Polymorphisms in the bovine tumour necrosis factor receptor type two gene (TNF-RII) and cell subpopulations naturally infected with bovine leukaemia virus

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Abstract

Introduction: Numerous mutations in the bovine tumour necrosis factor receptor type two (TNF-RII) gene have been identified, but their biological consequences remain poorly understood. The aim of this study was to determine whether polymorphism in the analysed loci of the bovine TNF-RII gene is linked with the size of cell subpopulations naturally infected with bovine leukaemia virus (BLV) which serve important immune functions in the host. **Material and Methods:** Samples originated from 78 cows. Polymorphisms in the studied gene were determined by PCR-RFLP and DNA sequencing by capillary electrophoresis. BLV infection was diagnosed by the immunofluorescence (IMF) technique and nested PCR. Cell subpopulations were immunophenotyped with IMF. **Results:** Similar and non-significant differences in the average percentages of TNFα+, IgM+TNFα+, and CD11b+TNFα+ cells infected with BLV were noted in individuals with various genotypes in the polymorphic sites g.-1646T > G and g.16534T > C of the TNF-RII gene, and significant differences in the percentages of these subpopulations were observed between selected microsatellite genotypes (g.16512CA$_{(n)}$). **Conclusion:** STR polymorphism and the number of CA dinucleotide repeats in intron 1 of the TNF-RII gene influence the frequency of TNF+, CD11b+TNF+, and IgM+TNF+ subpopulations naturally infected with BLV. Polymorphism in the gene's other two sites do not affect the size of these cell subpopulations.

Keywords: cattle, BLV, CD11b+TNFα+p24+ cells, IgM+ TNFα+p24+ cells, microsatellite DNA.

Introduction

Tumour necrosis factor receptor type two (TNF-RII) is a surface glycoprotein which forms a complex with tumour necrosis factor alpha (TNF-α) and participates in the host immune response. In cattle, the gene encoding TNF-RII has a length of approximately 33.5 kbp. It is composed of 10 exons and 9 introns and is localised in chromosome 16, but little is known about the functional areas of the gene's regulatory region (such as the promoter, enhancers, or silencers). Numerous mutations have been identified in various regions of the gene (dbSNP, NCBI, and Gene ID 338033), but their biological consequences remain poorly understood.

In the present study, single nucleotide polymorphisms (SNPs) were analysed in the polymorphic sites g.-1646T > G (rs42686838) of the 5' flanking region and g.16534T > C (rs42686850) of intron 1. A dinucleotide CA motif is a base unit of the short tandem repeat (STR) in intron 1 of the TNF-RII gene. In this DNA fragment two (CA)$_2$, five (CA)$_5$, or seven (CA)$_7$ repetitions of this motif can be observed. The polymorphism of this fragment which was identified for the first time in our previous study (26) was also analysed in intron 1 at position 16512.

Polymorphism g.-1646T > G in the regulatory region of the TNF-RII gene can influence the expression of the encoded protein and physiological functions, and it can ultimately modulate susceptibility to BLV infection. Intron 1 often plays an important role in gene transcription. The presence of the CA microsatellite in intron 1 was found in humans affected by various diseases (30), but its role in animals has been insufficiently investigated. The analysed polymorphic site g.16534T > C in intron 1 includes a sequence with an allele-specific ability to bind transcription factors of the E2F family which are eminent factors in regulating the cell cycle and cell proliferation (23).

The aim of this study was to determine whether polymorphism in the analysed loci of the bovine TNF-RII gene is linked with the size of cell subpopulations naturally infected with the bovine leukaemia virus (BLV) which play important immune functions in the host.

Material and Methods

Animals and blood collection. The study was performed on 78 Polish Holstein-Friesian Black-and-White cows which were diagnosed with a BLV infection. The animals originated from three herds that were free of tuberculosis and brucellosis. The herds were kept indoors in compliance with animal welfare standards.

Blood for analysis was sampled from the mammary or jugular vein with the consent of the Local Ethics Committee (decision no. 13/2008/N/T). Blood samples for the isolation of genomic DNA were collected into tubes containing EDTA, and heparin was used for the isolation of peripheral blood mononuclear cells (PBMCs) as anticoagulant. The BLV infection was diagnosed and cell subpopulations were identified in two or three replications. Animals which tested positive in both diagnostic tests were regarded as infected with BLV.

Determination of polymorphism in the TNF-RII gene

Isolation and evaluation of genomic DNA. Genomic DNA was isolated from whole peripheral blood with the Master Pure Purification Kit for Blood according to the manufacturer's protocol (Epicentre, USA). DNA was subjected to quantitative (NanoDrop ND-1000, Thermo Scientific, USA) and qualitative (1.5% agarose gel) evaluations.

Polymorphism of g.-1646T > G in the 5' flanking region. Polymorphism of g.-1646T > G was determined by the PCR-RFLP/*Oceanospirillum linum* I (OliI) method. The following primer sequences were designed in the Primer-Blast programme (29; NCBI, USA): F: 5'- ACACAGGGTATTTCGGCAGG-3' and R: 5'-CCAGCTCCTGGCTTTGGTAA-3'. The reference sequence NC-007314.4 from the NCBI database was used as the matrix. A selected fragment of the gene was amplified in a PCR mix with the composition given in Table 1. The following PCR programme was used: pre-denaturation for 3 min at 94°C (1 cycle), denaturation for 30 s at 94°C (35 cycles), primer annealing for 30 s at 68.8°C (35 cycles), elongation for 30 s at 72°C (35 cycles), and final elongation for 5 min at 72°C (1 cycle).

The PCR product was digested with the OliI enzyme (AleI) (Thermo Fisher Scientific, Lithuania) which recognises the restriction site in the 5'-CACNN/NNGTG-3' sequence. In individuals with the G/G genotype, the enzyme identified nucleotide G in the analysed fragment of the gene and cleaved that fragment into two segments with a length of 583 bp and 399 bp. In individuals with the T/T genotype, the restriction site was not identified, and one DNA fragment with the size of the PCR product (982 bp) was registered in an electropherogram.

Polymorphism of g.16512CA$_{(n)}$ in intron 1. CA$_{(n)}$ polymorphism was identified by capillary electrophoresis with the 3130 Genetic Analyser (Applied Biosystems, USA). The primer sequence was: F: 5'-TGGTGGCCTCGGTCACAGAGA-3' and R: 5'- CCCTGGGCGCTGACCCTTTG-3'. The forward primer at the 5' end was labelled with 6-FAM phosphoramidite (Applied Biosystems).

After amplification, the samples were diluted 1:1 in DEPC-treated deionised water (Ambion, USA). Next, 1 μL of the diluted PCR product was mixed with 0.5 μL of the GeneScan 1200 LIZ size standard (Applied Biosystems) and 13.5 μL of Hi-Di formamide (Applied Biosystems), denatured at 94°C for 2 min, and chilled on ice for 5 min.

Table 1. Composition of the PCR mix

| Ingredient	Initial concentration	Ingredients per sample volume (μL)	Manufacturer
Run reaction buffer with Mg^{2+} ions	10x	0.6x	A&A Biotechnology, Poland
Run polymerase	1U/ μL	0.028 U/μL	A&A Biotechnology, Poland
F primer	0.1 mM	1.6 μM	IBB PAN, Poland
R primer	0.1 mM	1.6 μM	IBB PAN, Poland
dNTPs mix	10 mM	0.6 mM	Epicentre, USA
Genomic DNA	± 75 ng/μL	± 6 ng/μL	
Distilled water	-	18.5 μL	
Total sample volume		25.0 μL	
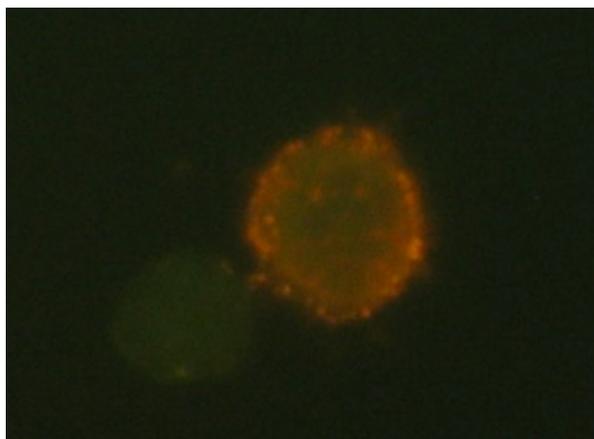
Fig. 1. Lymphocyte infected with BLV; BLV-positive and BLV-negative blood lymphocytes; (1,000×, PE staining); photo B. Bojarojć-Nosowicz

PCR products were separated using the 3130 Genetic Analyser (Applied Biosystems) and Genetic Analyser Data Collection 3.0 software (Applied Biosystems). The length of PCR products was measured with the 3130 Genetic Analyser in AFLP mode. A 36 cm capillary array (Applied Biosystems) and POP-7 polymer (Applied Biosystems) were used. The injection of samples onto capillaries lasted 11 s at 15 kV and the run lasted 100 min at 6.5 kV. Size calling of DNA fragments was performed with GeneMapper 4.0 software (Applied Biosystems).

Polymorphism of g.16534T > C in intron 1. The PCR-RFLP/*Acetobacter pasteurianus* (ApaLI) method was used. The composition of the PCR mix and thermal cycling profile were described in a previous study (26). The primer sequences were: F: 5'-TGGTGG CCTCGGTCACAGAGA-3' and R: 5'-CCCTGGGCG CTGACCCTTTG-3'. The ApaLI enzyme cleaves the identified 5'G↓TGCAC3' (allele T) sequence into two fragments (441 bp and 199 bp), whereas the T > C (allele C) substitution abolishes the restriction site, and a single DNA fragment (640 bp) is registered in an electropherogram.

Diagnosis of infection with the bovine leukaemia virus

Isolation of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated with Histopaque 1077 according to the manufacturer's procedure (Sigma, USA). The viability from each animal was determined in 0.2% trypan blue solution in a Bürker counting chamber and the number of isolated lymphocytes was counted with an Exigo veterinary haematology analyser (Boule Medical, Sweden). The cells were divided into portions with a concentration of 2×10^6 cells/ml for the diagnosis of BLV infection and immunophenotyping.

Immunofluorescence diagnosis of BLV infection. The BLV infection was identified in PBMCs based on the expression of p24, the main viral capsid protein. The epitope of protein p24 was identified and localised with the mouse anti-bovine monoclonal BLV3 primary antibody and goat anti-mouse IgG (H + L) secondary antibody labelled with the phycoerythrin (PE) fluorochrome (Invitrogen, USA), according to a previously described procedure (18). The presence of cells expressing p24 protein was indicative of a BLV infection (Fig. 1). The diagnoses were made based on at least 600 cells in the analysed smear.

Nested PCR. In the studied animals, BLV infection was confirmed by the nested PCR molecular test. The composition of the reaction mix and thermal cycling profile were described by Markiewicz *et al.* (21).

PBMC immunophenotyping. IgM+ and CD11b+ cells co-expressing TNFα (mTNFα) membrane protein and p24 viral protein were identified by the previously described indirect IMF technique (4). Epitopes were identified by double and triple staining on PBMCs. Cells of mTNFα+ type co-expressing protein p24 (TNFα+ p24+) were identified by double staining of both protein epitopes. Mouse anti-IgG2b monoclonal primary antibody (Acris GmbH, Germany) was used against bovine mTNFα (4) and mouse anti-BLV3 mAb (VMDR Inc., Pullman, USA) was used against BLV protein epitopes (4, 18). The mTNFα protein epitope was identified with biotinylated goat anti-mouse immunoglobulin as the secondary antibody (Dako Cytomation, Denmark) and streptavidin conjugated with Texas Red fluorochrome (red signal) (Vector Lab. Inc., USA) (Fig. 2). The p24 protein epitope in dual- and triple-colour reactions was identified with goat anti-mouse IgG (H+L) secondary antibody with fluorochrome FITC labelling (Invitrogen, USA) (4) (Fig. 2). The antibodies were applied sequentially in two IMF reactions. In the first reaction, the p24 protein epitope was detected and the mTNFα protein epitope was identified. IgM+ and CD11b+ cells co-expressing mTNFα and p24 proteins (IgM+TNFα+ p24+ and CD11b+TNFα+ p24+ immunophenotypes) were identified by triple epitope staining (Fig. 2). Primary and secondary antibodies binding IgM and CD11b molecules were applied according to the previously described procedure (4). The antibodies were applied sequentially in three IMF reactions. The epitope of each cell surface marker was identified in the first reaction, the p24 protein epitope was detected in the second reaction, and the mTNFα protein epitope was identified in the third reaction. All IMF reactions were carried out in the same portion of PBMCs isolated from the peripheral blood of the same animal. IMF reactions without the primary antibody were the control. The smears were stored at 4°C and analysed under a fluorescence microscope (Axiolab-Zeiss, Germany) with the appropriate filters, at 1,000 magnification. At least 600 cells (subject to epitope) were analysed in smears. The percentage of cells expressing a given immunophenotype was calculated.
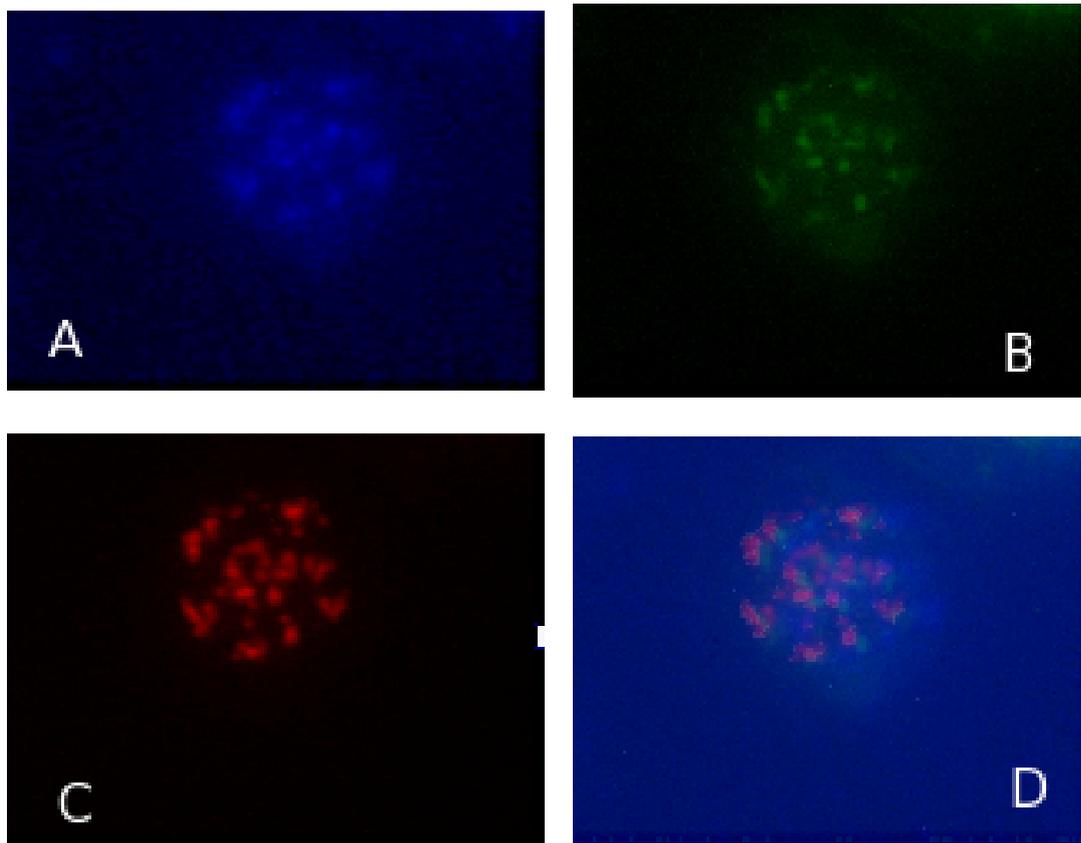
Fig. 2. Detection of epitopes: IgM (A), viral p24 protein (B), mTNF-alfa (C) and all together (D); photo B. Bojarojć-Nosowicz; A–Lymphocyte IgM+ (single-colour reaction, Cascade blue, 1,000×), B–Lymphocyte p24+ (BLV-positive cell) (single-colour reaction, FITC, 1,000×), C–Lymphocyte expressing mTNFα (single-colour reaction, Texas red, 1,000×), D–Lymphocyte IgM+ p24+TNFα+ (triple-colour reaction: Cascade blue, FITC, Texas red, 1,000×)

Statistical analysis. The frequency of genes and genotypes at every analysed polymorphic site of the TNF-RII gene was calculated. The chi-square test was used to check for deviations from Hardy-Weinberg equilibrium based on differences between the observed and the expected number of individuals. Data were processed statistically based on the arithmetic mean, standard error of the mean, and distribution fitting. The Kruskal-Wallis non-parametric test and the median test were used because the analysed values were not consistent with normal distribution. Differences between genotypes were verified by the multiple comparison procedure in a non-parametric test at $P < 0.05$ and $P < 0.01$. The calculations were performed in Statistica 12.0 software (StatSoft Inc., USA).

Results

Frequency of TNF-RII genes and genotypes in the studied population. The polymorphic sites g.-1646T > G and g.16534T > C (intron 1) in the studied animals were manifested by the presence of the two alleles and the three genotypes (T/T, T/G and G/G; T/T, T/C, and C/C, respectively) (Table 1a). The frequency of T and G alleles and T and C alleles and the frequency of homozygous genotypes were similar.

Heterozygous genotypes were most frequently identified at both analysed polymorphic sites. Their frequency was also similar (Table 1a).

The analysis of polymorphism of g.16512$(CA)_n$ (intron 1) revealed the presence of alleles with two $(CA)_2$, five $(CA)_5$, and seven $(CA)_7$ repeats with a size of 622 bp, 628 bp, and 632 bp, respectively. Five genotypes were identified in the analysed animals: $(CA)_2/(CA)_2$, $(CA)_2/(CA)_5$, $(CA)_2/(CA)_7$, $(CA)_5/(CA)_7$, and $(CA)_7/(CA)_7$ (Table 1b). Genotype $(CA)_5/(CA)_5$ was not detected. Allele $(CA)_5$ and genotypes with five dinucleotide repeats, $(CA)_2/(CA)_5$ and $(CA)_5/(CA)_7$, showed the lowest frequency of 0.08. The frequencies of alleles $(CA)_2$ and $(CA)_7$ was relatively high and similar (0.45 and 0.47, respectively). The heterozygous genotype $(CA)_2/(CA)_7$ (0.41) was most frequently identified, whereas $(CA)_2/(CA)_2$ and $(CA)_7/(CA)_7$ homozygotes were less frequently detected with frequencies of 0.21 and 0.23, respectively. At the analysed polymorphic sites, the observed and expected number of individuals with specific genotypes was largely consistent at position -1646 ($\chi^2 = 0.454$, $P = 0.50$) as well as at positions 16512 ($\chi^2 = 0.596$, $P = 0.89$) and 16534 ($\chi^2 = 0.799$, $P = 0.37$) of the TNF-RII gene. These results point to genetic equilibrium at the three analysed polymorphic sites of the TNF-RII gene in the studied population.

Polymorphism of the TNF-RII gene and the analysed cell subpopulations. Similar and non-significant differences in the percentages of TNFα+p24+, IgM+TNFα+p24+ and CD11b+TNFα+p24+ cells were observed in individuals with various genotypes at polymorphic sites g.-1646 and g.16534 of the TNF-RII gene (Tables 2 and 3). In contrast, the percentages of these subpopulations differed significantly between selected microsatellite genotypes (STR, position 16512 of the TNF-RII gene) (Table 4).

The percentage of subpopulations infected with BLV (TNFα+p24+, IgM+TNFα+p24+, and CD11b+TNFα+p24+ immunophenotypes) was the highest in $(CA)_7/(CA)_7$ homozygotes and lowest in individuals with the $(CA)_5/(CA)_7$ genotype (P = 0.0007, P = 0.0007, and P = 0.0009, respectively). In $(CA)_2/(CA)_7$ heterozygotes, the percentages of the above cells were significantly higher compared with the $(CA)_5/(CA)_7$ genotype (P = 0.004, P = 0.009, and P = 0.04, respectively), but they did not differ significantly from $(CA)_7/(CA)_7$ homozygotes (Table 4).

Table 1a. Frequency of alleles and genotypes in the TNFR-II gene locus

Location in the TNFR-II gene	Allele frequency		Genotype frequency		
g.-1646T > G (promotor)	T 0.49	G 0.51	T/T n = 20 0.26	T/G n = 36 0.46	G/G n = 22 0.28
g.16534T > C (intron 1)	T 0.48	C 0.52	T/T n = 20 0.26	T/C n = 35 0.45	C/C n = 23 0.29

n – number of animals

Table 1b. Frequency of alleles and genotypes in the TNF-RII gene locus

Location in the TNFR-II gene	Allele frequency							
	$(CA)_2$	$(CA)_5$	$(CA)_7$	$(CA)_2/(CA)_2$ n = 16	$(CA)_2/(CA)_5$ n = 6	$(CA)_2/(CA)_7$ n = 32	$(CA)_5/(CA)_7$ n = 6	$(CA)_7/(CA)_7$ n = 18
g.16512 $(CA)_n$	0.45	0.08	0.47	0.21	0.08	0.41	0.08	0.23

n – number of animals

Table 2. Polymorphism g.-1646T>G of the TNF-RII gene and the percentage of cell populations expressing mTNFα protein and co-expressing viral protein p24 in cows infected with BLV

Cell subsets (%)	Cow genotypes					
	T/T n = 20		T/G n = 36		G/G n = 22	
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM
TNF+p24+	2.10	0.21	1.86	0.15	1.81	0.21
IgM+TNF+p24+	1.84	0.20	1.48	0.13	1.34	0.18
CD11b+TNF+p24+	1.71	0.16	1.38	0.10	1.16	0.11

SEM – standard error of the mean
n – number of animals

Table 3. Polymorphism of 16534T>C (intron) of the TNF-RII gene and the percentage of cell populations expressing mTNFα protein and co-expressing viral protein p24 in cows infected with BLV

Cell subsets (%)	Cow genotypes					
	T/T n = 20		T/C n = 35		C/C n = 23	
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM
TNF+p24+	2.11	0.21	1.83	0.15	1.85	0.20
IgM+TNF+p24+	1.83	0.20	1.46	0.13	1.37	0.17
CD11b+TNF+p24+	1.71	0.16	1.38	0.10	1.17	0.11

SEM – standard error of the mean
n – number of animals

Table 4. STR polymorphism at position 16512 in the TNF-RII gene locus and the percentage of cell populations expressing mTNFα protein and co-expressing viral protein p24 in cows infected with BLV

Cells subsets (%)	Genotypes of cows									
	$(CA)_2/(CA)_2$ n = 16		$(CA)_2/(CA)_5$ n = 6		$(CA)_2/(CA)_7$ n = 32		$(CA)_5/(CA)_7$ n = 6		$(CA)_7/(CA)_7$ n = 18	
	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM	$\bar{X}$	SEM
TNF+p24+	1.57	0.21	1.96	0.50	2.06[B]	0.16	0.64[BC]	0.09	2.36[C]	0.21
IgM+TNF+p24+	1.21	0.19	1.37	0.35	1.63[D]	0.14	0.48[DE]	0.08	2.02[E]	0.20
CD11b+TNF+p24+	1.00[F]	0.13	1.21	0.24	1.47[g]	0.11	0.76[gH]	0.15	1.90[FH]	0.16

n – number of animals
Mean values followed by the same uppercase letter are significantly different at P < 0.01
Mean values followed by the same lowercase letter are significantly different at P < 0.05

Discussion

BLV is an aetiological factor of enzootic bovine leukosis (EBL), a chronic neoplastic disease which is still detected in many countries on different continents (12). BLV shares many similarities with human T-lymphotropic virus type 1 (HTLV-1) which is responsible for T-cell leukaemia in humans (1). The mechanism of pathogenesis induced by HTLV-1 is not fully understood and numerous similarities with BLV suggest that cattle with EBL can be used as an animal model for studying the pathogenesis of HTLV-1 infections in humans (1). Moreover, recent research has demonstrated that women with BLV are at higher risk of breast cancer (8).

This study analysed the differences in the size of BLV-infected populations of PBMCs which play important immune roles. Surface markers CD11b and IgM, and membrane protein TNFα (mTNFα) were detected in the above cells. Surface receptor CD11b, also known as Mac-1 (CD11b/CD18) and complement receptor type 3 (CR3), is a member of the β_2-integrin family. It is expressed on the surface of innate immune system cells such as macrophages and neutrophils. CD11b modulates many important functions in cells, including adhesion, migration, and phagocytosis (19). Our previous studies revealed that A(−824)G and C(−793)T polymorphism in the TNFα gene was linked with the size of the BLV-infected subpopulation of CD11b co-expressing mTNFα protein (CD11b+TNFα+p24+ immunophenotype) (4, 5). Homozygous individuals with gene mutations (G/G and T/T) were characterised by a significantly higher percentage of this subpopulation than opposing homozygotes, which suggests that a genotype with two copies of the mutated gene significantly increases the frequency of CD11b+TNFα+ cells infected with BLV. BLV mostly targets B lymphocytes which express surface immunoglobulin M. Our previous study also demonstrated a link between g.−824A > G polymorphism in the TNFα gene and a higher frequency of cells with the IgM+TNFα+p24+ immunophenotype (4). The percentage of cells with the above immunophenotype was also significantly higher

in cattle with chronic lymphocytosis than in animals with non-proliferative (aleukemic) leukosis, which suggests that TNFα gene polymorphism is linked with the expansion of IgM+TNFα+p24+ cells in the progression of EBL.

The TNFα protein is a pleiotropic cytokine which plays an important role in the elimination of infectious factors, but it can also promote the progression of disease (16). Many cellular responses induced by TNFα are modulated by receptor TNF-RI or TNF-RII. Viruses modify or disrupt the TNF/TNF-R signalling pathway to evade the host's immune system and to proliferate in the body (16). The proteins produced by some viruses, including EBL and HIV, can bind with TNFα and/or its receptor to inhibit or modulate the TNF/TNF-R signalling pathway (22). Some viruses, including HIV, rely on the complement system to gain easier access to a cell, maximise replication and proliferate (6). CR3 (molecule CD11b) and other adhesion molecules facilitate the penetration of target cells by HIV-1 (6). The mechanisms involving TNF/TNF-R signalling pathways which are used by BLV to infect cells and which facilitate the progression of EBL remain insufficiently investigated. Transactivator Tax protein encoded by the pX region of BLV and HTLV-1 genomes permanently activates the NF-κB signalling pathway, deregulates the expression of cellular genes (TNFα and others) and immortalises cells infected with HTLV-1 (15). It is also believed that TNFα and receptor TNF-RII play an important role in the progression of EBL in cattle (17). TNF/TNF-R signalling pathways participate in many cellular processes, including responses to viral infections (16). A complex equilibrium between the viral life cycle and the expression of cytokines and their receptors, in particular TNFα and TNF-R, plays a key role in the pathogenesis of many viral diseases (20), including diseases caused by HIV, HTLV-I, and BLV. TNF-RII mRNA levels and the percentage of TNF-RII+ cells have been linked with EBL progression (20, 27). Tumour cells from cows with the clinical stage (lymphosarcoma) of EBL harboured only receptor TNF-RII (17). It is believed that TNF-RII is responsible for the proliferative and anti-apoptotic effects of

TNF-α (2). Certain mutations in the TNF-RII gene could modify these processes. The consequences of mutations in the TNF-RII gene have been described in many human diseases (11, 24), but their role in BLV infection and EBL progression has not been fully elucidated.

The influence of polymorphism at three sites of the TNF-RII gene on the size of PBMC populations naturally infected with BLV was analysed for the first time in this study. Neither of the two SNPs (5' flanking region, position -1646; intron 1, position 16534) significantly differentiated the size of TNF+, IgM+TNF+ and CD11b+TNF+ cell populations infected with BLV. Significant differences between genotypes were noted only in the STR locus (intron 1, position 16512). The number of BLV-infected cells was the highest in $(CA)_7/(CA)_7$ homozygotes and $(CA)_2/(CA)_7$ heterozygotes and the lowest in $(CA)_5/(CA)_7$ heterozygotes. The frequency of allele $(CA)_5$ was also lowest in the studied animals. The absence of $(CA)_5/(CA)_5$ homozygotes, the low frequency of allele $(CA)_5$ and the lowest percentage of the analysed subpopulations in $(CA)_5/(CA)_7$ heterozygotes could indicate that individuals with this genotype are less susceptible to BLV infection. The highest frequency of BLV-infected cells in homozygotes with the highest number of CA repeats (genotype $(CA)_7/(CA)_7$) seems to indicate that individuals with this genotype are highly susceptible to BLV infection, and it suggests a potential link with the length of these alleles.

STRs are present both within and between genes. They are probably formed by the slippage of DNA polymerase during replication, which leads to the insertion or deletion (less frequently) of repeated units (28). Microsatellite sequences could also be formed due to recombination errors during unequal crossing-over between homologous chromosomes (28). Microsatellite polymorphism is manifested by differences in the number of common motif repeats and, consequently, differences in allele length. STRs participate in various mechanisms related to the expression of their genes, including modulation of transcription factor binding, spacing between promoter elements, enhancers, cytosine methylation, alternative splicing, mRNA stability, selection of transcription start and termination sites, and unusual structural conformations (3, 28). Microsatellites in gene promoters have the highest number of functions, whereas their role in introns has not been thoroughly investigated. STRs in introns could influence gene transcription, alternative mRNA splicing and its transport to the cytoplasm (3, 28). These microsatellites contribute to the development of many neurological diseases (13) and tumours (10, 25).

Dinucleotide STRs with the CA motif are highly numerous. Microsatellites in intron 1 play a very important role by regulating gene transcription (3, 28). Research has demonstrated that the number of CA repeats in intron 1 and gene expression were linked with several cancer- or disease-related genes in humans (9, 30). For example, the epidermal growth factor receptor (EGFR) gene encodes protein tyrosine kinase which plays a key role in signal transduction pathways and processes such as proliferation, differentiation and survival of normal and neoplastic cells. Intron 1 of the EGFR gene harbours a highly polymorphic CA microsatellite (14). This polymorphic region plays a regulatory function in the transcription process (14). The cited authors observed that the number of CA repeats could influence DNA conformation after transcription factor binding. They reported a decrease in transcriptional activity in individuals with a higher number of CA repeats. A similar trend was also observed with a decrease in EGFR expression in a clinical study of breast tumours (9). Mostly longer CA alleles in intron 1 of the EGFR gene were detected in young patients with a family history of breast cancer (7).

The results of our previous research (4, 5) and the present study indicate that both the genotypes with two mutated copies of the TNFα gene at positions −824 and −797 and the $(CA)_7/(CA)_7$ homozygotes in the STR locus of the TNF-RII gene are characterised by a high frequency of CD11b+TNF+ and IgM+TNF+ cells naturally infected with BLV. The above could suggest that these polymorphisms participate in the TNF/TNF-RII signalling pathway modified by BLV and, consequently, increase susceptibility to BLV infection. This suggestion appears to be highly probable based on the involvement of the analysed cell populations in major immune processes, but further research is needed to validate this observation.

The results of the present study indicate that STR polymorphism and the number of CA repeats in intron 1 of the TNF-RII gene influence the frequency of TNF+, CD11b+TNF+ and IgM+TNF+ subpopulations naturally infected with BLV. The study also revealed that single-nucleotide polymorphisms at the remaining two sites of the TNF-RII gene are not linked with the size of the analysed cell subpopulations. Due to the small distance between the analysed polymorphic sites in intron 1, the observed relationship with microsatellite polymorphism appears to be highly interesting and merits further more detailed research.

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