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Relationship between polymorphism in the tumour necrosis factor-alpha gene and selected indices and cell subpopulations in naturally bovine leukaemia virus-infected and healthy cows

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ABSTRACT: The bovine leukaemia virus (BLV) is the aetiological agent of enzootic bovine leukosis. The aim of this study was to determine whether polymorphism in the *TNFα* gene at positions –824 A→G and –793 C→T in the 5' flanking region, and g.1787 in exon 4 influences leukocyte counts, the percentages of monocytes and apoptotic cells and the sizes of $CD14^+$, $CD14^+TNF^+$, TNF^+p24^+ and $CD11b^+TNF^+p24^+$ cell subsets in BLV-infected and healthy cows. A total of 127 Polish black and white Holstein-Friesian cows at 3–8 years of age were analysed. SNPs in the *TNFα* gene were determined using PCR-RFLP. BLV infection was diagnosed with the use of indirect immunofluorescence (IMF) and nested PCR tests. Peripheral blood mononuclear cells (PBMCs) were identified using IMF. Apoptotic cells were detected with the Annexin-V-FLUOS Staining Kit. The data were analysed using the Kruskal-Wallis test and a multiple comparison test in Statistica 12.0 software. *TNFα* gene polymorphism was associated with significant differences in the sizes of $CD14^+$, $CD14^+TNF^+$, TNF^+p24^+ and $CD11b^+TNF^+p24^+$ subsets in BLV^+ cows, but not in healthy animals. $CD11b^+TNF^+$ cells homozygous for G/G (–824) and T/T (–793) in the 5' flanking region appear to be more prone to BLV infection than opposite homozygotes. *TNFα* gene polymorphism was correlated with leukocyte counts and the percentage of apoptotic cells, but only in healthy cows. In conclusion, different relationships were observed between the analysed SNPs and selected indices in BLV^+ and healthy animals. These findings could suggest that the interaction between *TNFα* gene polymorphism and BLV affects the size of $CD14^+$, $CD14^+TNF^+$, TNF^+p24^+ and $CD11b^+TNF^+p24^+$ subsets. Such an interaction was not observed for the other analysed parameters.

Keywords: apoptosis; BLV; enzootic bovine leukosis; p24; IMF; immunophenotypes; m*TNFα*$^+$ cells; $CD11b^+$ cells; $CD14^+$ cells

The tumour necrosis factor alpha (*TNFα*) gene has been localised on bovine chromosome BTA 23 (23q22). The *TNFα* gene comprises four exons separated by three introns, and it has a length of 3909 bp (GenBank Z14137). An analysis of the *TNFα* gene sequence in cattle revealed single nucleotide polymorphisms (SNPs) at positions –824, –793 and –627 in the 5' flanking region and g.1787 in exon 4 (rs109967811) (Konnai et al. 2006). Polymorphism in the regulatory region can influence transcription, mRNA stability and translation efficiency and can determine resistance/susceptibility to various diseases (Shastry 2009).

The bovine leukaemia virus (BLV) belongs to the Deltaretrovirus genus of the Retroviridae family. It is the aetiological agent of enzootic bovine leukosis (EBL), which is a chronic neoplastic disease. EBL is characterised by the aberrant proliferation and maturation of B lymphocytes (Frie and Coussens 2015).

Studies of cattle infected with BLV suggest that the *TNFα* gene plays a significant role in the de-

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velopment of BLV infection and the progression of EBL (Konnai et al. 2006). Most authors have analysed only the influence of the A→G polymorphism at position –824 of the *TNFα* gene, which limits the significance of their findings (Konnai et al. 2006; Bojarojc-Nosowicz et al. 2015; Lendez et al. 2015).

In the present study, we investigated three polymorphic sites: at positions –824 and –793 of the 5' flanking region and at position g.1787 in exon 4. Two of these sites have been poorly researched to date. The current understanding of the role played by $CD14^+$ and $CD11b^+$ cells in BLV infection and the progression of EBL in cattle also remains limited.

Our hypothesis was that *TNFα* gene polymorphism at positions –824 and –793 of the 5' flanking region and at position g.1787 in exon 4 significantly influences the selected indices and the size of the analysed cell subsets in cattle.

The aim of this study was to determine whether polymorphism in the *TNFα* gene influences biological parameters such as leukocyte counts, percentages of monocytes and apoptotic cells, and the size of $CD14^+$, $CD14^+TNF^+$, TNF^+p24^+ and $CD11b^+TNF^+p24^+$ cell subsets in BLV-infected and healthy cows.

MATERIAL AND METHODS

Animals and sampling. The study was performed on 127 Polish black and white Holstein-Friesian cows at 3–8 years of age. The animals were kept indoors, in three herds, in accordance with general welfare requirements. All herds were free of tuberculosis and brucellosis. Cows were fed diets which were balanced to meet their protein and energy requirements.

Blood was sampled from the mammary vein upon the consent of the Local Ethics Committee (decision No. 13/2008/N/T). EDTA and heparin were used as anticoagulants. The presence of BLV was determined twice or three times, at monthly intervals, beginning from the second half of the first month after calving. The animals that tested negative for BLV in all replications were classified as BLV-negative. The group of BLV-positive cows was divided into two subgroups of aleukaemic BLV-infected animals (BLV^+AL) (leukocyte counts $< 12 \times 10^9$/l, lymphocyte counts $< 8 \times 10^9$/l) and animals with persistent lymphocytosis (BLV^+PL) ($> 12 \times 10^9$/l and $> 8 \times 10^9$/l, respectively).

Isolation and evaluation of genomic DNA. Genomic DNA was isolated from whole peripheral blood using the Master Pure Purification Kit (Epicenter, USA) in accordance with the manufacturer's recommendations. The quantity (µg/ml) and purity (%) of isolated DNA was evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA). The quality of DNA was determined electrophoretically on 1% agarose gels.

Determination of *TNFα* gene polymorphism at positions –824 and –793 in the 5' flanking region and g.1787 in exon 4. Polymorphism in the *TNFα* gene at position –824 A→G was determined using PCR-RFLP with certain modifications (Bojarojc-Nosowicz et al. 2011).

A fragment of the *TNFα* gene at position –793 C→T was amplified with the use of the following primers: *TNF*1/–793 – 5' TCCAAGGCTGGGGACTAGAG 3' and *TNF*2/–793 – 5' CAGGGGCTGAAAGTAACGCT 3' (Sigma, USA). The PCR product was digested with the Fast Digest *Bsr*I enzyme which recognises the 5' A<u>C</u>TG/G 3' restriction site (New England Biolabs). In the analysed gene fragments, the enzyme identified the CC nucleotide (C/C genotype) sequence and cut it into two fragments with lengths of 203 bp and 97 bp. C→T substitution (T/T genotype) abolished the restriction site, and a single DNA fragment with the size of the PCR product (300 bp) was observed on agarose gels.

The fragment of the *TNFα* gene with the C→T substitution at position g.1787 in exon 4 was amplified with the following primers: *TNF*1/exon 4 – 5' GGTGGGACTCGTATGCCAAT 3' and *TNF*2/exon 4 – 5' ATAGTCCAGGTAGTCCGGCA 3' (Sigma, USA). The PCR product of 350 bp was digested with the Fast Digest *Cvi*QI enzyme which recognises the 5' G/TA<u>C</u> 3' restriction site (Thermo Fisher Scientific, Lithuania). This sequence was observed in cows with the C/C genotype, and it was cut into two fragments (260 bp and 90 bp). C→T substitution (T/T genotype) abolished the restriction site, and only a single gene fragment of 350 bp was observed.

Haematological indices. Total leukocyte counts and lymphocyte percentages were determined in a specialist laboratory with the use of a haematology analyser (Sysmex SF3000 or Sysmex XT1800, SYSMEX Corporation, Japan) and based on blood smears stained with May-Grunwald-Giemsa solutions. The results were used to classify BLV-infected cows into BLV^+AL and BLV^+PL groups.

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Isolation of peripheral blood mononuclear cells (PBMCs) from whole peripheral blood. PBMCs were isolated from whole peripheral blood using the density gradient method with the Histopaque 1077 reagent (Sigma, USA) in accordance with the manufacturer's recommendations. The viability of the isolated PBMCs was controlled in a Burker counting chamber with 0.2% trypan blue solution, and cell counts were determined in a haematology analyser (Exigo-Boule Medical, Sweden). Cells with a minimum viability of 98% were suspended in RPMI medium (Sigma, USA) with the addition of penicillin and streptomycin (Sigma, USA). They were incubated for 18 hours at a temperature of 4 °C, divided into portions with a concentration of 2×10^6 cells/ml, and used for BLV detection and immunophenotyping.

Immunofluorescent (IMF) detection of BLV-infected lymphocytes. Cells infected with BLV were identified based on the expression of p24, the main viral capsid protein, detected with the use of monoclonal antibody BLV3 (anti-BLV p24, IgG1, VMDR Inc. Pullman, USA) and the method described by Kaczmarczyk et al. (2008). The IMF method was applied for BLV detection and immunophenotyping. A minimum of 600 cells were analysed in each smear.

Nested PCR. Detection of BLV was also confirmed by molecular means using nested PCR. The reaction mixture and the thermal profile of the reaction were previously described by Markiewicz et al. (2003).

Detection of CD14. PBMCs containing CD14 surface markers ($CD14^+$) were identified with mouse monoclonal M-M9 primary antibody (anti-CD14) against bovine epitopes of surface markers (working dilution of 1.0 µg/50 µl) (VMDR Inc. Pullman, USA). CD14 cell surface marker was detected with goat anti-mouse IgG (H^+L) secondary antibody labelled with Cascade Blue fluorochrome (blue signal) (Invitrogen, USA).

Detection of CD11b. The CD11b epitope was identified with mouse anti-bovine IgG2b M-M12A monoclonal antibody (anti-CD11b) (working dilution of 1.0 µg/50 µl) (VMDR Inc. Pullman, USA). CD11b cell surface marker was detected with goat anti-mouse IgG (H^+L) secondary antibody labelled with Cascade Blue fluorochrome (Invitrogen, USA).

Detection of TNFα membrane protein. The TNFα membrane protein (mTNFα) epitope was identified with mouse anti-bovine IgG2b monoclonal antibody (diluted 0.5 µg/50 µl) (Acris GmbH, Germany). mTNFα protein was detected with biotinylated goat anti-mouse immunoglobulin (Dako Cytomation, Denmark) as a secondary antibody and streptavidin conjugated with Texas Red fluorochrome (red signal) (Vector Lab. Inc., USA).

The subsets of PBMCs were labelled in accordance with a previously described method (Bojarojc-Nosowicz et al. 2015).

CD14 and cell surface markers on PBMCs were detected in a single-colour reaction. The expression of mTNFα protein on those cells was detected in dual and triple-colour reactions. A triple-colour reaction was used to detect individual PBMC subsets co-expressing p24 and mTNFα proteins. Antibodies were applied sequentially in three IMF reactions. The epitope of every cell surface marker was identified in the first reaction. The second reaction detected the p24 protein epitope. The mTNFα protein epitope was detected in the third reaction. All three IMF reactions (detection of cell surface markers, p24 and mTNFα protein epitopes) were performed on the same portion of PBMCs isolated from the peripheral blood of the same individual.

In the control smears, the above procedures were performed without the primary antibody. Smears were stored at a temperature of 4 °C and analysed under a fluorescence microscope (Axiolab-Zeiss, Germany) equipped with suitable filters, at × 1000 magnification. A minimum of 600 cells (subject to epitope) per smear were analysed. The percentage of cells expressing a given epitope/epitopes was calculated.

Apoptosis. Apoptotic cells were identified with the Annexin-V Fluos Staining Kit according to the manufacturer's recommendations (Roche Diagnostics). Annexin V binds phospholipids in a Ca^{2+}-dependent manner and shows affinity for phosphatidylserines, which allows detection of early apoptosis. Analyses were conducted with the use of a fluorescence microscope (Axiolab-Zeiss, Germany) with a suitable filter. Apoptotic cells emitted a green signal and were registered at × 1000 magnification. In each smear, 600 cells were analysed, and the percentage of apoptotic lymphocytes was calculated.

Statistical analysis. The results were analysed statistically using the Kruskal-Wallis non-parametric test in Statistica 12.0 software. Differences between groups were verified by multiple comparisons in a non-parametric test at $P < 0.05$, $P < 0.01$ and $P < 0.001$.

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Table 1. TNFα gene polymorphism and the analysed indices in the studied cows

		BLV⁻ cows (n = 49)						BLV⁺ cows (n = 78)					
Genotypes		A/A (n = 11)		A/G (n = 19)		G/G (n = 19)		A/A (n = 22)		A/G (n = 29)		G/G (n = 27)	
Locus	indices	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	leukocyte (× 10^9)	8.56^{A}	1.45	8.41^{B}	1.83	7.44^{AB}	1.43	11.29	6.46	10.70	5.65	10.10	3.55
–824 A→G	monocyte (%)	3.72	2.63	4.36^{d}	2.33	3.24^{d}	2.00	3.14	2.22	3.09	1.96	3.50	2.07
	apoptosis (%)	1.71^{e}	1.05	2.13^{e}	0.93	1.70	0.62	1.12	0.45	1.52	0.91	1.38	0.80
Genotypes		C/C (n = 14)		C/T (n = 19)		T/T (n = 16)		C/C (n = 20)		C/T (n = 40)		T/T (n = 18)	
Locus	indices	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	leukocyte (× 10^9)	8.80^{Ab}	1.51	7.94^{b}	1.78	7.58^{A}	1.45	12.34	8.09	10.22	4.03	9.98	3.85
–793 C→T	monocyte (%)	4.23	2.72	3.95	2.13	3.20	2.08	3.12	2.01	3.12	2.03	3.60	2.18
	apoptosis (%)	1.71^{d}	0.96	2.12^{d}	0.95	1.70	0.62	1.15	0.48	1.46	0.85	1.38	0.85
Genotypes		C/C (n = 17)		C/T (n = 21)		T/T (n = 11)		C/C (n = 25)		C/T (n = 29)		T/T (n = 24)	
Locus	indices	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	leukocyte (× 10^9)	7.72^{a}	1.54	8.00	1.86	8.71^{a}	1.28	9.87	3.47	11.00	5.79	11.05	6.13
Exon 4 C→T	monocyte (%)	3.18	2.04	3.95	2.27	4.42	2.65	3.30	1.80	3.12	2.20	3.20	2.18
	apoptosis (%)	1.69	0.62	2.18^{B}	1.08	1.55^{B}	0.60	1.42	0.76	1.48	0.97	1.17	0.44

BLV⁻ = bovine leukaemia virus-negative, BLV⁺ = bovine leukaemia virus-infected

Mean values followed by the same capital letter are significantly different at $P < 0.001$ or $P < 0.01$

Mean values followed by the same small letter are significantly different at $P < 0.05$

Table 2. The size of cell subpopulations with different TNFα genotypes in the studied cows

		BLV⁻ cows (n = 49)						BLV⁺ cows (n = 78)					
Genotypes		A/A (n = 11)		A/G (n = 19)		G/G (n = 19)		A/A (n = 22)		A/G (n = 29)		G/G (n = 27)	
Locus	cell subset (%)	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	$CD14^+$	5.77	1.47	5.39	1.35	5.85	1.45	4.84^{Ad}	1.61	5.51^{Bd}	1.30	7.21^{AB}	1.42
	$CD14^+TNF^+$	3.44	0.94	3.42	0.81	3.37	0.96	2.95^{E}	1.14	3.12^{F}	0.86	4.10^{EF}	0.94
–824 A→G	TNF^+p24^+	0.00	0.00	0.00	0.00	0.00	0.00	1.20^{GH}	1.06	1.92^{H}	1.21	2.48^{G}	1.51
	*$CD11b^+TNF^+p24^+$	0.00	0.00	0.00	0.00	0.00	0.00	0.95^{Ij}	0.78	1.42^{j}	0.88	1.79^{I}	0.96
Genotypes		C/C (n = 14)		C/T (n = 19)		T/T (n = 16)		C/C (n = 20)		C/T (n = 40)		T/T (n = 18)	
Locus	cell subset (%)	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	$CD14^+$	5.69	1.50	5.70	1.48	5.56	1.32	5.57^{A}	1.76	5.46^{B}	1.50	7.24^{AB}	1.48
	$CD14^+TNF^+$	3.44	0.93	3.51	0.87	3.24	0.88	3.20^{D}	1.18	3.16^{E}	0.96	4.18^{DE}	0.98
–793 C→T	TNF^+p24^+	0.00	0.00	0.00	0.00	0.00	0.00	1.45^{F}	1.21	1.77^{G}	1.27	2.72^{FG}	1.42
	*$CD11b^+TNF^+p24^+$	0.00	0.00	0.00	0.00	0.00	0.00	0.93^{Hi}	0.83	1.43^{ij}	0.92	1.87^{Hj}	0.86
Genotypes		C/C (n = 17)		C/T (n = 21)		T/T (n = 11)		C/C (n = 25)		C/T (n = 29)		T/T (n = 24)	
Locus	cell subset (%)	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
	$CD14^+$	5.50	1.31	5.72	1.52	5.78	1.42	6.38^{A}	1.75	6.10^{B}	1.56	5.15^{AB}	1.66
	$CD14^+TNF^+$	3.18	0.90	3.52	0.86	3.53	0.92	3.66^{d}	1.22	3.46	0.93	3.07^{d}	1.04
Exon 4 C→T	TNF^+p24^+	0.00	0.00	0.00	0.00	0.00	0.00	1.94^{e}	1.42	2.47^{F}	1.33	1.18^{eF}	1.00
	*$CD11b^+TNF^+p24^+$	0.00	0.00	0.00	0.00	0.00	0.00	1.51^{G}	1.00	1.74^{H}	0.87	0.90^{GH}	0.75

BLV⁻ = bovine leukaemia virus-negative, BLV⁺ = bovine leukaemia virus-infected

*Cell subpopulations whose size was also analysed by Bojarojc-Nosowicz et al. (2015)

Mean values followed by the same capital letter are significantly different at $P < 0.001$ or $P < 0.01$

Mean values followed by the same small letter are significantly different at $P < 0.05$

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RESULTS

SNPs in the *TNFα* gene were analysed at positions –824 (A→G) and –793 (C→T) in the 5' flanking region and g.1787 in exon 4 (C→T). All three genotypes were identified in each of the analysed sites (Tables 1 and 2). A BLV analysis revealed that 78 cows were infected with the virus, of which 83.3% were aleukaemic (BLV^+AL). Forty-nine animals were free of BLV (BLV^-) (Tables 3 and 4).

An analysis of the results obtained for the BLV^+ group revealed significant differences between *TNFα* genotypes in the examined sites and the percentages of cells with $CD14^+$, $CD14^+TNF^+$, TNF^+p24^+ and $CD11b^+TNF^+p24^+$ phenotypes (Table 2). The highest percentages of the analysed cell subpopulations were noted in cows with two copies of the gene mutated at G/G (–824) and T/T (–793) ($P < 0.001$) (Table 2). C→T polymorphism in exon 4 resulted in different changes in the percentages of the analysed cell subpopulations. The percentage of cells with $CD14^+$ and $CD14^+TNF^+$ phenotypes was significantly higher in C/C than in T/T homozygotes. Additionally, C/T heterozygotes in exon 4 were characterised by a significantly higher percentage of $TNFα^+$ and $CD11b^+TNF^+$ cells expressing viral protein p24 (TNF^+p24^+ and $CD11b^+TNF^+p24^+$ phenotypes) than T/T homozygotes (Table 2).

The above relationships were not observed in the BLV-negative group (Table 2). However, significant differences were noted in leukocyte counts and the percentages of apoptotic cells (Table 1).

Table 3. The analysed indices in BLV-infected and healthy cows

Indices	BLV^- (n = 49)		BLV^+ (n = 78)			
			AL (n = 65)		PL (n = 13)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Leukocytes ($\times 10^9$)	8.06^{AB}	1.67	8.87^{AB}	1.89	20.47^{A}	6.86
Monocytes (%)	3.79	2.32	3.20	2.00	3.46	2.40
Apoptosis (%)	1.87^{D}	0.88	1.45^{DE}	0.812	0.96^{DE}	0.39

AL = aleukaemic, BLV^- = bovine leukaemia virus-negative, BLV^+ = bovine leukaemia virus-infected, PL = persistent leukaemia

Mean values followed by the same capital letter are significantly different at $P < 0.001$ or $P < 0.01$

An analysis of mutated alleles at positions –824 and –793 of the *TNFα* gene revealed significantly lower ($P < 0.001$) leukocyte counts in G/G and T/T homozygotes than in A/A and C/C homozygotes. The opposite was observed for the C→T polymorphic site in exon 4, where the highest values were noted in T/T homozygotes and the lowest values in C/C homozygotes (Table 1). Heterozygotes were characterised by less regular variations in leukocyte counts than homozygotes. Significant differences in the percentages of apoptotic PBMCs as well as relatively low and generally non-significant differences in the percentages of monocytes were also observed (Table 1). The percentages of apoptotic cells were significantly higher ($P < 0.05$) in A/G and C/T heterozygotes at polymorphic sites of the 5' flanking region than in A/A and C/C homozygotes. In the analysed locus of exon 4, differences were observed between C/T and T/T genotypes ($P < 0.01$). The percentage of apoptotic cells was significantly lower in T/T homozygotes than in C/T heterozygotes (Table 1). Such differences were not observed in BLV^+ cows (Table 1).

The influence of BLV infection and EBL progression on selected indices was also investigated (Tables 3 and 4). Significant differences were observed only in leukocyte counts and the percentages of apoptotic cells. As expected, leukocyte counts were highest in BLV^+PL cows and lowest in healthy animals ($P < 0.001$) (Table 3). The percentage of apoptotic cells exhibited a different trend: it was

Table 4. The size of the analysed cell subpopulations in the studied cows

Cell subset (%)	BLV^- (n = 49)		BLV^+ (n = 78)			
			AL (n = 65)		PL (n = 13)	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
$CD14^+$	5.65	1.42	6.00	1.72	5.38	1.71
$CD14^+TNF^+$	3.40	0.89	3.42	1.09	3.35	1.09
TNF^+p24^+	0.00	0.00	1.71^{a}	1.29	3.08^{a}	1.22
*$CD11b^+TNF^+p24^+$	0.00	0.00	1.37	0.97	1.68	0.64

AL = aleukaemic, BLV^- = bovine leukaemia virus-negative, BLV^+ = bovine leukaemia virus-infected, PL = persistent leukaemia

*Cell subpopulations whose size was also analysed by Bojarojc-Nosowicz et al. (2015)

Mean values followed by the same small letter are significantly different at $P < 0.05$

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lowest in BLV$^+$PL cows and highest in BLV$^-$ animals ($P < 0.001$) (Table 3). The percentage of monocytes was similar in all animals (Table 3). In BLV$^+$ cows, an immunophenotyping analysis revealed a significantly higher percentage of TNF$^+$p24$^+$ cells in BLV$^+$PL cows than in BLV$^+$AL animals (Table 4). The percentages of the remaining cell subsets were similar in all groups (Table 4).

DISCUSSION

EBL remains a threat in Europe and in other parts of the world (Frie and Coussens 2015). The disease is most frequently diagnosed in the subclinical aleukemic stage (AL) or as persistent lymphocytosis (PL). Lymphosarcoma (LS), the clinical stage of EBL, is diagnosed in only 0.1–10% of individuals infected with BLV, and it has lethal consequences (Frie and Coussens 2015).

The correlation between *TNFα* gene polymorphism and resistance/susceptibility to mastitis (Ranjan et al. 2015), tuberculosis (Cheng et al. 2016) and EBL has been investigated in a limited number of studies (Konnai et al. 2006; Bojarojc-Nosowicz et al. 2015; Lendez et al. 2015).

In the present study, *TNFα* gene polymorphism was correlated with the percentage of CD14$^+$ and CD14$^+$TNF$^+$ cells in BLV$^+$ cows. No such correlations were found in healthy animals. The above results could point to the presence of interactions between SNPs in the promoter region of the *TNFα* gene and BLV protein/proteins that modify the expression of that gene. Other authors have also demonstrated that viral factors can modify regulation of the promoter region of the *TNFα* gene (Fu et al. 2002).

Membrane-bound CD14 (mCD14) is a glycoprotein synthesised mainly by monocytes and macrophages, and, less frequently, by granulocytes (Wright et al. 1990). In ruminants, mCD14 is a specific surface marker for monocytes and macrophages (Sopp et al. 1996), but its role has been insufficiently investigated.

In this study, the observed differences in the percentages of CD14$^+$ and CD14$^+$TNF$^+$ cells between animals with various genotypes in all analysed polymorphic sites of the *TNFα* gene could indicate that the *TNFα* polymorphism is correlated with the expression of mCD14 and the coexpression of mCD14 and mTNFα on PBMCs in BLV$^+$ cows. The percentage of mCD14$^+$ cells was higher than the percentage of monocytes, which could suggest that in cattle, as well as in healthy humans (Ziegler-Heitbrock et al. 1994) and patients with B-cell chronic lymphocytic leukaemia (B-CLL) (Morabito et al. 1987), this molecule is also synthesised by B cells. According to Schumann et al. (1994), mCD14 expression on human lymphocytes could represent a link between non-specific and specific immunity to facilitate the elimination of pathogens. CD14 binding by lymphocytes could be an important element of the lymphocyte regulation mechanism with the involvement of monocytes (Jabara and Vercelli 1994). However, the observed correlations were difficult to interpret at this stage of research.

No significant differences in the percentages of CD14$^+$ and CD14$^+$TNF$^+$ cells were noted between healthy cows and animals with both subclinical forms of EBL (Table 4), which could suggest that mCD14 is not involved in the pathogenesis of EBL.

The results of a preliminary study (Bojarojc-Nosowicz et al. 2015) and the present experiments also revealed a correlation between *TNFα* gene polymorphism and the size of the TNF$^+$p24$^+$ cell subpopulation. *TNFα*$^+$ cells homozygous for G/G (–824) and T/T (–793) were more susceptible to BLV infection than A/A and C/C homozygotes. A different direction of changes in the percentage of TNF$^+$p24$^+$ cells was observed in the analysed site of exon 4 (Table 2). In the preliminary study and the present experiments, the percentage of TNF$^+$p24$^+$ cells was nearly two-fold higher in BLV$^+$PL than in BLV$^+$AL cows (Table 4), which indicates that m*TNFα* is involved in the progression of EBL.

TNFα participates in the elimination of infectious factors, but it can also speed up the progression of disease (Herbein and Khan 2008). Some viruses, such as HIV, EBV, cowpox and poxvirus, produce proteins that can bind to TNFα and/or TNFR to inhibit TNFα or modulate the TNF/TNFR signalling pathway and control viral replication (Rahman and McFadden 2006; Herbein and Khan 2008). The mechanisms associated with the TNF/TNFR signalling pathway that lead to BLV infection and the progression of EBL have not yet been explored. However, the existing evidence indicates that *TNFα* gene polymorphism and mTNFα protein play an important role in these processes. The mTNFα glycoprotein could be used by viral protein/proteins to modulate the TNF/TNFR signal-

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ling pathway and control the replication of BLV in *TNFα*$^+$p24$^+$ cells, which has been observed in humans infected with HIV. In early stages of infection, viral proteins (mainly Nef, Vpr and Tat) modulate the TNF/TNFR signalling pathway, which enhances HIV replication and virion production in infected CD4$^+$ T cells and macrophages (Herbein and Khan 2008). In humans infected with HIV, viral Tat protein participates in the regulation of *TNFα* gene expression in monocytes and macrophages via NF-κB-dependent transcription (Chen et al. 1997). The results of a preliminary study (Bojarojc-Nosowicz et al. 2015) and the present experiments suggest that *TNFα* gene polymorphism and mTNFα play important roles in the pathogenesis of EBL.

In a preliminary study, A→G polymorphism at position –824 in the *TNFα* gene was correlated with the percentage of CD11b$^+$ cells co-expressing *TNFα* and viral protein p24 (CD11b$^+$TNF$^+$p24$^+$ immunophenotype) (Bojarojc-Nosowicz et al. 2015). The above correlations were confirmed in the present experiments. The percentage of CD11b$^+$ cells was higher in G/G (–824) and T/T (–793) homozygotes than in opposite homozygotes. The above index was also correlated with C→T polymorphism in exon 4, but the direction of changes in the percentage of CD11b$^+$TNF$^+$p24$^+$ cells was somewhat different (Table 2).

CD11b or Mac-1 (CD11b/CD18) is an important complement receptor type 3 (CR3) which participates in cellular adhesion and phagocytosis of molecules or cells that bind complement receptors (Ghannam et al. 2008). CD11b also plays an important role in antigen presentation to B-cells and T-cells (Ghannam et al. 2008). Some viruses, including HIV, rely on the complement system (such as complement C3) to enter cells, maximise replication and spread in the body (Bouhlal et al. 2007).

The current study demonstrated a clear correlation between *TNFα* gene polymorphism and the percentage of CD11b$^+$TNF$^+$p24$^+$ cells. The obtained results could also indicate that CD11b$^+$TNF$^+$ cells are more susceptible to BLV infection in G/G (–824) and T/T (–793) homozygotes in the 5' flanking region. The CD11b$^+$TNF$^+$p24$^+$ subset could also participate in the initiation of BLV infection, but not in the progression of EBL, because the size of these subpopulations did not differ between BLV$^+$AL and BLV$^+$PL cows. The higher sensitivity of CD11b$^+$TNF$^+$ cells that were homozygous for G/G (–824) and T/T (–793) in the 5' flanking region to BLV infection could be associated with the fact that G/G and T/T individuals are more likely to have a high proviral load (HPL) than A/A and C/C homozygotes where the frequency of the CD11b$^+$TNF$^+$ subset was significantly lower ($P < 0.001$). Similar results were reported by Lendez et al. (2015) in whose study cows with the G/G genotype of *TNFα* polymorphism at position –824 (A→G) were significantly more often classified as BLV$^+$ with HPL than BLV$^+$ with a low proviral load (LPL).

SNPs located in the 5' flanking region of the *TNFα* gene can affect gene expression by changing transcription factor binding sites. An *in silico* analysis performed with Matinspector 8.2 Genomatix Software GmbH (Cartharius et al. 2005) revealed new putative binding sites for PAX-2/5/8 and PRDM transcription factors at position –824 G in the 5' flanking region of the *TNFα* gene, which were not recognised at the same position in the *TNFα* sequence with the A allele. The paired box transcription factor Pax-5, known as a B-cell-specific activator protein, is a key regulator of lineage-specific gene expression and differentiation in B lymphocytes (Fitzsimmons et al. 2001). PRDM (PRDI-BF1 and RIZ homology domain containing) proteins have a pivotal role in the transduction of signals that control cell proliferation and differentiation and, consequently, neoplastic transformation (Zazzo et al. 2013). An analysis of position –793 (C→T) in the 5' flanking region of the *TNFα* gene revealed a connection between CCAAT binding factors for the T allele. The CCAAT transcription factor belongs to the family of CCAAT/enhancer-binding proteins (C/EBP α, β and δ). C/EBP β plays an important role in promoting proliferation, and its levels are increased in different types of tumours (Ramji and Foka 2002). C/EBP β-deficient mice are characterised by impaired expression of serum amyloid A and P proteins, a1-acid glycoprotein, complement component (C3) and *TNFα* (Ramji and Foka 2002).

In this study, correlations were not observed between the SNPs of the *TNFα* gene and the analysed cell subpopulations in healthy (BLV$^-$) cows. However, the SNPs of the *TNFα* gene were correlated with leukocyte counts and the percentages of apoptotic cells, but the direction of the observed changes varied between gene fragments harbouring

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the analysed SNPs. These findings are difficult to interpret at this stage of research.

C→T SNP in exon 4 of the *TNFα* gene does not result in an amino acid substitution (p.TYR192TYR) (NP_776391.2). However, recent studies have revealed several mechanisms by which synonymous changes may affect protein function and cause numerous diseases (Sromek et al. 2017). Synonymous mutations, similarly to pathogenic nonsense and missense mutations, change the dynamics of the corresponding proteins in the *TNFα* signalling network, leading to a significant increase in the critical dose of *TNFα* necessary for cell death (Sromek et al. 2017).

The percentage of apoptotic cells was two-fold lower in BLV$^+$PL cows than in healthy animals, whereas in BLV$^+$AL individuals, the percentage of apoptotic cells was significantly higher than in BLV$^+$PL cows, but lower than in healthy animals (Table 3).

Retrovirus infections contribute to spontaneous and uncontrolled proliferation of lymphocytes which do not undergo apoptosis (Cantor et al. 2001). In BLV$^+$PL cows, B cells undergo apoptosis in the G_2/M phase of the cell cycle, whereas B cells expressing the virus are more likely to remain in the G_0/G_1 phase and avoid apoptosis (Stone et al. 2000). The above mechanisms have not been elucidated in animals infected with BLV. The observed significant differences in leukocyte counts and the percentages of apoptotic cells between BLV$^+$ and healthy cows are characteristic of EBL.

In conclusion, the results of this study indicate that *TNFα* gene polymorphism induced significant differences in the sizes of CD14$^+$, CD14$^+$TNF$^+$, TNF$^+$p24$^+$ and CD11b$^+$TNF$^+$p24$^+$ subpopulations in BLV$^+$ animals, but not in healthy cows. These findings could suggest that the interaction between *TNFα* gene polymorphism and viral protein/proteins could influence the expression of CD14, mTNFα and CD11b molecules in BLV$^+$ animals. They also point to a clear correlation between *TNFα* gene polymorphism and the percentage of CD11b$^+$TNF$^+$p24$^+$ cells. It appears that in homozygotes with two mutated genes G/G (–824) and T/T (–793) in the 5' flanking region, cells are more prone to BLV infection than in opposite homozygotes. This study demonstrates that *TNFα* gene polymorphism, mTNFα and the CD11b$^+$TNF$^+$p24$^+$ subset play important roles in BLV infection. It was also demonstrated that *TNFα* gene polymorphism is correlated with leukocyte counts and the percentage of apoptotic cells only in healthy cows, thus suggesting the presence of different relationships between the analysed SNPs and selected indices in BLV$^+$ and healthy animals.

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