-like receptors in cows not infected with BLV (figure 3A and 3B). Negative cows showed a statistically significant difference ($p=0.0001$) between the relative expression level of TNF-α RI mRNA and the relative expression level of TNF-α RII mRNA, finding a lower expression level of RII-type receptors in cows not infected by BLV. No statistically significant difference ($p>0.05$) was found between the relative expression level of TNF-α RI mRNA and TNF-α RII mRNA when bovines exhibited the infection regardless of their AL or PL status. The group of AL cows showed a lower level of expression of RI and RII type receptors compared to TNF-α RI receptors in cows not infected by BLV ($p<0.05$) (figure 3B).

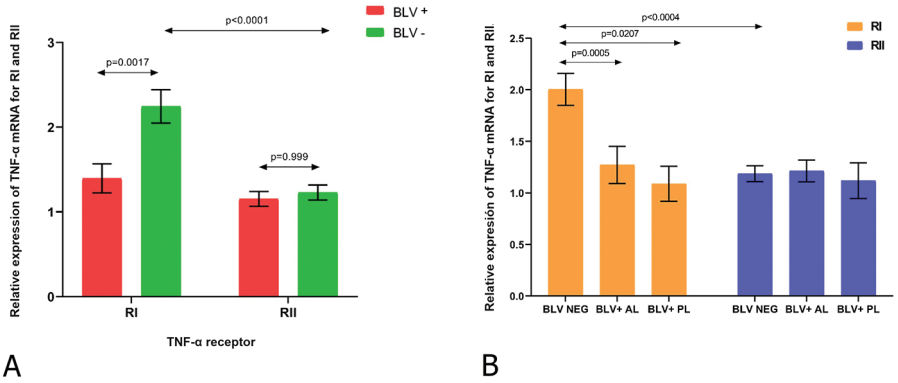

Figure 3. Relative expression levels of TNF-α RI and RII mRNA in BLV-negative and BLV-positive cattle. (A) Red bars represent BLV+ cows and green bars represent BLV– cows. Data are shown as mean ± standard error of the mean (SEM). *p*-values indicating differences between BLV+ and BLV – groups, as well as between RI and RII, are displayed above the bars. Statistical significance was set at $p<0.05$. (B) Orange bars correspond to TNF-α RI expression, and blue bars correspond to TNF-α RII expression. Data are presented as mean ± SEM. *p*-values indicating differences among BLV-, BLV+ AL, and BLV+ PL groups are shown above the bars. Statistical significance was set at $p<0.05$.

No significant association was observed between the relative expression level of TNF-α RI mRNA and the number of cells/μl in BLV-infected cattle (Figure 4a). Similarly, no correlation was detected between TNF-α RI mRNA expression and proviral load (Figure 4b), as indicated by a low correlation coefficient ($\varrho=0.08$) and a non-significant *p*-value ($p>0.05$).

In contrast, no association was observed between the relative expression level of TNF-α RII mRNA in BLV-infected cows and either the number of cells/μl (figure 4c) or the proviral load (figure 4d). In both cases, the correlation coefficients were negative and low ($\varrho=-0.17$ and $\varrho=-0.23$ for cells/μl and proviral load, respectively), and these correlations were not statistically significant ($p>0.05$).

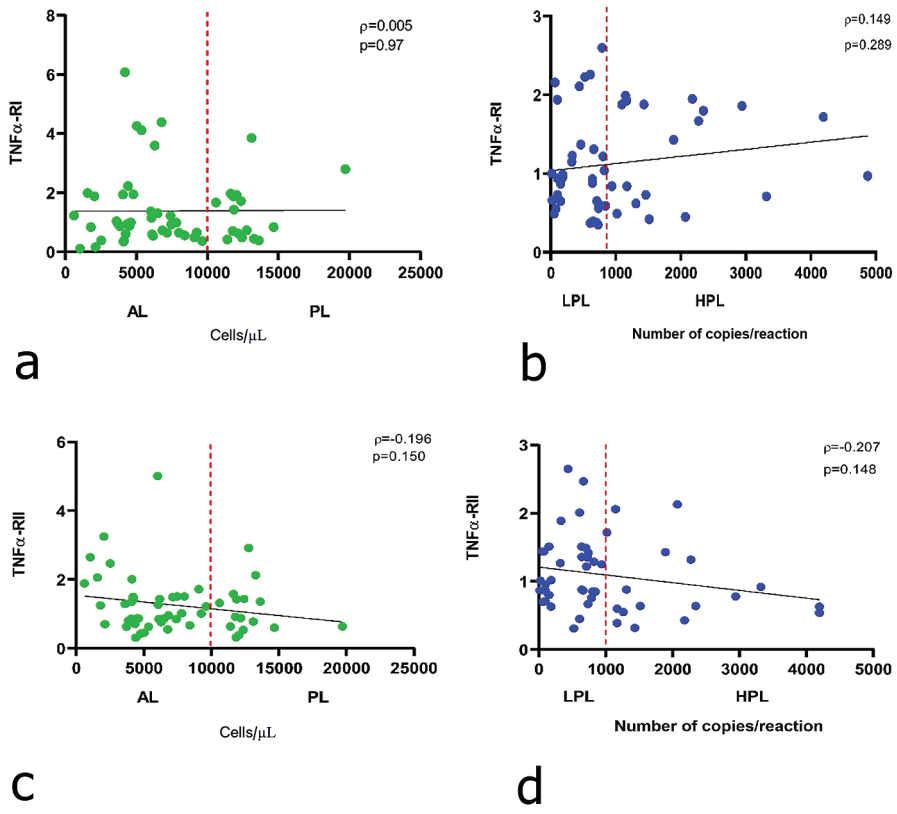

Figure 4. Pearson's correlation between the relative expression of TNF-α RI and RIImRNA in peripheral blood leukocyte count (cells/μL) from BLV-positive cows and total lymphocyte count (a, c) and proviral load (b, d). The *p*-values are shown on each graph. Values of $p<0.05$ were considered statistically significant. ϱ=Pearson's correlation coefficient

DISCUSSION

Several SNPs located upstream in the promoter region, including – 824 A/G, – 793 C/T, and – 627 C/G, have been evaluated for their potential involvement in BLV infection dynamics. Among these, the – 824 A/G SNP has been the most extensively studied due to its association with TNF-α expression and BLV propagation [18]. This polymorphism may influence not only the quantity and quality of TNF-α mRNA but also its translational efficiency, thereby affecting the host's antiviral response. In the present study of Holstein cows from a specialized dairy herd, the A/G genotype was the most frequent (76%), followed by G/G (12%) and A/A (11%), in agreement with the findings of [11]. Notably, the frequency of the A/G genotype was similar between BLV-infected and uninfected animals, suggesting that this heterozygous state may not be differentially associated with BLV infection status. The homozygous

G/G genotype of the TNF-α – 824 SNP has been reported in association with BLV infection. In the present study, a higher frequency of the G/G genotype was observed in BLV-positive cows compared with uninfected animals. This supports the hypothesis that the presence of the G allele in homozygosity may compromise the host's antiviral defense, potentially by altering TNF-α expression levels or function. Cows carrying the G/G genotype were therefore classified as more susceptible to BLV infection. Previous studies have reported a significantly higher proportion of BLV-infected peripheral blood mononuclear cells (PBMCs) in cattle with the G/G genotype (17.3%) compared to heterozygous A/G animals (10.9%, $p < 0.05$), suggesting a genotype-dependent influence on viral burden [18]. Functional single nucleotide polymorphisms (SNPs) in cytokine genes, such as TNF-α, may affect both viral replication and immune evasion mechanisms. Similar genotype-dependent effects have been observed in other retroviral infections; for instance, only a subset of individuals infected with human T-lymphotropic virus type 1 (HTLV-1) develop clinical disease, largely determined by host immune responses. Polymorphisms in cytokine genes, such as the IFNG +874 A/T SNP, can modulate IFN-γ production and thereby influence the efficiency of viral control [19].

BLV-positive cows carrying the homozygous G/G genotype exhibited a significantly higher proviral load measured as the number of copies per PCR reaction compared to animals with at least one A allele (A/G or A/A genotypes). Moreover, when comparing BLV-positive cows classified as having either aleukemic (AL) or persistent lymphocytosis (PL) forms of the disease, those with the PL condition and the G/G genotype showed markedly elevated proviral loads (>1,500 copies/reaction), whereas AL cows with the same genotype had considerably lower viral loads (<500 copies/reaction), a difference that was statistically significant ($p=0.0028$) (Figure 2A). These results suggest a strong association between the G/G genotype and disease progression. Additionally, a significant difference ($p=0.0013$) was found in total lymphocyte counts among G/G cows: AL cows had an average of 5,182 lymphocytes/µl, whereas PL cows averaged 14,047 lymphocytes/µl (Figure 2B). Consistent with these findings, previous studies have shown that TNF-α protein levels in PBMCs were significantly higher in G/G homozygous cows than in A/A homozygous individuals ($p<0.0001$), reinforcing the role of this genotype in immune dysregulation and viral persistence [6]. Other studies have shown that single nucleotide polymorphisms (SNPs) in cytokine promoter regions can modulate the host inflammatory response and viral replication, as seen with IL-1β rs16944 and IL-17 rs2275913, whose variants are associated with increased severity and susceptibility to influenza A (H1N1) infection [20].

Similar presence percentages were found among animals infected with the A/A genotype with respect to healthy bovines with the same genotype (Figure 1). However, the A/A genotype showed an OR=1.082, indicating a potential association with BLV infection status; however, this association was not statistically significant ($p>0.05$). Fewer cells are infected with BLV when the cows are genotype A/A compared to the number of cells infected by BLV in cows with genotypes A/G and G/G [18].

No animal was found in the group of cows positive for BLV PL with genotype A/A. In addition, BLV+ AL cows with genotype A/A have a lower viral load than BLV+ PL cows with genotype G/G (p=0.0028). This would be related to a higher rate of transcription of the TNF-α gene in cows that have A alleles (without mutation) and, therefore, a higher cytotoxic immune response to control the proliferation of infected B cells and control or prevent PL development. Moreover, the A/A genotype has been reported in relation to BLV infection dynamics; however, confirmation of this pattern requires larger sample sizes, as only 15 cows in the present study carried this genotype.

Experimental studies in BLV-infected sheep have shown that elevated TNF-α mRNA expression is associated with effective viral control and clearance, whereas low TNF-α expression correlates with active viral replication and disease progression [21]. These findings highlight the critical role of TNF-α in modulating the host immune response to BLV. Consistent with this, the present study did not identify any bovines with persistent lymphocytosis (PL) carrying the homozygous A/A genotype. This absence may reflect a protective effect conferred by the A/A genotype, potentially linked to a more robust or better regulated TNF-α mediated immune response that limits viral replication and favors an aleukemic (AL) state. Supporting this hypothesis, previous research has indicated that specific promoter SNPs in proinflammatory cytokine genes, including TNF-α, can significantly influence susceptibility or resistance to retroviral infections by modulating gene expression and downstream immune activity [22].

In the case of A/G heterozygotes, animals that developed PL exhibited a significantly higher viral load than those that remained AL (p=0.027). This difference may be attributed to the presence of the G allele, as well as to other immune-related factors in bovines, such as the expression levels of TNF-α RI and TNF-α RII receptors. The role of TNF-α in the progression or control of BLV infection is closely linked to the expression of these membrane receptors. Both TNF-α RI and TNF-α RII are expressed on the surface of white blood cells and can be activated by either the soluble or transmembrane forms of TNF-α. Upon receptor binding, TNF-α signaling triggers a range of inflammatory responses, including apoptosis, cell proliferation, and cytokine production [23].

In the present study, BLV-positive cows showed significantly lower TNF-α RI mRNA expression compared with BLV-negative cows (p=0.0017). In contrast, no significant difference was observed in TNF-α RII mRNA expression between BLV-negative and BLV-positive animals (p=0.999) (Figure 3A). These findings suggest that BLV infection may downregulate the expression of TNF-α RI, potentially as a viral strategy to evade host immune responses mediated through this receptor. Since TNF-α RI is primarily involved in apoptosis and proinflammatory signaling, its reduced expression could impair the elimination of infected cells, favoring viral persistence. Konnai et al. [5] proposed a potential relationship between the expression levels of TNF-α RI and TNF-α RII receptors and the progression of BLV infection. An imbalance in mRNA expression between these receptors appears to influence the fate of infected cells. Specifically, cells exhibiting higher TNF-α RII expression levels tend to enter a state

of proliferation, which may contribute to the development of PL. Conversely, when TNF-α RI expression is equal to or higher than that of TNF-α RII, cells are more likely to undergo apoptosis, thereby limiting viral expansion. The lack of variation in TNF-α RII expression indicates that BLV may selectively modulate specific components of the TNF-α signaling pathway rather than inducing a generalized suppression. Similar regulatory effects on TNF-α receptors have been reported in other retroviral infections, where altered receptor expression contributes to immune tolerance and chronic infection. Supporting this hypothesis, studies on T cells infected with HTLV-1, a retrovirus with genomic organization similar to BLV have shown strong resistance to TNF-α-mediated apoptosis when TNF-α RII expression is upregulated [24].

In this study, uninfected cows exhibited significantly higher mRNA expression levels of TNF-α type I receptors (TNF-α RI) compared to type II receptors (TNF-α RII), with a highly significant difference ($p<0.0001$) (Figure 3B). In contrast, BLV-infected cows showed no marked difference between TNF-α RI and RII expression levels, suggesting a potential dysregulation of TNF-α receptor signaling during infection. Elevated TNF-α RI expression in B lymphocytes has been associated with a more effective cytotoxic immune response against BLV, mediated through enhanced activation of apoptotic pathways and increased pro-inflammatory cytokine production [23]. Conversely, the balanced or reduced TNF-α RI-to-RII expression ratio observed in BLV-infected animals may attenuate cytotoxic responses and favor cellular proliferation over apoptosis, thereby creating an environment conducive to viral persistence and the progression to persistent lymphocytosis. This shift in receptor balance could reflect a viral immune-evasion mechanism aimed at dampening proapoptotic signaling while sustaining cell survival pathways that support viral replication. Recent evidence further supports that modulation of TNF-α receptor expression critically influences disease outcomes in retroviral infections, underscoring the importance of maintaining TNF-α signaling balance for effective immune control [25].

When comparing cows classified as AL and PL, no significant differences were observed in the expression levels of TNF-α RI or TNF-α RII receptors between the two groups. In both cases, similar mRNA expression levels of these receptors in white blood cells appeared to have no measurable impact on the development of PL or the maintenance of an AL state. This finding suggests that the transition from aleukemic to persistent lymphocytic stages may not be directly driven by differences in TNF-α receptor transcription, but rather by other host or viral regulatory mechanisms influencing immune cell dynamics. It is possible that post-transcriptional regulation, receptor signaling efficiency, or interactions with other cytokine pathways play a more decisive role in determining disease progression.

The results of this study revealed significantly higher TNF-α RI mRNA expression in BLV-negative cows compared with both BLV+ AL ($p=0.0005$) and BLV+ PL animals ($p=0.0207$), suggesting that elevated TNF-α RI expression may contribute to a more effective cytotoxic immune response against BLV infection. This enhanced

receptor expression could facilitate stronger proinflammatory and apoptotic signaling, promoting the clearance of infected cells and preventing viral persistence.

No significant correlation was observed between TNF-α RI or TNF-α RII mRNA expression levels and total lymphocyte count in BLV-positive cows. Likewise, no association was detected between TNF-α receptor expression and proviral load. Although previous studies have reported that elevated TNF-α RII mRNA expression is associated with increased proviral load [5], the present findings suggest that comparable expression levels of TNF-α RI and RII are not decisive factors in the transition to persistent lymphocytosis (PL) or in maintaining an aleukemic (AL) state and therefore may not directly contribute to lymphocyte proliferation. Consistently, both low proviral load (LPL) and high proviral load (HPL) animals exhibited similar receptor expression profiles, supporting the notion that TNF-α receptor transcription alone does not account for differences in viral burden or disease progression. Taken together, these data suggest that while TNF-α receptor mRNA levels remain relatively stable across infection states, BLV may exert subtler regulatory effects at the post-transcriptional or signaling level. BLV infection may induce specific downregulation of TNF-α RI mRNA expression, increased mRNA degradation, or both. Retroviruses are known to modulate host mRNA stability to favor their replication, thereby dampening immune responses and facilitating viral persistence and cellular transformation [25]. A decrease in TNF-α RI expression skews signaling toward TNF-α RII-mediated pathways, which are primarily associated with anti-apoptotic and cell survival responses rather than cytotoxic or proinflammatory effects [26, 27]. Such receptor imbalance could therefore represent a key mechanism by which BLV circumvents apoptosis, promotes lymphocyte survival, and establishes long-term persistence in infected cattle.

A limitation of the present study is the use of a single reference gene for normalization of gene expression data. Although β-actin has been widely employed as a housekeeping gene in bovine immunological and infectious disease studies and showed stable Ct values across the experimental groups analyzed here, the use of multiple validated reference genes is generally recommended, particularly under inflammatory conditions, to improve normalization accuracy. In addition, TNF-α receptor expression was assessed only at the transcriptional level. Because mRNA abundance does not necessarily reflect protein concentration or receptor surface expression, the results should be interpreted as indicative of transcriptional associations rather than functional receptor activity. Future studies incorporating multiple reference genes, as well as protein-level and functional analyses, will be necessary to further clarify the role of TNF-α signaling during BLV infection.

In summary, the TNF-α G/G genotype was significantly associated with higher odds of BLV infection in this population. In addition, BLV-negative cows showed higher TNF-α RI mRNA expression than infected animals, whereas TNF-α RI and RII expression levels did not differ between aleukemic and persistent lymphocytosis profiles among BLV-positive cows. Together, these results indicate statistical associations between host TNF-α genetic variation, receptor expression patterns, and

BLV infection status. However, due to the cross-sectional design of the study and the exploratory nature of several comparisons, causal relationships or mechanistic directionality cannot be established. The observed expression differences may therefore reflect infection-related modulation rather than determinants of disease progression. Further longitudinal and functional studies are required to clarify the role of TNF-α signaling in BLV infection dynamics.

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Authors' contributions

CUM contributed to formal analysis, investigation, original draft preparation, visualization, and funding acquisition. ALH contributed to supervision and project administration. Both authors contributed to conceptualization, methodology, and writing, review, and editing.

Declaration of conflicting interests

The authors declare that they have no conflicts of interest.

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Data availability

All the data analyzed during the current study are available from the corresponding author upon request.

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VARIJANTE GENA TNF-A I EKSPRESIJA RECEPTORA POVEZANIH SA BLV INFEKCIJOM KOD KOLUMBIJSKIH GOVEDA HOLŠTAJNSKE RASE

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Virus goveđe leukemije (BLV) je jednolančani RNK retrovirus čija perzistencija kod zaraženih goveda ukazuje na to da urođeni imunski odgovor, posebno antivirusni citokini, nije dovoljan za kontrolu infekcije. Faktor tumorske nekroze alfa (TNF-a) je ključni proinflamatorni citokin koji može doprineti antivirusnoj odbrani, delujući preko dva membranski vezana receptora: TNF-a receptor I (TNF-a RI) i TNF-a receptor II (TNF-a RII). Oba receptora učestvuju u apoptotskim i imunoregulatornim putevima. Cilj ove studije bio je da istraži polimorfizam -824 A/G u promotorskom regionu gena TNF-a i da kvantifikuje nivoe transkripta TNF-a RI i TNF-a RII, procenjujući njihovu povezanost sa provirusnim opterećenjem i perzistentnom limfocitozom (PL) kod goveda holštajnske rase. Uzorci krvi od 140 krava analizirani su na genotipove TNF-a i ekspresiju mRNK receptora. Podaci su normalizovani i analizirani korišćenjem jed-

nofaktorske analize varijanse (ANOVA) kako bi se procenile razlike u ekspresiji gena između eksperimentalnih grupa. Genotip TNF-a G/G bio je značajno povezan sa povećanom verovatnoćom infekcije BLV ($p=0,006$). Ekspresija mRNK TNF-aRI se značajno razlikovala između BLV-pozitivnih i BLV-negativnih krava ($p=0,0017$), dok ekspresija TNF-aRII nije pokazala razlike u zavisnosti od statusa infekcije ($p=0,999$). Kod BLV-negativnih životinja, nivoi ekspresije TNF-aRI i TNF-aRII su se značajno razlikovali, sa nižom ekspresijom RII ($p<0,0001$); ovaj obrazac nije primećen kod inficiranih krava, bez obzira na aleukemijski ili perzistentni limfocitozni status. Aleukemijske krave su pokazale smanjenu ekspresiju RI i RII u poređenju sa nivoima RI kod neinficiranih životinja ($p<0,05$). Nisu otkrivene značajne korelacije između ekspresije TNF-aRI ili TNF-aRII i broja PBMC ili provirusnog opterećenja kod inficirane goveda ($r\leq 0,23$; $p>0,05$). Rezultati ukazuju na vezu između genetske varijacije TNF-a, obrazaca ekspresije receptora i statusa BLV infekcije, što sugeriše da TNF-a signalizacija može doprineti interakciji domaćina i virusa tokom BLV infekcije. Potrebna su dalja longitudinalna i funkcionalna istraživanja kako bi se razjasnili biološki mehanizmi koji povezuju regulatorne polimorfizme TNF-a sa perzistencijom virusa i progresijom bolesti.