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Original article

Blood dendritic cells in cattle infected with bovine leukemia virus (BLV): isolation and phenotyping

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Abstract

Dendritic cells (DCs) are most potent antigen presenting cells (APCs) with unique ability to prime effective immune responses. They express higher levels of MHC class II and accesory molecules on their surface, than other professional APCs. The investigations were performed on DCs generated from blood with the use of microbeads magnetically labeled with mouse anti human CD14. Flow cytometry was applied for determination of DCs immunophenotype in healthy and naturally infected with BLV cattle. For immunophenotyping mouse monoclonal antibodies anti bovine: CD11a, CD11b, CD11c, MHC-I and MHC-II were used. Our results demonstrated that dendritic cells infected with BLV expressed very high percentage of determinants: CD11a, CD11b, CD11c, MHC-I and MHC-II class. Leukaemic DCs exhibited DCs morphology and had a phenotype of mature DCs. The expression of gp51 glycoprotein of BLV on leukaemic DCs was detected in flow cytometry investigations.

Key words: cattle, bovine leukemia, dendritic cells, immunophenotype, flow cytometry

Introduction

Bovine leukaemia virus (BLV) belongs to the genus *Deltaretroviridae*, family *Retroviridae* and is closely related by genomic organisation and disease progression to human T-cell leukaemia viruses (HTLV-1 and HTLV-2) and simian T-cell leukaemia virus. BLV infection remains subclinical in the majority of cattle, but about one third of infected animals develop persistent lymphocytosis (PL). About 1-5% of infected animals develop lymphosarcoma with or without prior PL (Ferrer et al. 1980). The primary cellular target of BLV is the B-lymphocyte (Paul et al. 1977). The susceptibility of cells other than B lymphocytes to BLV infection is less clear.

Monocytes were first implicated as potential carriers of BLV in sheep on the basis of cell morphology and *in situ* hybridisation. Some authors reported that

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BLV was present in 5% to 40% of adherence purified monocytes, but not in T cells or granulocytes from BLV infected cattle with or without PL. T cell susceptibility for BLV infection was evidenced, when immunoaffinity depletion of B cells and monocytes from peripheral blood or positive selection of T cells with immunomagnetic beads were performed (Schwartz et al. 1994).

Dendritic cells (DCs) are professional antigen--presenting cells, responsible for eliciting an efficient response against pathogens. DCs were first detected in the spleen of mice and described by Steinman and Cohn in 1973. They sample foreign pathogens in the periphery and migrate to lymphoid tissues, where they present processed antigens to naïave T cells, initiating an immune response (Banchereau et al. 1998). These cells are distributed especially in tissues that interface with an external environment, such as the skin, gut and lungs (Sertl et al. 1986, Nestle et al. 1993, Nelson et al. 1994). Due to these locations, they can perform a sentinel function for incoming pathogens, and have the capacity of recruiting and activating cells of the innate immune system upon inflammation (Sallusto et al. 1998, Fernandez et al. 1999, Rescigno et al. 1999).

Uptake of pathogens by DCs induces a state of activation, which leads to the migration of an antigen-loaded DCs to lymphoid organs, where the cells of an adaptive immune response can be excited (Moll et al. 1993). DCs show a high degree of phenotypical and functional heterogeneity. Different DC lineages have been described, found at various anatomical locations and showing a distinct set of surface molecular markers. For example, CD8α-negative myeloid DCs were found in peripheral, nonlymphoid tissues and in the secondary lymphoid organs including Langerhans cells of the skin, interstitial dendritic cells in various organs, and marginal zone DCs in the spleen. DCs with CD8α molecule, called lymphoid DCs, are exclusively found in the T cell areas of the secondary lymphoid organs and in the thymus (Vremec et al. 1997).

Whereas the origin, development and function of myeloid DCs are well recognized, both developmental pathways and functions of lymphoid dendritic cells are still not exactly defined. DCs that circulate in blood or reside in solid tissues are immature (O'Doherty et al. 1994, Weissman et al. 1995). They can effectively endocytose antigens, although they express the accessory molecules needed for T cell activation at low levels. However, after antigen uptake and exposure to stimulatory signals, DCs mature. As a consequence of maturation, DCs express molecules that enable them to effectively bind and activate T cells in a manner that promotes immune responses (Banchereau et al. 1998), including those that are necessary for effective immunotherapy. The application of DCs ex-vivo transduced with a virus coding for one or multiple TAA (Tumour Associated Antigens) might have several potential advantages over the technically easier direct vaccination (Toes et al. 1999).

Two methods have been used to investigate dendritic cells in cattle: the cannulation of pseudoafferent lymphatic ducts and generation from monocytes cultured in the presence of GM-CSF and IL-4 (Sallusto et al. 1998).

The aim of the present study was generation of dendritic cells from bovine blood monocytes and determination of subpopulations and morphology of these cells in healthy and leukaemic cattle.

Materials and Methods

Animals

Investigations were performed on 19 cows naturally infected with BLV and 12 healthy cows, at the age of 4-7 years, polish black and white lowland breed.

Generation of dendritic cells from monocytes

Investigations were performed on two groups of cattle: healthy and naturally infected with BLV, positive in ELISA and PCR tests. For dendritic cells preparation, peripheral blood mononuclear cells were isolated from 100 ml of whole blood treated with EDTA-K2, by standard density centrifugation in Histopaque (Sigma). After centrifugation cells were collected and CD14 positive cells were separated by magnetic sorting using VarioMACS technique (Miltenyi Biotec GmbH) according to manufacturer's protocol. Briefly, blood samples were centrifuged for 30 min, at 4°C, 2300 rpm. After that buffy-coat was harvested and AutoMACS Rinsing Solution was added to the volume of 35 ml. This cells suspension was divided in two parts, loaded on the Histopaque gradient (1.077) and centrifuged for1h, 4°C, 2000 rpm. Then, cells from interphase were transferred to the new tubes, the AutoMACS Rinsing Solution was added and cells were centrifuged 3 times, 10 min each, 2000 rpm. The supernatant was discarded, cells were counted and concentration of 10^7/ml was prepared. To the suspension of 10^7 cells, the amount 80 µl of AutoMACS Rinsing Solution supplemented with 0,5% FCS was added, gently mixed and then 20 µl of immunomagnetic microbeads conjugated with monoclonal mouse anti human CD14 antibody was added and cells were incubated 15 min at temperature 4-8°C. Then, cells

were washed, centrifuged at 1500 rpm for 10 min. Supernatant was discarded and 10^8 of the cells pellet was resuspended in 500 µl of AutoMACS Rinsing Solution with 1% of FCS. The cells separation was performed on the MACS LS Column (Miltenyi Biotec). The column was placed in the magnetic field of MACS Separator and column was prepared by filling and rinsing with 60 ml of buffer AutoMACS. Then 3 ml of AutoMACS Rinsing Solution with 1% of FCS was added on a magnetic column and cells suspension was placed into the column. After that, column was washed 3 times with 3 ml each of AutoMACS Rinsing Solution with 1% FCS. The eluted fluid containing unlabeled cells was discarded. The labeled cells were eluted from the column by removal the magnetic column from the magnetic source, then 5 ml of the AutoMACS Rinsing Solution with 1% of FCS was placed on the column and cells were flushed out by firmly applying the plunger supplied with the column.

Dendritic cells culture

Cells were cultivated, according to the procedure modificated and described by Szczotka (Szczotka et al. 2009). Briefly, CD14+ isolated cells were cultured at a concentration of 1 x 10^6 cells/ml in standard culture flasks, in RPMI 1640 medium containing 10% FCS, glutamax and supplemented with antibiotics. The cells were incubated at 37°C, in a humidified atmosphere with 5% CO_2, in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). On day 3, about half of the culture medium was removed from flasks and replaced with a fresh complete medium containing GM-CSF and IL-4. The cells were fed with fresh medium every 3 days, for 4 weeks. Every week one part of cells was analysed in flow cytometer and under a microscope: cells from cultures were collected, washed and submitted for light and scanning microscopy examination, as well as flow cytometry.

Flow cytometry of dendritic cells

Dendritic cells were counted and a concentration of cells 600 000/ml was prepared. Then, cells were coupled with 1 µl of mouse monoclonal antibodies directed against human CD14 (Miltenyi Biotec), and mouse anti bovine CD11a, CD11b, CD11c, MHC-I, MHC-II and anti-BLV-gp51 (VMRD Pullman) and incubated for 15 min at room temperature. Next, cells were 3 times washed in phosphate-buffered saline (PBS) containing γ globulin- free horse serum, centrifuged and the cell pellet was incubated with 1 µl of goat-anti- mouse (IgG+IgM) H+L-FITC conjugate (Biosource Camarillo, CA) for 15 min, at room temperature, in darkness. After 3 washings in PBS buffer with horse serum and another 3 washings in PBS buffer without horse serum, the cell pellet was resuspended in PBS buffer containing formaldehyde and flow cytometry analysis was performed.

Determination of dendritic cells morphology

Morphology of dendrittic cells was determined with a light and scanning electron microscopes. For the analysis with the light microscope smears of cells were fixed and stained according to the May-Grünwald method. For scanning microscopy, the cells were attached to the plastic coverslip that had been coated with 15 poly-L-lysine in water for 15 min. The cells were fixed with 1.2% glutaraldehyde in 0.1 M PBS, pH 7.4, passed through an alcohol gradient, dried and prepared for examination with scanning electron microscope (SEM).

Results

After immunomagnetic separation procedure we obtained positively enriched (95%-97%) labeled CD14 cells and 98% of viability (estimated by trypan blue exclusion). The CD14 positive cells had typical monocytes morphology, with large nucleus and small cytoplasm. These cells cultured in the presence of GM-CSF and IL-4 in growth medium generated to the dendritic cells. These cells had characteristic for blood and lymph dendritic cells dendrites and veiled cytoplasm. In the cell culture we observed dendritic cells on different levels of maturity. There were immature cells with characteristic Birbeck granules, as well as mature cells, in which granules were small or absent, but they had dendrites-like processes and lamellipodia (Fig. 1, 2). DCs observed in SEM had delicate veiles and edges divided on the end (Fig. 3, 5). These structures enable to take up and process many particles of antigens in the same time and to present them to the lymphocytes (Fig. 6). In the 72 h culture of monocytes almost all cells had typical for DCs shape, veiles and edges (Fig. 3). Fig. 4 presents dendritic cells harvested from the cell culture and prepared for investigations in flow cytometry.

The results of dendritic cells immunophenotyping and the fluorescence intensity of cells investigated with flow cytometer are presented in Fig. 7 and Fig. 8.

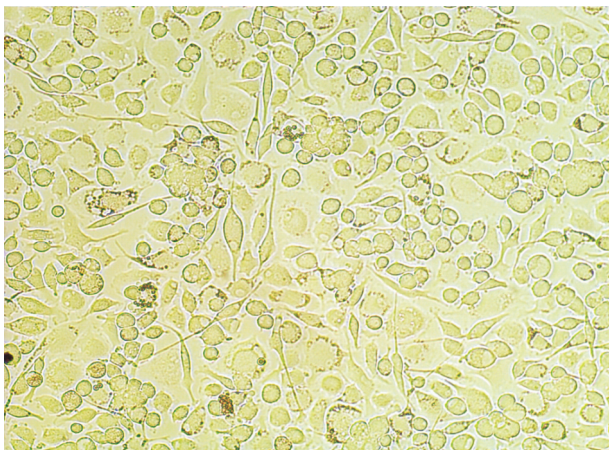


Fig. 1. Formation of dendritic cells in monocytes cell culture (24 h cell culture, x20).

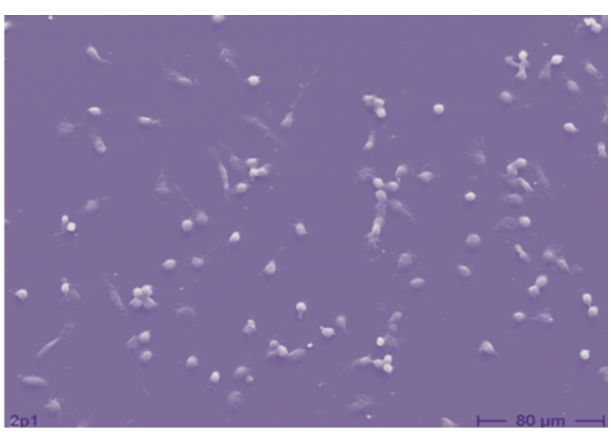


Fig. 2. The 24 h monocytes cell culture (SEM x1000).

Fig. 3. The 72 h cell culture of DCs (SEM x2000).

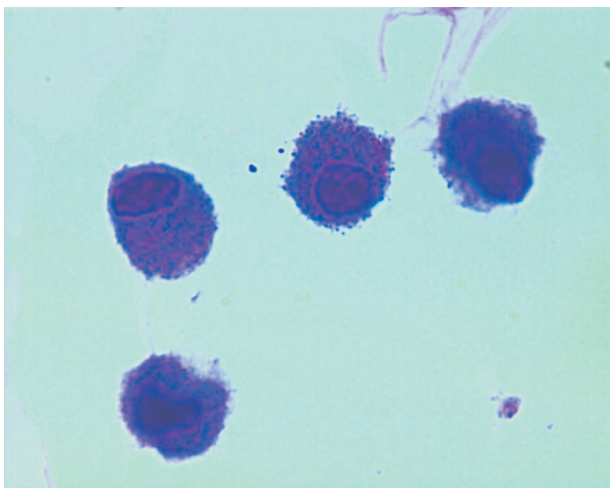


Fig. 4. Dendritic cells harvested from cell culture for flow cytometry (x100).

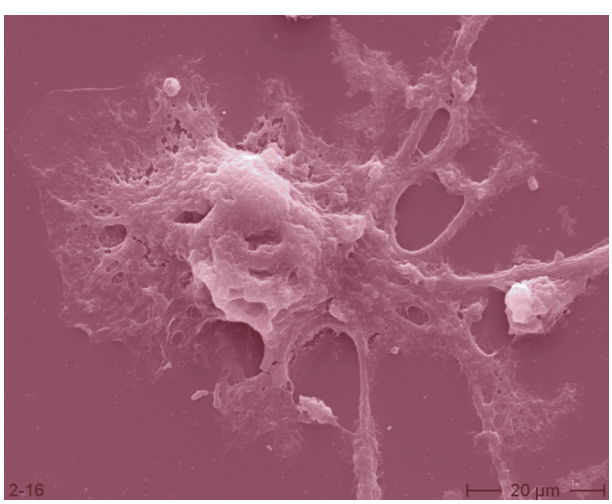


Fig. 5. Mature blood dendritic cell (SEM x10000).

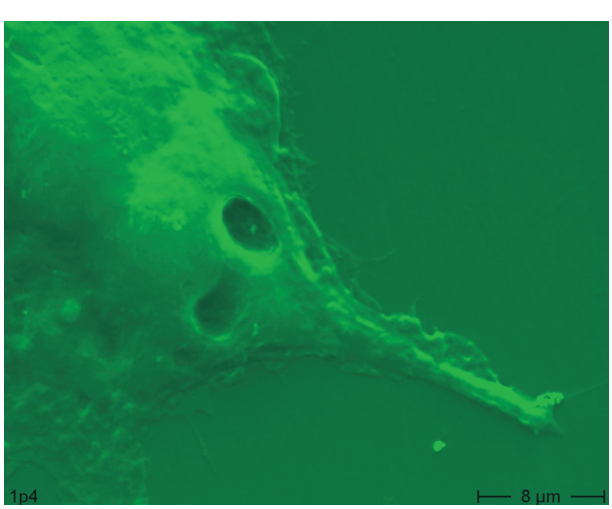


Fig. 6. Blood dendritic cell with typical morphology: long dendrite and veil (SEM x10000).

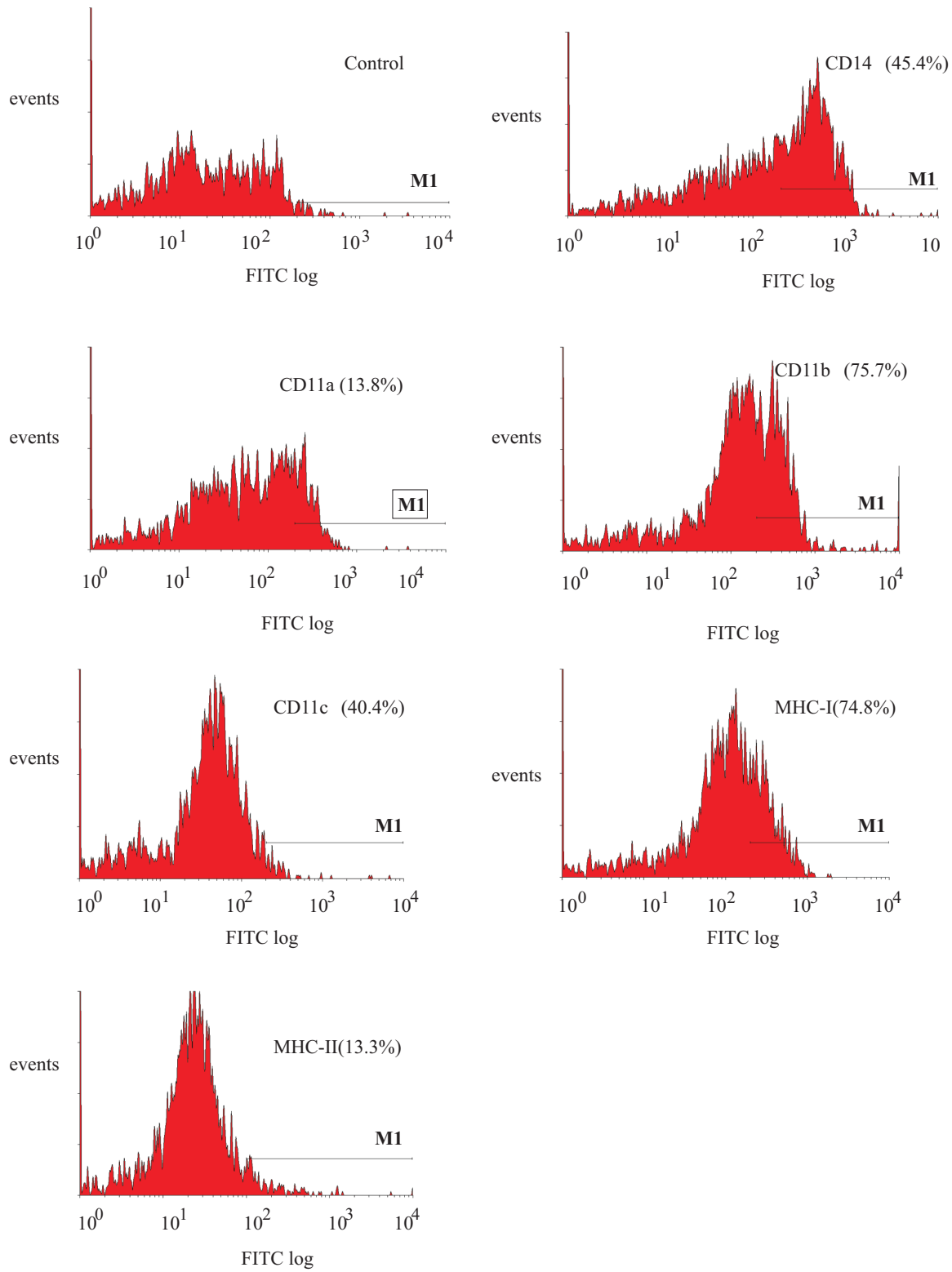


Fig. 7. Expression of surface molecules related to dendritic cells generated from monocytes of a healthy cow.

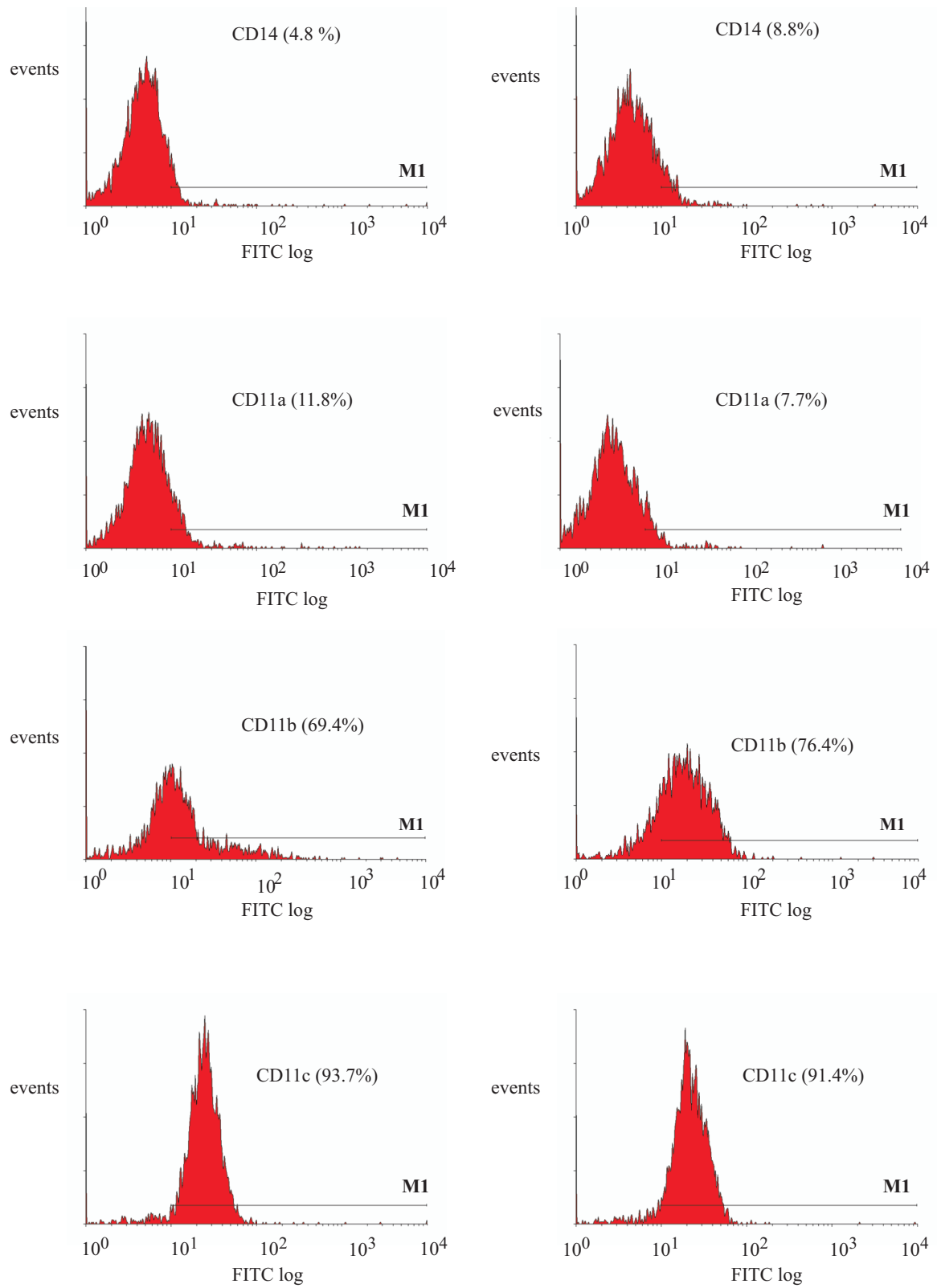

CD14 (4.8 %)
CD14 (8.8%)
CD11a (11.8%)
CD11a (7.7%)
CD11b (69.4%)
CD11b (76.4%)
CD11c (93.7%)
CD11c (91.4%)
events
M1
10^0 10^1 10^2 10^3 10^4
FITC log

Fig. 8

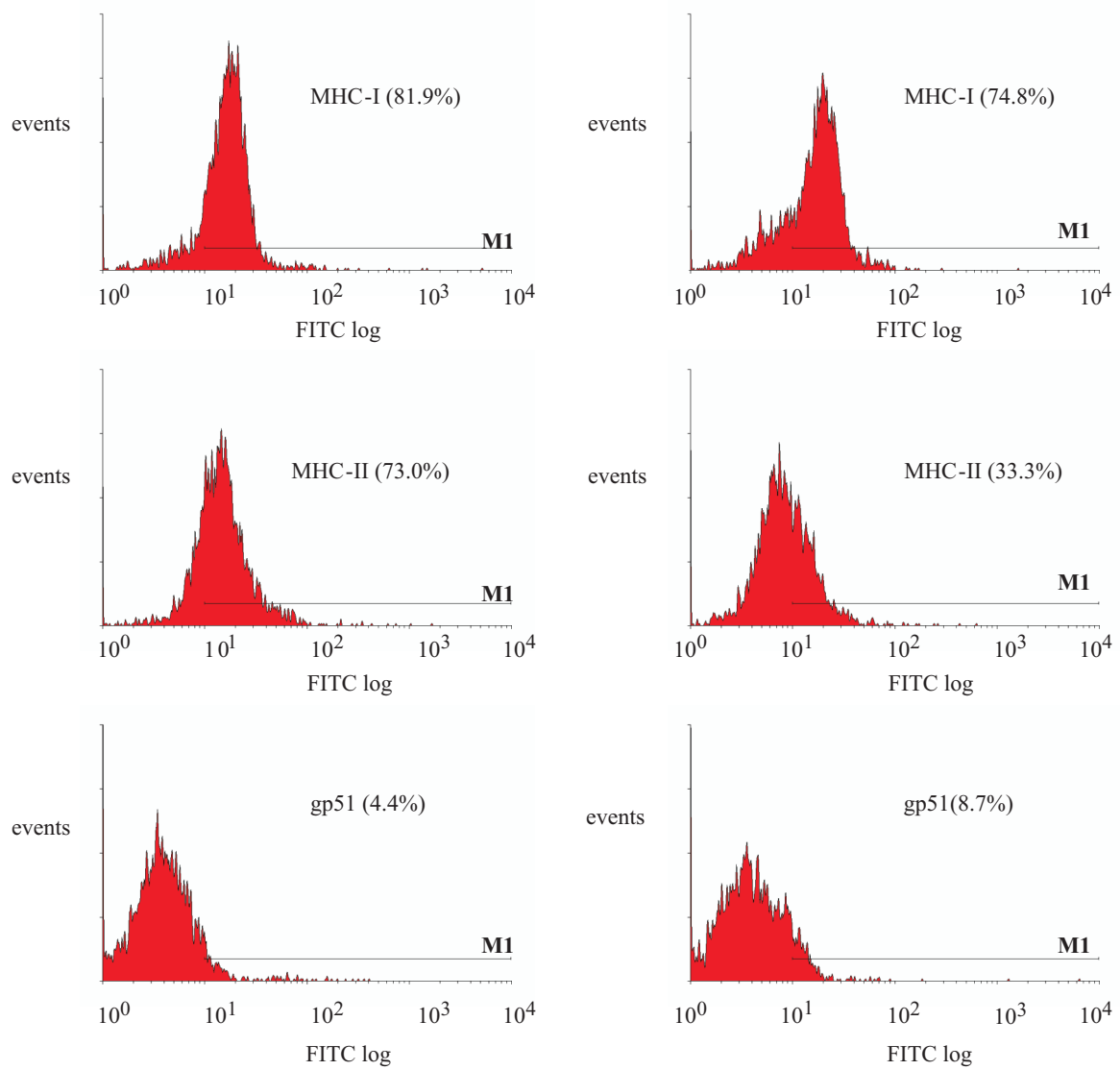


Fig. 8. Expression of BLV-gp51glycoprotein and surface molecules related to dendritic cells generated from monocytes of two cows naturaly infected with BLV.

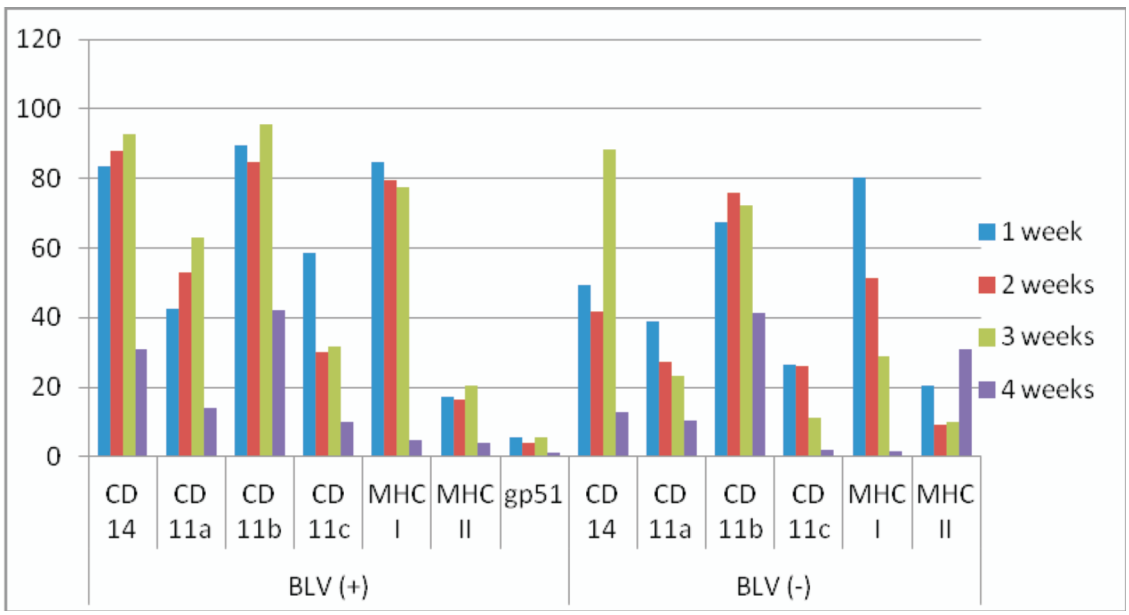
Table 1. The immunophenotype of dendritic cells in 19 leukaemic and 12 control cows (% cells – mean values).

BLV(+)	CD 14	CD 11a	CD 11b	CD 11c	MHC I	MHC II	gp51
1 week	83.6	42.5	89.5	58.4	84.6	17.0	5.6
2 weeks	87.9	53.0	84.9	30.0	79.5	16.4	4.1
3 weeks	92.7	63.2	95.7	31.6	77.5	20.5	5.4
4 weeks	31.0	13.8	42.1	10.1	4.7	4.0	1.2

BLV(-)	CD 14	CD 11a	CD 11b	CD 11c	MHC I	MHC II	gp51
1 week	49.4	38.8	67.3	26.5	80.4	20.5	–
2 weeks	41.6	27.3	75.7	26.0	51.3	9.2	–
3 weeks	88.4	23.4	72.2	11.2	28.9	10.0	–
4 weeks	12.7	10.4	41.3	2.1	1.4	30.7	–

Mann-Whitney U Test was used for statistical analysis, significant at ($p<0,05$).

Table 2. Immunophenotype of dendritic cells and BLV-gp51 expression in leukaemic and healthy cattle (%).



Dendritic cells isolated from leukaemic cattle had high expression of CD14. In the first week of incubation the cells culture was composed of 83.6% of these cells. We observed that these values rised in the next two weeks up to 92.7% with decrease to 31% one week later. In the control group this percentage was significantly lower (49.4%), but in the third week of culture it increased almost twice (up to 88.4%), then rapidly decreased to 12.7%. The level of cells with CD11a marker expression elevated from 42.5% in the first week of culture to 63.2% in the third week, then was only 13.8%. The percentage of CD11a cells in the third week was much higher in infected cows and was statistical significant ($p<0.05$), but in the fourth week the percentages of these cell decreased. In the control animals the percentage of these cells was lower (38.8%) and had tendency to constantly diminish, up to 10.4%. The percentage of cells with CD11b marker was high in BLV-positive animals in the first week of culture, reaching (89.5%) and increased in the third week to the value of 95.7% (statistical significant). In the control animals the percentage of these cells was 67.3% in the first week of culture, then a little elevated, but was lower after four weeks. We observed that in the first week the level of CD11c cells was higher (statististical essential) in leukaemic animals than in the control group (58.4% and 26.5%, respectively), but in both groups of animals in the next weeks there was readily visible tendency to decrease, with statistical essential difference in the third week. When the cells with MHC-I expression were analysed, it was found that in infected animals these population was more abundant three weeks of investigations than in healthy animals, in which this value was still lower – from 80.4% to 2.1% in the fourth week. We observed, that percentage of MHC-II cells in the first week was higher in the healthy group than in infected animals (20.5% and 17%, respectively), but after decrease in the next week, it showed tendency to elevation in both groups of cattle during cell culture. The expression of the BLV glycoprotein gp51 in dendritic cells was present in infected animals during all the time of the experiment (4.1% – 5.6%). The statistical significance of higher percentages of DCs with expression of CD11c in infected cows was found in the first week of culture. In the third week of culture the statistical significance between CD11a, CD11b and CD11c was observed. We found that expression of CD markers on dendritic cells rapidly decreased in both group of animals in the fourth week of DCs culture, only expression of MHC-II in the control group was much higher than earlier. The explanation of this phenomenon may be found in the results of further studies on this subject.

The present study revealed that bovine leukaemia virus very readily influenced on the immunity of the infected animals. The immunophenotype of monocyte generated dendritic cells in leukaemic animals was different than that found in the control group. Dendritic cells in the leukemic animals showed significantly higher expression of surface antigens: CD14, CD11a, CD11c MHC-I and MHC-II. Statistical analysis showed essential differences in the expression of CD14 (% of cells) and CD11a, CD11b and CD11c (mediana values) in both experimental groups of cows.

Discussion

For many years it has been very difficult to obtain large numbers of DCs. With the development of recombinant cytokines and culture techniques it is now possible to generate large numbers of DCs *in vitro* (Ye et al. 1996). These cells can be adapted as antigen carriers for tumour vaccination (Weissman et al. 1995, Wischatta et al. 2000, Yi et al. 2002). Due to their high expression levels of costimulatory and adhesion molecules and their exquisite ability to produce cytokines, such as IL-12 and IL-18, they are the most effective T-cell activators (Xiao et al. 2003, Xiao et al. 2004). These characteristics make them of particular interest as adjuvant agents in cancer vaccine preparations. Promissing clinical results have even been obtained by using DCs fused with whole tumour cells, by *ex vivo* transduction of DCs using either RNA or replication-defective recombinant viral vectors to introduce genes encoding antigen. Generation of dendritic cells from the blood monocytes was very useful for DCs immunophenotype determination in flow cytometry and microscopical analysis.

Our observations performed during 4 weeks showed that CD surface antigens presented on the DCs were different and dependent on the maturity of these cells. We observed statistical essential differences in the expression of CD11a, CD11b and CD11c on dendritic cells in third week of culture *in vitro* and rapid decrease of the expression of almost all CD markers in the fourth week in both group of cows. Mature DCs are more immunogenic than immature DCs in mice (Inaba et al. 2000), and there is good evidence that this also applies to humans. Mature DCs express a high number of costimulatory molecules and more MHC-peptide complexes with a longer half-life (Kukutsch et al. 2000). In direct comparison in melanoma patients, intranodally injected peptide-pulsed mature DCs led to a potent T cell response whereas immature DCs failed to do so (Jonuleit et al. 2001). Recent studies have shown that immature DCs can even silence the immune system. Repetitive stimulation of naïve CD4+ T cell with immature DCs results in IL-10 producing regulatory T cells (Jonuleit et al. 2000). The experiments performed by Dhodapkar (Dhodapkar et al. 1999) *in vivo* on healthy volunteers and advanced melanoma patients with fully mature DCs have demonstrated that both antigen-specific CD8+T cells and IFN-γ Th1 T cells can be rapidly induced (Schuler-Thurner et al. 2002). They recommended the use of mature DCs for cancer therapy. Mature DCs exhibit a stable phenotype and are more immunogenic, easier to cryopreserve and even resistant to CTL-mediated lysis (Moll et al. 1993). The results presented in this report demonstrate that bovine in *vitro-derived* DCs can be generated using methods available for human DC generation. These DCs will aid further characterization of bovine dendritic cells biology and their relations with infectious agents.

DCs possess a unique ability to environmental stimuli, leading to different functional phenotypes based on cell surface markers and production of cytokines such was observed in Langerhans cells, plasmacytoid and myeloid DCs. In a clinical perspective this is of interest since immunomodulation of specific DCs subset leads to activation of different DCs involved in the generation of T cell mediated anti-tumour immunity. In patients with a high risk of metastatic disease or in a state of minimal residual disease, potentiation of systemic or local T cell specific immunity by modulation of DCs in tumour draining lymph nodes may establish effective immunosurveillance (Zhou et al. 2002).

DCs play a very promissing role in therapy of leukemias and tumors or in immunomodulation, but still questions remain over what is the best type of subtype of DCs to be used, route of administration, dose of antigen required to induce a response, and maintenance of therapy and control.

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