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Cytokine secretion in stem cells of cattle infected with bovine leukaemia virus

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Abstract

Introduction: Bovine leukaemia virus (BLV) is a Deltaretrovirus responsible for enzootic bovine leukosis, the most common neoplastic disease of cattle. It deregulates the immune system, favouring secondary infections and changes in the blood and lymphatic tissues. Blood homeostasis depends on functional haematopoietic stem cells (HSCs). Bone marrow is populated by these cells, which express $CD34^+$ and $CD35^+$ surface antigens and produce and release cytokines involved in the maintenance of haematopoiesis. The aim of the study was determination of the profile of cytokine production by $CD34^+$ stem cells of cattle naturally infected with BLV. **Material and Methods:** The HSCs were generated from the blood and lymphoid organs of cows infected with BLV and healthy control cows with immunomagnetic separation and anti-$CD34^+$ monoclonal antibodies. Isolated $CD34^+$ cells were cultivated for two weeks with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor. The levels of IL-6, IL-10, IL-12p40, IL-12p70, interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) were determined in culture fluid by flow cytometry. **Results:** The expression of IL-6, IL-12p70 and TNF-α in blood HSCs was higher in BLV^+ cows than in the control animals. In bone marrow HSCs of infected cows, IL-12, TNF-α and IFN-γ were more concentrated, but in these cows' spleen HSCs only expression of IL-10 was elevated. In HSCs isolated from the lymph nodes of leukaemic cows, only TNF-α secretion was lower than in control cows, the other cytokines being more potently secreted. **Conclusion:** Infection with BLV caused statistically significant differences in cytokine expression by HSC $CD34^+$ cells.

Keywords: bovine leukaemia virus (BLV), stem cells $CD34^+$, cytokines, flow cytometry.

Introduction

Bovine leukaemia virus (BLV) is an oncogenic Deltaretrovirus that belongs to the *Retroviridae* family. The virus naturally infects cattle and induces a persistent infection that remains mainly asymptomatic but can also lead to leukaemia or lymphoma. It is closely related to human T-lymphotropic viruses 1 and 2 (HTLV-I and HTLV-II) and to simian T-cell leukaemia viruses (STLVs) (1). Human T-lymphotropic virus I causes T-cell leukaemia, and its type 2 counterpart has been found in dermatopathic lymphadenopathy, hairy T-cell leukaemia and prolymphocytic leukaemia cases. Bovine leukaemia virus has been used as a model of HTLV infection and the course of the disease. It integrates into the host genome, and in many cases no outward effect of infection or clinical disease is observed (11). The BLV envelope (env) glycoprotein, which is composed of the gp 51 outer membrane and the gp 30 transmembrane glycoprotein, is directly involved in infectivity events, and like the p24 major structural protein, can elicit a strong immune response in infected cattle. Diagnosis of BLV infection is commonly performed by tests for antibodies recognising the p24 capsid protein and structural glycoprotein gp 51.

The virus can be detected by *in vitro* culture of peripheral blood mononuclear cells (PBMCs), where BLV is present when the host is infected, and can also be observed in tumour cells as provirus integrates into the DNA of infected cells. It was also found in the cellular fraction of various body fluids (nasal and bronchial fluids, saliva and milk). Viral antigens and proviral DNA can be identified in the semen, milk and colostrum of infected animals. This Deltaretrovirus has been transmitted horizontally by direct contact between animals, iatrogenic procedures or insect bites effecting the transfer of cells infected with virus from the milk, blood and body fluids of infected dams. Infection with BLV is distributed worldwide; in warm climates there are indications of insect-borne propagation of the virus. The genetic material of BLV in biting flies can be detected by PCR; however, despite the presence of

PCR-positive insects, natural transmission to Holstein or Aberdeen Angus steers and heifers was not confirmed (25).

Bovine leukaemia virus infection can be experimentally transmitted to sheep, goats and alpacas (12, 19). The primary targets of BLV are B lymphocytes, but tropism to T-cells has been indicated for HTLV-I. Infection with BLV induces a non-neoplastic persistent lymphocytosis of B-lymphocytes in about 30% of infected animals. Only a small proportion of infected animals develop symptoms of enzootic bovine leukaemia; the less than 1% typical prevalence of persistent BLV in peripheral blood cells is the reason for infection being asymptomatic in the majority of infected cattle. The infection can progress to fatal leukaemia/lymphoma and to formation of tumours. The virus carries the classical genes (*gag*, *pro*, *pol* and *env*, the latter of which has an adjacent X region before the long terminal repeat sequence as do HTLV-I and -II) which are required to complete the viral cycle: genesis and budding of a virion, and infection, reverse transcription and integration into the host cell chromosome. Infection with BLV, similarly to infection with HTLV-I, caused infected B-cells to be arrested in the G0/G1 phase of the cell cycle and protected from apoptosis (29). Some authors suggested that B-cell progenitors with the $CD5^+$ marker are more susceptible to infection and suggested a relationship of B-cell phenotype to virus tropism (22). The infected progenitor is detected early after viral infection and could be a contributory factor to clonal expansion and genetic instability, which is a feature of cancer cells. Clear sequence homologies are shown by BLV, HTLV-I and HTLV-II. The pathology of the BLV-induced disease, most notably the absence of chronic viraemia, a long latency period and lack of preferred proviral integration sites in tumours, is similar to that of the adult T-cell leukaemia/lymphoma induced by HTLV-I (18). Infections with BLV and HTLV-I are both characterised by low or undetectable viral expression *in vivo*, but cells isolated from an infected animal during the pre-malignant phase spontaneously expressed viral proteins *in vitro* (18).

Vaccination of cattle against BLV infection is still being researched. Retroviruses such as BLV have a relatively stable genome, and thus an effective vaccine may be possible. However, many previous attempts at developing a vaccine against BLV infection have been unsuccessful. Many research teams are engaged in developing a vaccine with its antigen attenuated by targeted mutations and deletions. This vaccine ought to effectively protect animals against infection, and it will not be difficult to distinguish vaccinated cattle from naturally infected animals.

Stem cells are precursor cells that have the capacity to self-renew and to generate multiple mature cell types. They may be categorised into two major classes: pluripotent and multipotent stem cells. The first kind are cells in early embryos and they are able to replenish all cell types in the animal. Multipotent stem cells are present in various organs in the body – blood, bone marrow, adipose tissue and cord blood – and they are able to replenish specific tissues. The current classification of these cells is by their ability to differentiate themselves and by their origin. Haematopoietic stem cells (HSCs) present in bone marrow are among the best-known adult stem cells (16). The haematopoietic system has evolved to generate billions of mature cells every day. In adult organisms the developmental process takes place in bone marrow.

In physiological conditions haematopoietic homeostasis is maintained by the normal balance of proliferation and self-renewal or apoptosis of HSCs. In pathological conditions, stress state or infection, the levels of cytokines increase, and this can enhance differentiation and proliferation (27). Abnormal haematopoiesis, which is the result of signalling pathway disorders, can trigger the development of cancer or leukaemia. Viral infections can cause deregulation of normal haematopoietic signalling pathways (35). Some authors indicated that retrovirus-mediated leukaemogenesis demonstrated similarities with the development of other types of tumour (2).

Haematopoietic stem cells are pluripotent cells that generate new cells of all blood lineages *via* progenitor and precursor cells. The population of HSCs is characterised by the surface expression of $CD34^+$ and they can proliferate and differentiate into individual mature haematopoietic cells (10). In adult organisms HSCs are maintained within bone marrow and can differentiate into two haematopoietic progenitors: the first is a common lymphoid progenitor population and generates B, T and natural killer cells, and the second is a common myeloid progenitor and generates granulocytes, neutrophils, eosinophils, erythrocytes and macrophages (7). Haematopoietic stem cells are self-renewing cells, which are indispensable to haematology and transplantation studies (5). All types of blood cells can develop from a single haematopoietic stem cell, which indicates their possibilities. These cells ensure a constant supply of mature blood cells. They can be used for optimising engraftment as a therapeutic procedure to treat patients suffering with haematopoietic diseases such as leukaemia (30). Isolation of biologically homogenous populations of HSCs is essential for functional and molecular investigations of the mechanisms that regulate their self-renewal and lineage restriction and differentiation (9). Identification of HSCs on the basis of their functional and phenotypic characteristics is also important for accurate estimation of HSC numbers and evaluation of patient responses to cytokine therapy (15), *ex vivo* expansion or other manipulations aimed at promoting haematopoietic recovery after cytoreductive therapy or transplantation (8).

Similarly to physiological haematopoiesis, leukaemia is also hierarchically organised and the subpopulation of leukaemic cells – leukaemic stem cells (LSCs) – is responsible for initiation of disease,

progression, relapse, the maintenance of malignant cells and the induction of their greater differentiation. There are cancer stem cells, which present the characteristics of both physiological stem cells and tumour cells. It is established that stem cells can be potential therapeutic agents in many diseases such as cancer, neurodegenerative diseases, cardiac insufficiency, orthopaedic problems and diabetes (20, 34). Currently, haematooncological disorders are treated with HSC transplantation (3, 15). In veterinary medicine therapeutic procedures with the use of multipotent stem cells were described in horses and dogs (14). Adipose tissue can be also the source of stem cells which are used as regenerative therapy (3).

Leukaemia stem cells are a small subpopulation of leukaemic cells which have stem-cell properties. They can propagate leukaemia *in vivo* while being able to differentiate into committed progenitors. Their capacity for disease initiation also earns them the description as leukaemia-initiating cells (LICs). These cells remain unaffected by many therapeutic procedures and represent the major cause of disease relapse (19).

Haematopoietic stem cells were functionally defined by their ability to serially engraft recipients and replenish all myelolymphoid lineages because of their activity to self-renew and differentiate (26). These cells are rare and represent only 0.005% to 0.01% of all nucleated cells in bone marrow. They can be isolated on the basis of the distinct pattern of cell surface marker expression. The leukaemic stem cells predominantly belong to the $CD34^+CD38^-$ compartment, but LICs are not restricted to $CD34^+CD38^-$ cells and are also present in the $CD34^-$ fractions (5). Abnormal activation of these cell-signalling pathways induces cell cycling and development of leukaemia (13). The main driver of LSCs and chronic myelogenous leukaemia is the BCR-ABL1 fusion protein encoded by an abnormal gene, which is present in more than 90% of patients suffering this type of leukaemia (23).

The several cell types in various body compartments are integrated into an immune response by cytokines, which are pleiotropic proteins or small glycoproteins with a molecular weight less than 30 kDa (<200 amino acids). Cytokines are the intercellular messengers in the immune system. They are produced by and cause the response from every cell except erythrocytes. They are categorised by their producers, which are either Th1 cells or Th2 cells. They may be useful biomarkers for health and disease and can act as diagnostic, prognostic and therapeutic agents (28). Cytokines have a key role in many diverse processes including differentiation, inflammation, angiogenesis, tumorigenesis, neurobiology and viral pathogenesis (29). Cytokines are secreted by stem cells generated from different lymphatic tissues of BLV-infected and noninfected cattle. The population of HSCs is characterised by the surface expression of $CD34^+$, and they can proliferate and differentiate into individual mature haematopoietic cells.

The aim of the study was determination of cytokine secretion in $CD34^+$ cells isolated from the blood, bone marrow, spleen and lymph nodes of BLV-infected and healthy cows.

Material and Methods

Animals. The experiment was performed on a group of Black-and-White Lowland cattle at the age of 4–6 years. The experimental group was composed of 35 cows infected with BLV (BLV^+), the immunological status of which was determined in ELISA and real-time PCR tests. Fifteen uninfected, healthy cows (BLV^-) served as the control group. Samples of blood and lymphatic organs were taken in an abattoir, and no experiments on live animals were conducted, which meant that no permission from an Ethics Committee was required.

Analysis of cytokine secretion in haematopoietic stem cells. Stem cells which were $CD34^+$ were isolated from the blood, bone marrow, spleen and lymph nodes of cows infected with BLV. Stem cells were also isolated from the same tissue and organs of uninfected cows as control material. Briefly, the samples of organs were taken aseptically, cut with scissors and forceps, stirred and pressed by steel mesh and suspended in Roswell Park Memorial Institute (RPMI) 1640 culture medium with 20% of foetal calf serum (FCS), 0.3 mg/mL of L-glutamine and antibiotic–antimycotic solution at 1:100 dilution. The cellular suspensions were filtered by sterile gauze, cells were counted and $CD34^+$ cells were isolated with the use of magnetic microbeads coated with anti-CD34 monoclonal antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). The tubes were incubated for 1 h at room temperature, then cells were separated by magnet and washed three times. Separated cells at a concentration of 1×10^6 cells/mL of RPMI medium complemented with 10% FCS and antibiotic–antimycotic solution were cultured in plastic flasks in an incubator at 37°C and atmosphere of 5% CO_2 for 7 d. After that time, the supernatant from cell cultures was collected, lyophilised and stored at −20°C before the planned assay.

Determination of the interleukin (IL)-6, IL-10, IL-12p40, IL-12p70, tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) cytokine levels was achieved with a BD Cytometric Bead Array Kit and Flex Set multiplexed system (Biosciences PharMingen, San Diego, CA, USA) according to the producer's recommendations. An advantage of this method is that a reaction requires a very small amount of the investigated material (25–50 μL).

This method is very sensitive and is able to detect a concentration of an investigated substance of only 0.274 pg/mL.

In the cytometric bead array, molecules of a particular size were used, which were capable of laser light dispersion in individually determined manners. These molecules were used for fixation of different soluble compounds and their quantitative analysis by

fluorescence emission detection and cytometric analysis. Each molecule (capture bead) was coated with antibodies specific for one analysed protein and had its own fluorescence, making it possible to prepare a mixture of these beads and analyse multiple proteins at the same time. The mixture of these capture beads permitted simultaneous detection of up to 30 different compounds in one individual liquid sample.

The addition of detecting antibodies coupled with phycoerythrin to the mixture caused the formation of complexes analogical to ELISA. All procedures of capture bead mixture and cytometry standard preparation were performed exactly to the producer's instructions. Samples were incubated with a mixture of anti-allophycocyanin and anti-allophycocyanin with cyanine 7 conjugate (APC-Cy7) microbeads for IL-6, IL-10, IL-12p40, IL-12p70, TNF-α and IFN-γ, and with a detecting antibody conjugated with phycoerythrin (PE).

The measurement and concentration analysis of compounds present in the tube were performed in a Navios Flow Cytometer (Beckman Coulter, Brea, CA, USA) equipped with FCAP software for CBA analysis (BD Biosciences) and 488 nm, 532 nm and 633 nm lasers. Reads were taken in 10 channels and compensation was used on FL2/FL7 (PE/APC-Cy7). The results of cytokine expression by $CD34^+$ cells were expressed in pg/mL. The results of cytometric analysis were calculated from a standard curve with the use of the WinMDI 2.8 application. Statistical analysis was made with the use of Statistica 10 software (StatSoft, now TIBCO, Palo Alto, CA, USA).

Determination of the BLV gp 51 expression in stem cells. The presence of gp 51 expression in stem cells isolated from the blood and lymphatic tissues of BLV-infected cattle was determined with the use of immunofluorescence reaction. Foetal lamb kidney cells permanently infected with BLV served as positive controls and $CD34^+$ cells isolated from healthy animals were used as negative controls. Cells were stained with an anti–BLV-gp 51 monoclonal antibody conjugated with fluorescein isothiocyanate (VMRD, Pullman, WA, USA). Smears of cells were fixed for 10 min in buffered formalin and washed, and the monoclonal antibody was dropped onto the surface of slides and staining was performed for 1 h at room temperature in the darkness. After washing, Vectashield Antifade mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Newark, CA, USA), which inhibits rapid photobleaching of fluorescent proteins and dyes, was dropped on the smears and the cells were analysed under a fluorescent UV microscope. Additionally the cellular nuclei were counterstained with a fluorescent dye for DNA-DAPI (dihydrochloride) staining (Thermo Fisher Scientific, Waltham, WA, USA), both pictures were merged and the slides were observed under the UV microscope. The $CD34^+$ HSCs isolated from the blood and lymphoid organs of healthy animals were used as a negative control.

Statistical analysis. For estimation of the differences in the analysed cytokine levels between the BLV^+ and BLV^- groups, two-way analysis of variance including interaction was used.

Results

The cytokine levels in the culture fluids of $CD34^+$ stem cells isolated from blood and different organs are presented in Table 1.

Table 1. Mean values of cytokine secretion by bovine leukaemia virus–infected and uninfected $CD34^+$ stem cells cultured *in vitro*

Sample type and presence of provirus ($^+/^-$)		Mean secretion (pg/mL)					
		IL-6	IL-10	IL-12p40	IL-12p70	TNF-α	IFN-γ
Blood	BLV^+	1.90	3.42	27.01	5.30	3.36	2.22
	BLV^-	1.45	5.44	29.13	2.44	2.33	2.44
Bone marrow	BLV^+	1.63	2.72	18.84	2.13	2.94	2.74
	BLV^-	1.89	2.70	17.12	1.13	2.47	2.26
Spleen	BLV^+	0.33	1.87	53.03	1.46	3.82	1.67
	BLV^-	1.68	0.86	103.20	3.53	4.40	3.67
Lymph node	BLV^+	1.21	1.36	49.81	2.19	3.72	1.93
	BLV^-	0.78	0.71	40.52	2.09	3.89	1.90

IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

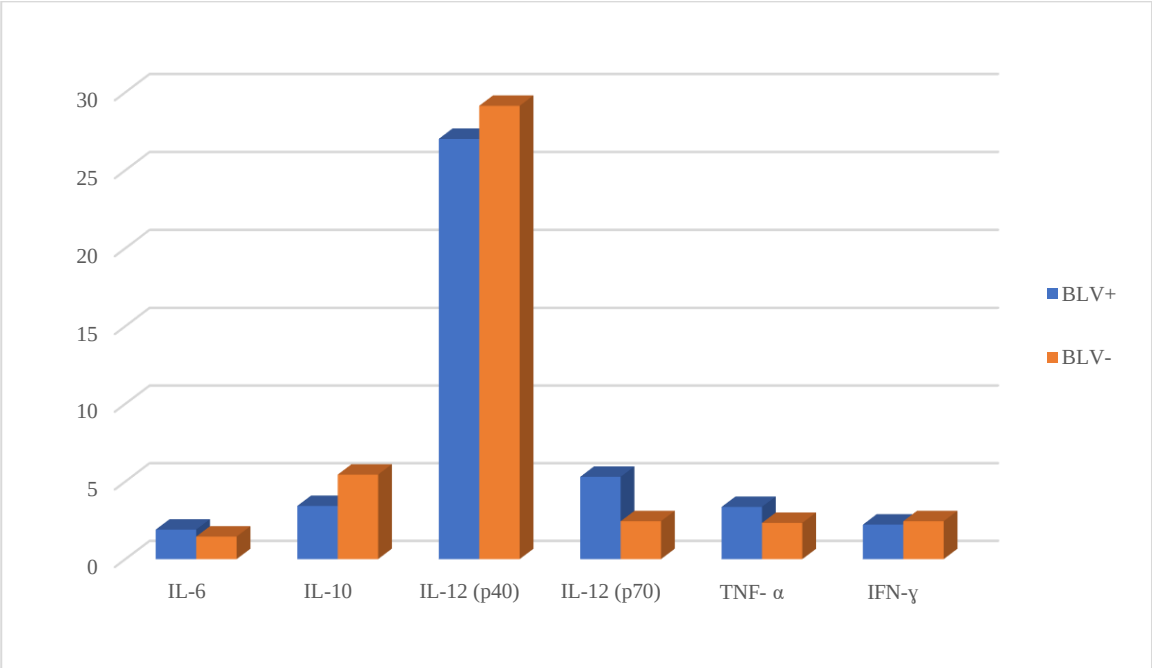

Fig. 1. Cytokine expression of CD34$^+$ stem cells isolated from the blood of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle in *in vitro* cell culture (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

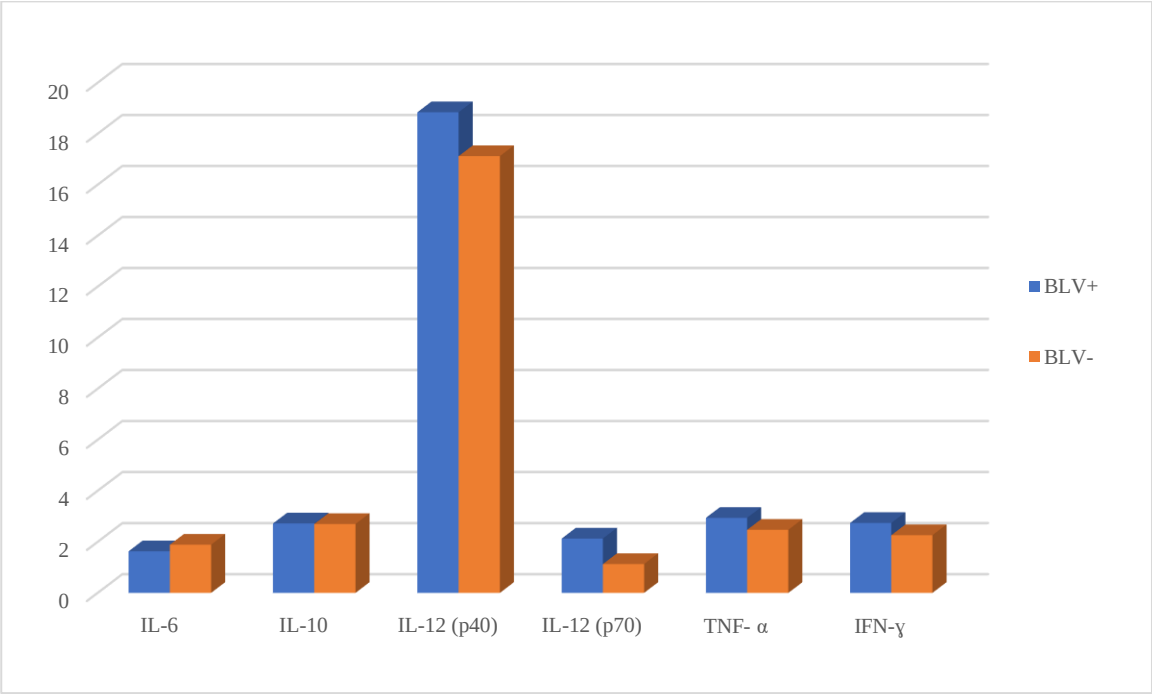

Fig. 2. Cytokine expression in CD34$^+$ stem cells isolated from the bone marrow of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle in *in vitro* cell culture (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

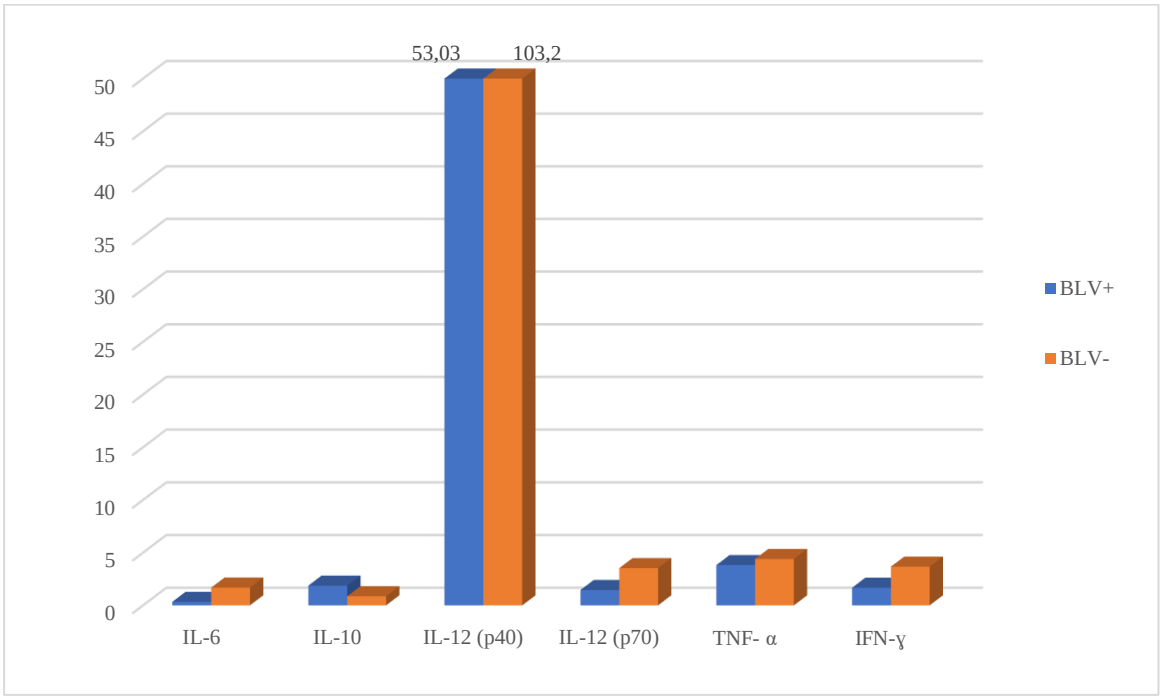

Fig. 3. Cytokine expression in CD34$^+$ stem cells isolated from the spleen of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle in *in vitro* cell culture (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

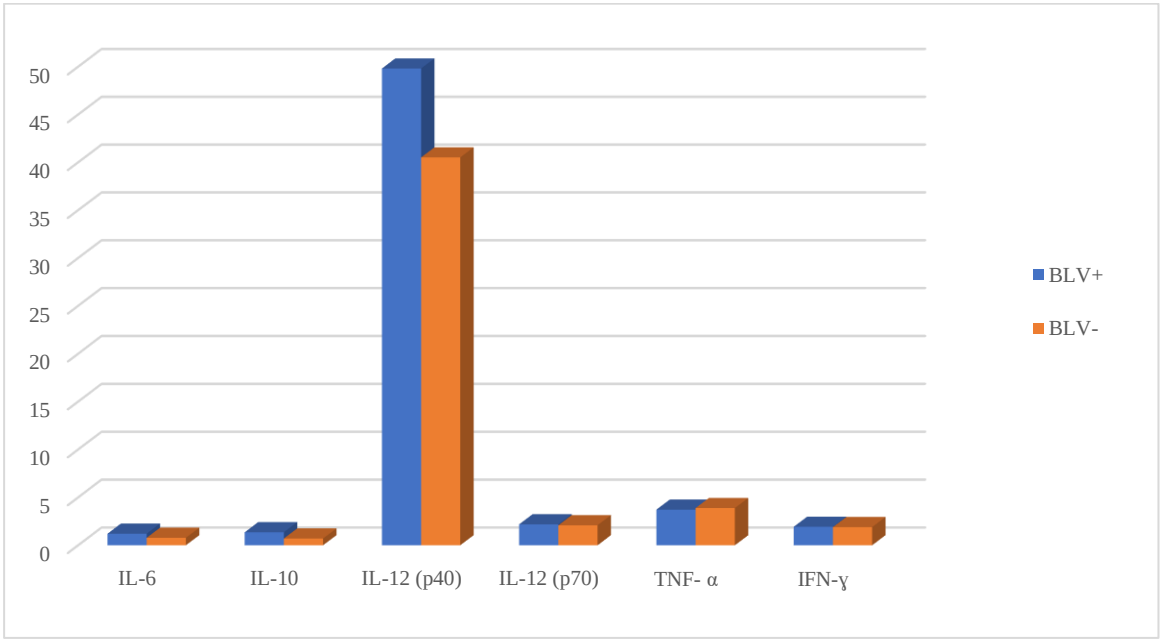

Fig. 4. Cytokine expression in CD34$^+$ stem cells isolated from the lymph nodes of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle in *in vitro* cell culture (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

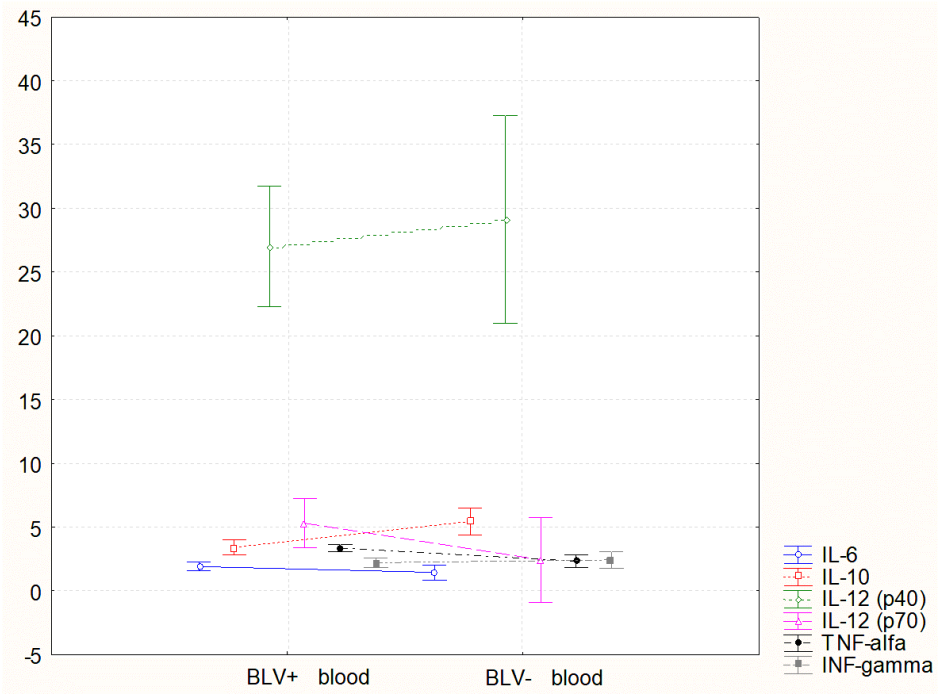

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Fig. 5. The cytokine levels in culture of CD34⁺ stem cells isolated from the blood of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

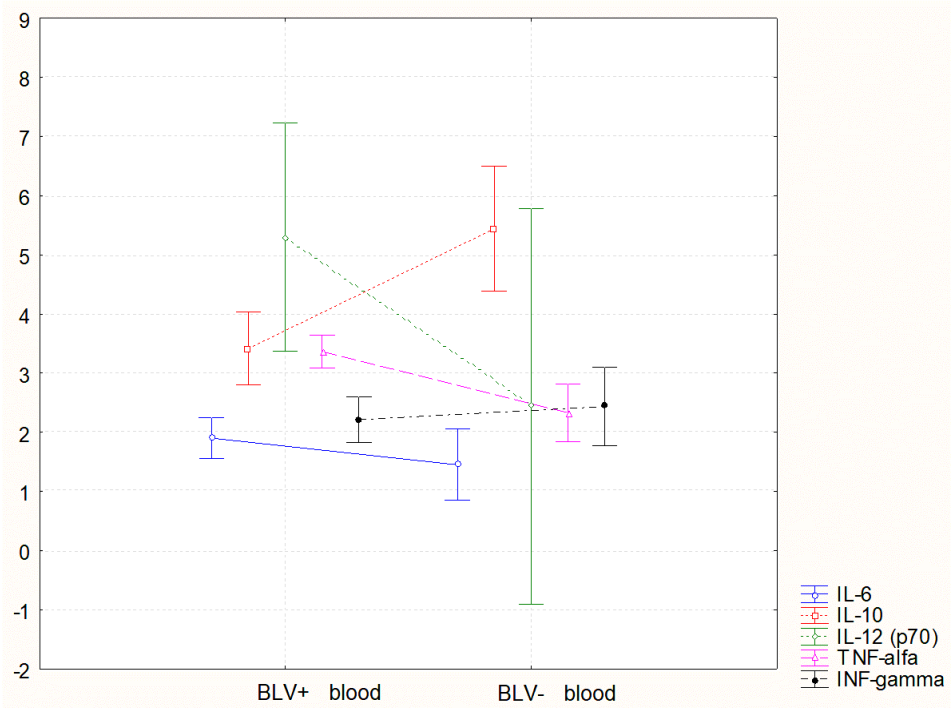

Fig. 6. Results of two-way analysis of variance of the cytokine levels in culture of CD34⁺ stem cells isolated from the blood of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

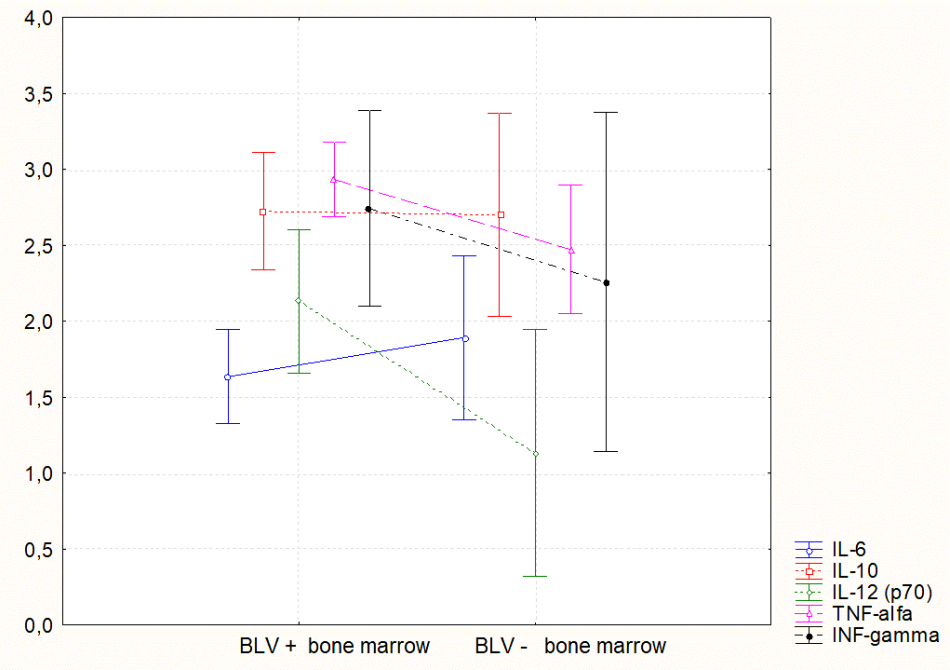

Fig. 7. Results of two-way analysis of variance of the cytokine levels in culture of CD34$^+$ stem cells isolated from the bone marrow of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

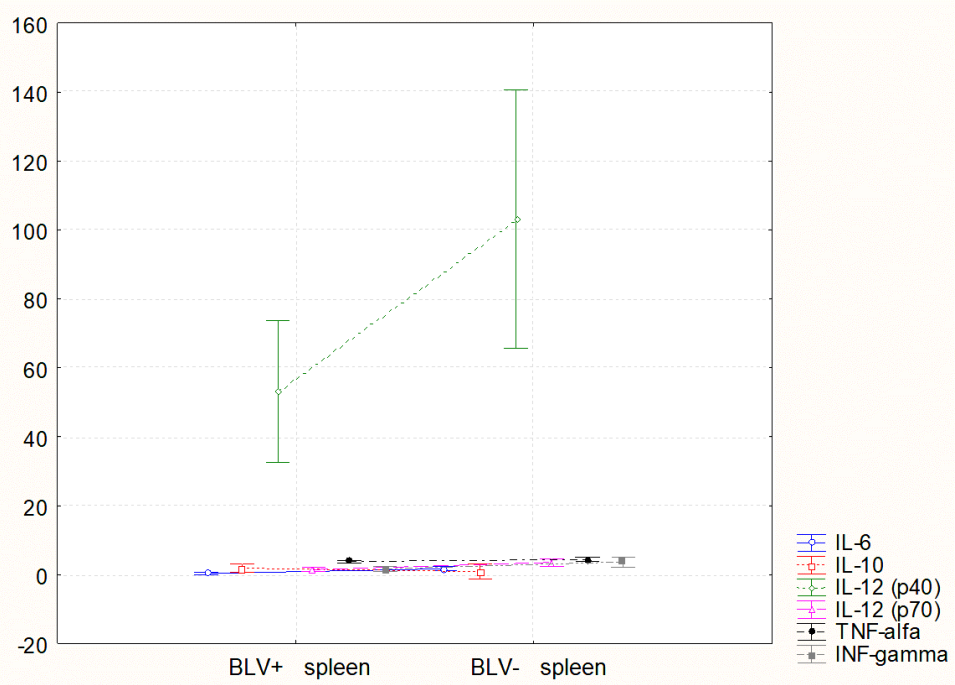

Fig. 8. The cytokine levels in culture of CD34$^+$ stem cells isolated from the spleens of bovine leukaemia virus–infected (BLV$^+$) and uninfected (BLV$^-$) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

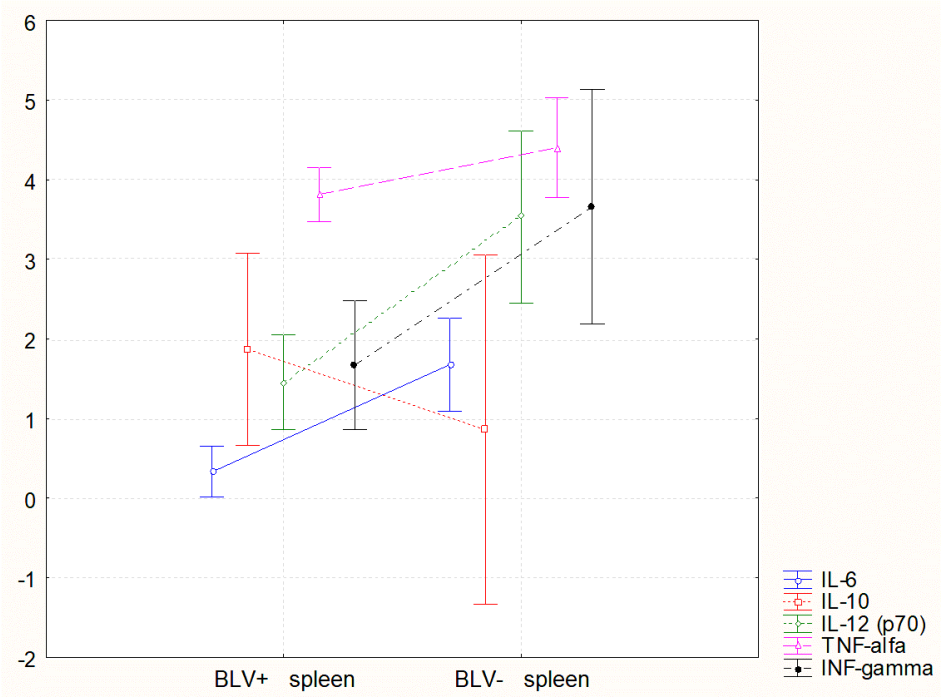

Fig. 9. Results of two-way analysis of variance of the cytokine levels in cultures of CD34$^+$ stem cells isolated from the spleens of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

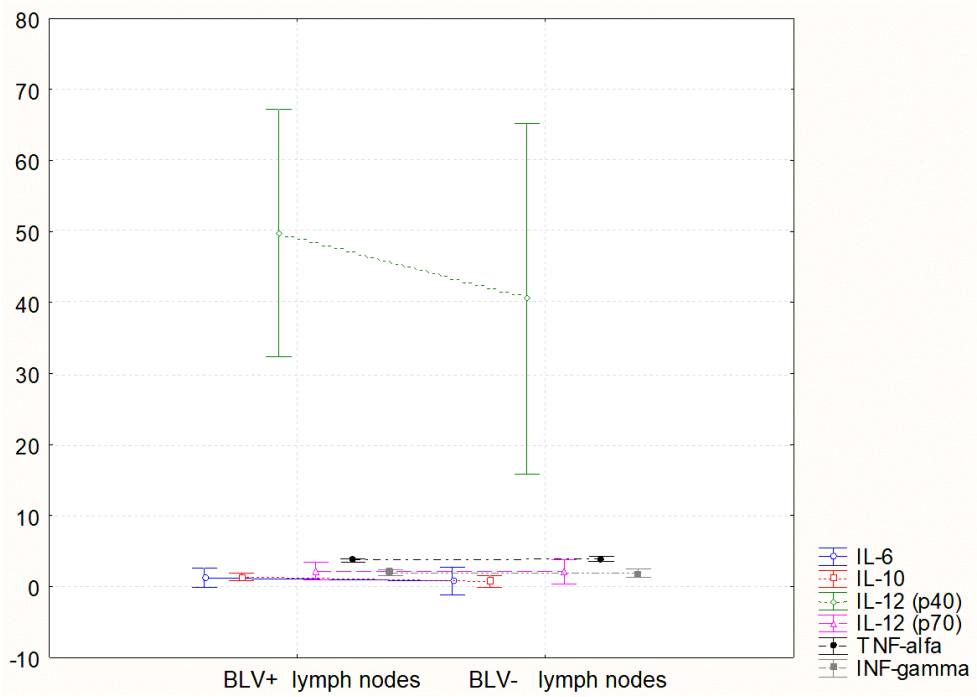

Fig. 10. The cytokine levels in cultures of CD34$^+$ stem cells isolated from the lymph nodes of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p40 – interleukin 12 subunit p40; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

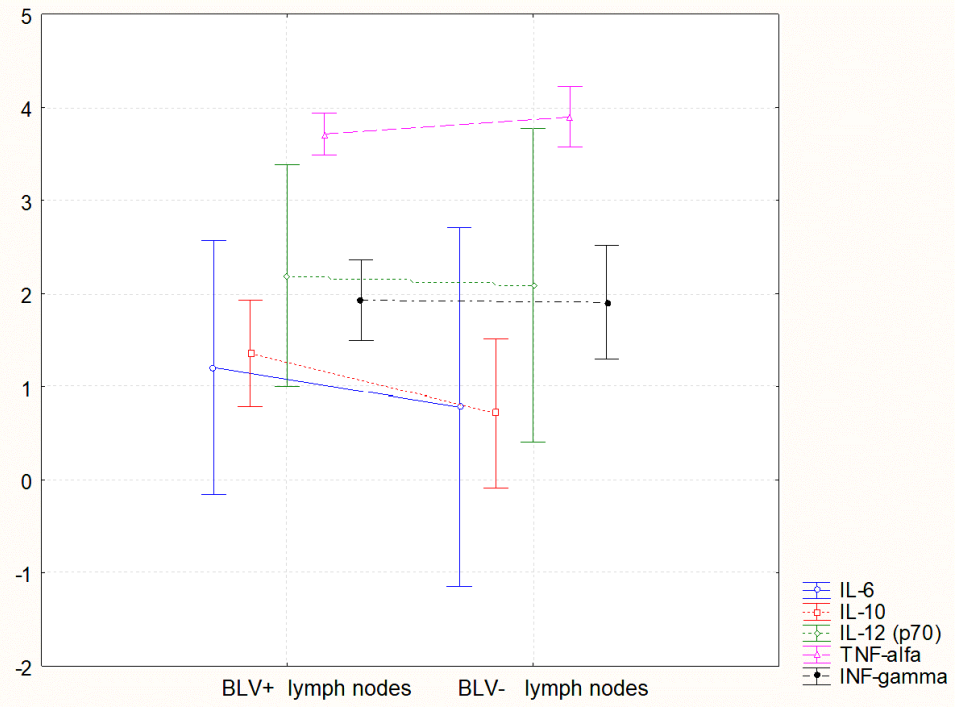

Fig. 11. Results of two-way analysis of variance of the cytokine levels in cultures of CD34$^+$ stem cells isolated from the lymph nodes of bovine leukaemia virus–infected (BLV+) and uninfected (BLV−) cattle (pg/mL)
IL-6 – interleukin 6; IL-10 – interleukin 10; IL-12p70 – interleukin 12 subunit p70; TNF-α – tumour necrosis factor alpha; IFN-γ – interferon gamma

The obtained results indicated statistically significant differentiation in cytokine levels between the infected group of animals and the uninfected group in CD34$^+$ cells isolated from the blood: the Wilks' lambda value was 0.61942, the F-distribution value with 6 and 33 degrees of freedom was 3.3793 and the P-value was 0.01042. Statistically significant differences were detected in cytokine levels between the described groups, with a P-value of <0.05 being regarded as the threshold. The levels of IL-10 (P-value 0.0084), IL-12p70 (P-value 0.0058) and TNF-α (P-value 0.0020) were the ones which differed significantly in infected cattle's samples from the levels in uninfected animals' samples.

Two-way analysis of variance including interactions detected differences in the levels of cytokines between BLV$^+$ and BLV$^-$ animals. The results are shown in Figs 6, 7, 9 and 11.

The results indicated statistically significant differentiation (P-value of 0.00007) in cytokine levels between CD34$^+$ stem cells isolated from the bone marrow of BLV$^+$ cows and those isolated from the bone marrow of BLV$^-$ cows. Statistically significant differences (P-value < 0.05) were found between the groups in the levels of the following cytokines: IL-12p70, with a P-value of 0.0473, and TNF-α, with a P-value of 0.0271.

An indication of statistically significant differentiation of cytokine levels was also given by the results for cultured CD34$^+$ stem cells isolated from the spleen between the BLV$^+$ group and the BLV$^-$ group, denoted by a P-value of 0.00442. Statistically significant differences (P-value < 0.05) in cytokine levels were found between the groups in IL-6, with a P-value of 0.0106; IL-12p40, with a P-value of 0.0425; and IL-12p70, with a P-value of 0.0139.

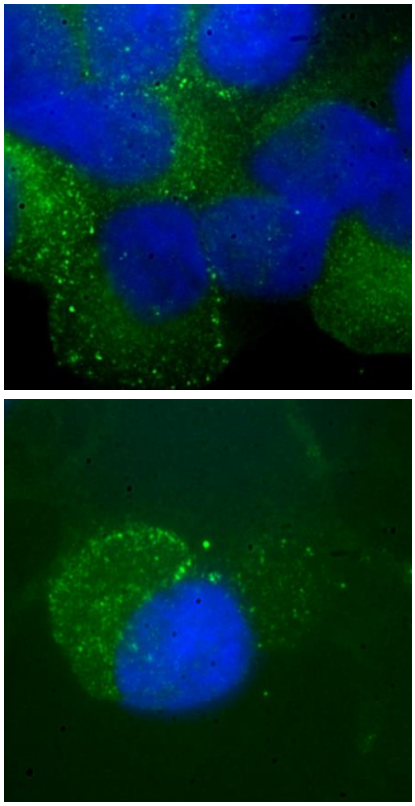

Fig. 12. The expression of bovine leukaemia virus glycoprotein 51 in foetal lamb kidney cells permanently infected with BLV

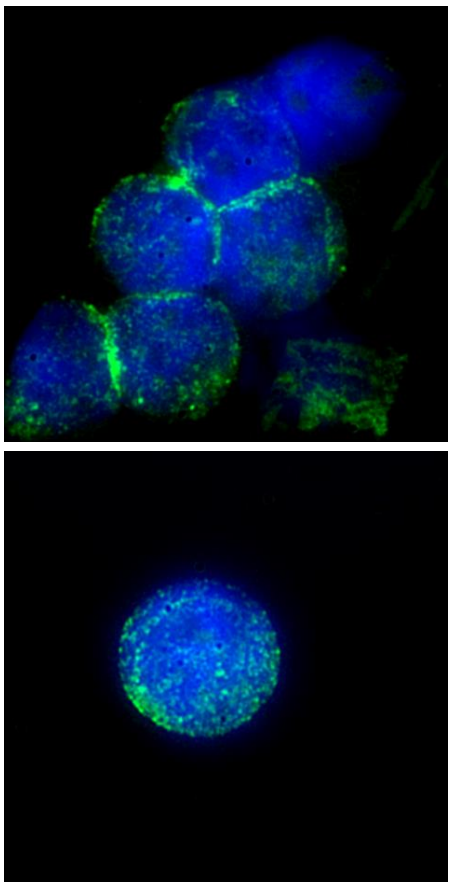

Fig. 13. The expression of bovine leukaemia virus glycoprotein 51 in $CD34^+$ blood stem cells

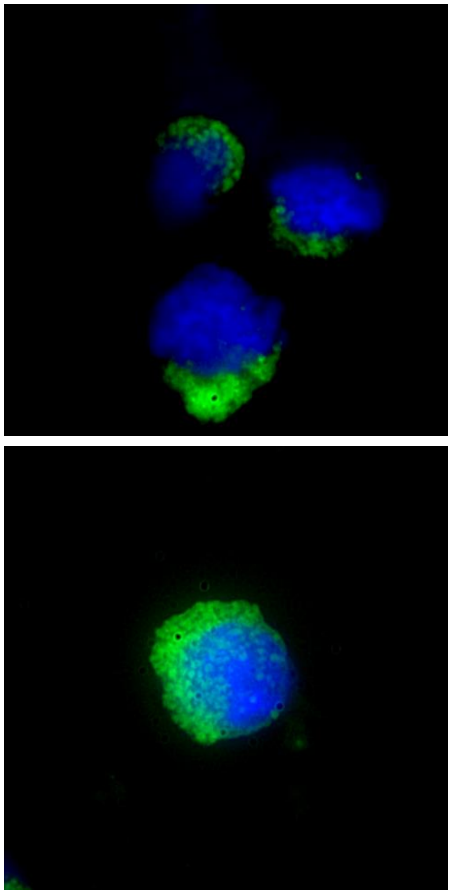

Fig. 14. The expression of bovine leukaemia virus glycoprotein 51 in $CD34^+$ bone marrow stem cells

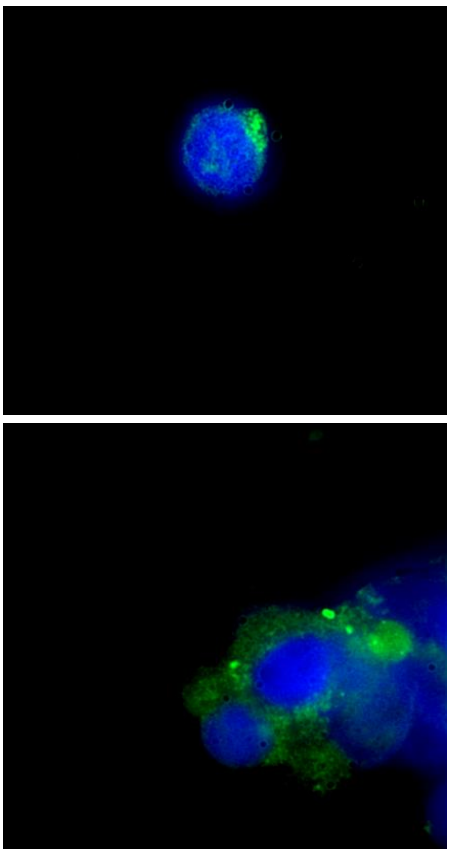

Fig. 15. The expression of bovine leukaemia virus glycoprotein 51 in $CD34^+$ spleen stem cells

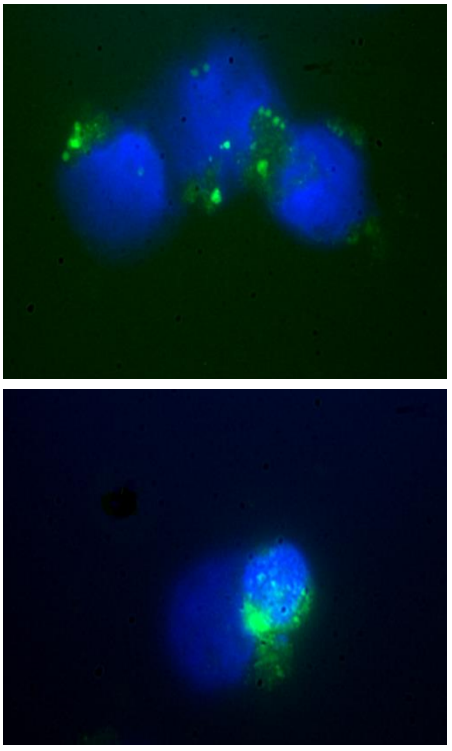

Fig. 16. The expression of bovine leukaemia virus glycoprotein 51 in $CD34^+$ lymph node stem cells

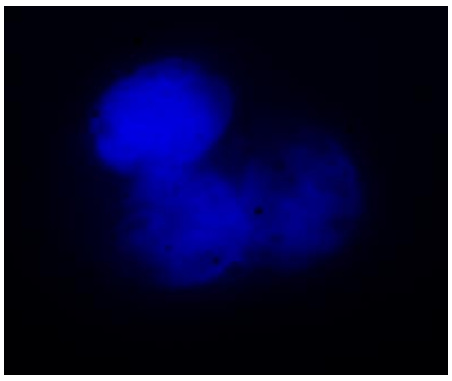

Fig. 17. Lack of fluorescence in the control cells

In contrast to the results for the cytokine levels in cell cultures of CD34+ stem cells isolated from the blood, bone marrow and spleen, those for levels in cultures of $CD34^+$ stem cells isolated from the lymph nodes indicated only statistically insignificant difference between BLV^+ and BLV^- animals and gave a P-value of 0.71015. A statistically significant difference (P-value <0.05) in one cytokine level was nevertheless found: for IL-10, with a P-value of 0.0269.

Immunofluorescence results. The results of immunofluorescence staining are presented in Figs 12–17. The presence of glycoprotein BLV-gp 51 in HSCs isolated from the blood and lymphatic tissues of BLV-infected cows was detected.

Discussion

It is known that immune cells are potent cytokine producers after bacteria and virus infection has begun. When cytokines produced by immune cells and non-haematopoietic tissues accumulate in sufficient amount, they circulate back to the bone marrow niche with blood circulation and activate haematopoietic progenitor/haematopoietic stem cells (HP/HSCs). The HP/HSCs residing in the bone marrow are able to leave it and reach the peripheral organs that are the spleen, liver, lymph nodes, gut and adipose tissues in blood and lymphatic circulation (27, 32). Interleukin 6, TNF-α, INF-α, INF-γ and other cytokines with the ability to regulate proliferation and differentiation of HP/HSCs have been identified (23). Haematopoietic stem cells survive inflammatory stress that kills other blood cells, but the mechanism of their survival remains poorly understood (33). It was detected that TNF-α acts differently on HSCs and progenitors, thus facilitating hematopoietic clearance and promoting regeneration.

Some authors suggested that viral infection may influence the process of normal haematopoiesis (17). Viral infection of HP/HSCs may adversely affect the levels of cytokines and transcription factors necessary for proliferation and differentiation. These infections are able to induce such reactions as apoptosis, cytolysis or disruption of progenitor cells, which finally suppresses haematopoiesis. Infected HP cells may take part in pathogen dissemination and spread the infection in the organs, and these cells may then be the targets for cellular transformation in specific organs. Stem cells of the $CD34^+$ type are susceptible to infection with numerous viruses including HIV, HTLV-I, parvoviruses, hepatitis C virus (HCV), human herpesvirus and human cytomegalovirus (HCMV). It has been suggested that other viruses such as HTLV-I and Kaposi's sarcoma herpesvirus can also infect $CD34^+$ HP/HSCs and cause latent infection within the cells resident in bone marrow (2).

Alterations in the cytokine secretion of bone marrow stromal cells (BMSCs – cells producing cytokines, adhesion molecules and growth factors, which are necessary for differentiation and maturation of HSCs) from patients with chronic myelomonocytic leukaemia (CMML) were found by Shi *et al.* (28). This form of leukaemia is a clonal disorder of HSCs and the principle characteristic is monocytosis in peripheral blood. It was observed that after co-cultivation of BMSCs isolated from patients with CMML with cord blood $CD34^+$, secretion of multiple cytokines was decreased from the level secreted by healthy BMSCs, which may result in the reduction in haematopoietic supportive activity. The IL-6, IL-8 and C-X-C motif chemokine ligand 2 cytokine secretion levels were decreased significantly. It has been shown that IL-6 and IL-8, which are secreted by BMSCs, can promote HSC stemness (4). Interleukin 6 induces haematopoietic cell proliferation, functional maturation and differentiation, and deficiency of IL-6 can affect BMSC function and impair haematopoietic differentiation and supportive activity. In our investigations, low levels of IL-6 secretion were found in cell cultures of bone marrow- and spleen-originated BLV-infected stem cells, which is in agreement with other authors' findings and indicative of the stemness of these bone marrow and spleen cells when infected with BLV (31). Contrastingly, IL-6 secretion was higher in the blood and lymph node stem cells.

Interleukin 10 has substantial regulatory properties; in humans and cattle it mostly appears to have a downregulating effect on expression of proinflammatory cytokines. The presented study indicates an evident increase of IL-10 levels in HSCs from BLV-infected bone marrow, spleen (values statistically insignificant) and lymph node tissue (statistically significant). The data reported by others indicated an increase in the secretion of IL-10 during the progression of BLV infection. Elevated concentrations of this cytokine were noted in experimentally infected sheep, and this was associated with progression of the disease (31). Interleukin 10 actively blocked BLV replication in adherent cultures of monocytes/macrophages, which suggested involvement of this cytokine in mechanisms of viral latency (11).

Interleukin 12 is a heterodimer (p70) combined of two subunits (p35 and p40). In our study elevated secretion of IL-12p40 was found in BLV-infected cows' bone marrow and lymph node HSCs; however, in both organs the differences were statistically insignificant.

Elevated values of subunit IL-12p70 were found in the blood, lymph node and bone marrow and their differences from control cells' secretion of this heterodimer were statistically significant.

Interferon gamma, as a cytokine of the Th1 response, takes part in polarisation of cytotoxic cells, activates macrophages and induces functional maturation of antigen-presenting cells. It also creates a positive feedback loop with IL-12 and exhibits similar antiviral and antineoplastic characteristics. In our investigations changes of this cytokine's secretion in $CD34^+$ stem cells were statistically insignificant. The existence was described of a connection between IFN-γ expression, being an indicator of strong immune response, and suppression of virus spread in the early stage of BLV infection (24). Evidence was also shown of a correlation between a higher viral load and a decrease in production of IFN-γ and an ability of this cytokine to suppress BLV replication *in vivo* (24).

Tumour necrosis factor alpha is a cytokine which is both pro-inflammatory and regulatory, and which is involved in processes of the immune response in viral and bacterial infections. The main function of TNF-α as an acute response cytokine is the upregulation of many pro-inflammatory chemokines, cytokines, growth factors and adhesion molecules. A link was shown between TNF-α expression and proliferation of BLV-infected B cells: TNF-α was considered to be a cytokine inducing the death of B cells and proliferation of B cells infected with BLV (35). We observed statistically significant higher values of this cytokine's expression in the blood and bone marrow of BLV-infected stem cells, which indicated their engagement in pathological leukaemogenesis. In the stem cells of other lymphoid organs, TNFα expression was lower in leukaemic cows than in the control animals.

Statistically significant differences in cytokine levels were noted in cell cultures of $CD34^+$ stem cells generated from the blood from the described groups (P-value < 0.05). Statistically significant differentiation between the BLV^+ and BLV^- groups in cytokine levels in cell culture of $CD34^+$ stem cells isolated from bone marrow was also observed, but this differentiation was found between other cytokines than those determined as differentially secreted by the blood $CD34^+$ cells. A similar phenomenon was observed in stem cells from the spleen and lymph nodes, which may be the result of different concentrations or profiles of the analysed cytokines in each organ or tissue even in the BLV^- control group.

Persistent infection and chronic inflammation produce a tumour-supporting microenvironment, which is essential for oncogenesis. The results of epidemiological and clinical investigations indicated a clear association between chronic infection, inflammation and cancer. Cytokines produced by activated innate immune cells are important factors in these pathological processes.

The presence of BLV gp 51 was detected in $CD34^+$ stem cells isolated from all examined lymphatic tissues. Strong green fluorescence was found in cytoplasm and cellular nuclei were blue through counterstaining with DAPI, which confirmed infection with BLV. In the control cells only blue-coloured nuclei were visible.

In bovine leukaemia, B-lymphocytes are the primary target of BLV infection, and as infected lymphocytes they are arrested in G_0/G_1 and protected from apoptosis. It was suggested by Mirsky *et al.* (22) that $CD5^+$ progenitors are more sensitive to BLV infection and that this observation indicates a relationship between BLV tropism and B-cell phenotype. The authors suggested the existence of a pre-malignant clone. This infected progenitor can be detected early after viral infection and could cause genetic instability and clonal expansion, two characteristics of cancer cells. It can be speculated that the infection of progenitor populations by BLV may result in the establishment of an infectious leukaemic stem cell/infectious cancer stem cell and ultimately in the development of leukaemia. Our results are in agreement with those of other authors, which also indicated the suppressive effect of retroviral infections on stem cell secretion and their generation of changes in cytokine profiles (28).

On the basis of the obtained results it is evident that retroviral infections may influence normal haematopoiesis and cytokine secretion, and our results are in agreement with those of other authors. Viral infections impair HSCs by altering their stroma, and may affect the levels of cytokines and transcription factors, which was found in the results of our experiments. This type of infection may also cause apoptosis, and the destruction of progenitor cells by cytolysis, which has the consequence of impeding haematopoiesis. Haematopoietic stem cells may be a target for cellular transformation by some viruses and are susceptible to infection by HIV-1, HTLV-I, human herpesviruses, parvoviruses, HCV and HCMV. It was demonstrated that some viruses, HTLV-I and Kaposi's sarcoma herpesvirus among them, can infect, invade and remain latent in bone marrow (17). As a retrovirus, HTLV-I along with HIV-1 and Moloney murine leukaemia virus causes an infection which potentially arrests the cell cycle, increases cell susceptibility to apoptosis, and as a result suppresses haematopoiesis.

Conclusion

Cytokines are active in stem cell differentiation, allograft rejection and vaccine efficacy and provide a crucial role as regulators of immune homeostasis. Our investigations demonstrated that infection with BLV influenced HSCs activity and caused changes in cytokine secretion and cytokine profiles.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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