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(54) Title: MONOCLONAL ANTIBODIES THAT SPECIFICALLY RECOGNIZE CANINE DLA-DR ANTIGEN AND THEIR USES

(57) Abstract: Disclosed are monoclonal antibodies and their fragments that specifically recognize canine DLA-DR antigen and their use in the treatment, prevention, or diagnosis of leukemias and lymphomas, especially canine.

Monoclonal antibodies that specifically recognize canine DLA-DR antigen and their uses

The invention relates to monoclonal antibodies, and fragments thereof, which specifically recognize canine DLA-DR antigen and their use in the treatment, prevention or diagnosis of leukemias and lymphomas, especially canine.

Lymphoid diseases (leukemia, lymphoma) in dogs account for about 30% of all diagnosed types of cancer in this animal species. The most common are B-cell non-Hodgkin lymphomas, equivalent to human non-Hodgkin lymphomas (NHL), which account for about 20% of all canine tumors and about 85% of lymphoid tumors. The incidence of lymphomas in the dog population is higher than in humans. Dogs of different races and ages are affected, but the largest group of patients are dogs aged about 10 years.

Traditional treatment regimens for human lymphomas are used, based on classical cytostatic drugs (doxorubicin, vincristine, prednisolone, etoposide) administered alone or in combination. A disadvantage of such a therapy is small specificity with large undesirable effects. A more comprehensive therapy offers faster recovery and longer survival time, but this procedure is more costly and more toxic. In turn, chemotherapy with single drugs is cheaper but less effective. Despite the initial remission of the disease, conventional chemotherapy results in resistance development and relapse. A serious problem is the financial cost that must be borne by the dog owner. At the moment there are no other effective treatment methods that at the same time are mild and affordable. Due to the anatomical and physiological similarities, dogs have become a useful research model in development of drugs and treatments for humans.

Also naturally occurring cancers in dogs show a number of common features.

Humanized monoclonal antibodies recognizing CD20 antigen on lymphoid B cells (Rituximab, Ofatumumab) or CD33 antigen on myeloid cells (Gemtuzumab), B and T cells (CD52) (Alemtuzumab) are used to treat human lymphomas and leukemias. The disadvantage of these antibodies is that they affect normal lymphoid cells, but the natural process of renewal circulating B cells from the bone marrow prevents permanent immune deficiencies associated with deletion of these cells by the anti-CD20 immunotherapy. Attempts to use Rituximab in dogs were unsuccessful due to the lack of cross-reactivity with canine CD20. Novel monoclonal antibodies that specifically recognize the canine CD20 were obtained by Rue S.M. *et al.* and tested in the context of treatment canine B-cell lymphoma (Sarah M. Rue, Brendan P. Eckelman, Jem A. Efe, Kristin Bloink, Quinn L. Deveraux, David Lowery, Marc Nasoff, Veterinary Immunology and Immunopathology 164 (2015) 148-159).

In addition to the CD20 and CD33 antigens, the HLA-DR antigen, which belongs to the main histocompatibility complex MHC II, is considered as a target for anticancer therapy. Its expression is significantly increased in B-cell leukemias and lymphomas and in a number of autoimmune diseases, including rheumatoid arthritis (Malone DG, Wahl SM, Tsokos M, Cattell H, Decker JL, Wilder RL., J Clin Invest. 1984 Oct;74(4):1173-85) and multiple sclerosis (Olerup O, Fredrikson S, Olsson T, Kam-Hansen S. Lancet, 1987 Aug 8;2(8554):327).

Currently, human clinical trials are underway for humanized antibody against alpha subunit of human HLA-DR (hL243) (Goldenberg *et al.*, US Patent 8992917B2). HLA-DR antigen is released from the cell surface by proteolytic digestion and is detectable in serum and other body fluids. Determination of the soluble form (s-HLA-DR) may be an important diagnostic marker for lymphoproliferative and autoimmune diseases, and blocking the interaction of s-HLA-DR with Tirc7 receptors on the surface of CD4+ T cells may affect their activation (Frischer J, Reindl M, Künz B, Berger T, Schmidt S, Milford E, Knosp E, Lassmann H, Utek N.Mult Scler. 2014 Feb 13).

Sarmiento and Valli have described a monoclonal antibody recognizing T200 antigen (LCA, CD45) that may be used in immunohistochemistry for detection of canine lymphoid tumor cells (Sarmiento UM, Valli V. A canine Lymphocyte Surface Antigen detectable by a Monoclonal antibody (DT200). Can J Vet Res 1987,51, 110-116).

Steplewski and co-workers have described several monoclonal antibodies obtained against canine lymphoma cells and recognizing unidentified surface antigens on canine lymphoid cells. These antibodies reacted to varying degrees with normal lymphoid cells (Z. Steplewski, K. A. Jeglum, C. Rosales, N. Weintraub. Canine lymphoma-associated antigens defined by murine monoclonal antibodies. Cancer Immunol. Immunother. 1987,24, 197-201; CA1340898 "Monoclonal Antibodies Against Lymphoma-associated Antigens, Hybrid Cell Lines Producing These Antibodies, and Use Therefore").

The object of the invention is to provide new substances suitable for the treatment, prevention and diagnosis of leukemias and lymphomas in dogs.

Surprisingly, the problem described above is solved in the present invention.

The subject of the invention is a polypeptide that is a heavy chain variable region of an antibody specifically interacting with canine lymphoma and leukemia cells, comprising CDR region having a sequence selected from SEQ ID Nos. 1-6, preferably comprising CDR

regions designated as SEQ ID Nos. 1-3 or comprising CDR regions designated as SEQ ID Nos. 4-6, especially having the amino acid sequence designated as SEQ ID No. 7 or 8.

A further subject of the invention is a polypeptide that is a light chain variable region of an antibody specifically interacting with canine lymphoma and leukemia cells, comprising CDR region having a sequence selected from SEQ ID Nos. 9-14, preferably comprising CDR regions designated as SEQ ID Nos. 9-11 or containing CDR regions designated as SEQ ID Nos. 12-14, especially having the amino acid sequence designated as SEQ ID No. 15 or 16.

The polypeptides of the invention as defined above may be used to obtain antibodies recognizing specifically the canine DLA-DR antigen, preferably suitable for the treatment, prevention or diagnosis of leukemias and lymphomas, especially canine.

A further subject of the invention is an antibody specifically interacting with canine lymphoma and leukemia cells having:

A) antibody heavy chain containing CDR regions designated as SEQ ID Nos. 1-3, especially comprising heavy chain variable region having an amino acid sequence designated as SEQ ID No. 7 and

antibody light chain containing CDR regions designated as SEQ ID Nos. 9-11, especially comprising light chain variable region having an amino acid sequence designated as SEQ ID No. 15

or

B) antibody heavy chain containing CDR regions designated as SEQ ID Nos. 4-6, especially comprising heavy chain variable region having an amino acid sequence designated as SEQ ID No. 8 and

antibody light chain containing CDR regions designated as SEQ ID Nos. 12-14, especially comprising light chain variable region having an amino acid sequence designated as SEQ ID No. 16.

Preferably, the antibody of the invention is a chimeric murine-canine antibody having: antibody heavy chain containing heavy chain constant region derived from a canine immunoglobulin, especially from a canine antibody, and antibody light chain containing light chain constant region derived from a canine immunoglobulin, especially from a canine antibody. An example of such a chimeric caninized antibody is the cE11 antibody described

in Example 7. In an analogous manner, a caninized antibody containing CDR regions derived from B5 antibody can be obtained from polypeptides disclosed in the application.

Preferably, the antibody of the invention is an antibody produced by a hybridoma selected from cell lines deposited in DSM under access numbers DSM ACC3287 and DSM ACC3288.

A further subject of the invention is an antibody as defined above for use in the treatment or prevention of leukemia, especially canine, or lymphoma, especially canine.

A further subject of the invention is an antibody as defined above for use in the diagnosis of leukemia, especially canine, or lymphoma, especially canine.

A further subject of the invention is a hybridoma selected from cell lines deposited in DSM under access numbers DSM ACC3287 and DSM ACC3288.

Light (Vk) and heavy (VH) chain variable regions of two antibodies named B5 and E11 are disclosed, which specifically interact with antigens on canine lymphoma and leukemia cells. The B5 antibody contains CDRs of the Vk and VH chains whose amino acid sequences and their corresponding coding nucleotide sequences are disclosed in Fig. 1 as B5Vk and B5VH, while the E11 antibody contains CDRs of the Vk and VH chains whose amino acid sequences and their corresponding coding nucleotide sequences are disclosed in Fig. 2 as E11Vk and E11VH.

B5 and E11 antibodies are produced by hybridoma cells deposited in DSM under access numbers DSM ACC3287 (for B5 antibody) and DSM ACC3288 (for E11 antibody).

Surprisingly, as a result of the selection of monoclonal antibody clones directed against canine B-cell leukemia, clones have been identified which specifically recognize cells of canine B-cell, T-cell and mixed B/T leukemias and lymphomas expressing canine MHC class II antigens (DLA-DR). These antibodies interact with normal T and B lymphocytes, but the expression level of the molecules recognized on these cells is much lower than that of leukemia and lymphoma cells. The antibodies obtained can be used both in diagnosis and treatment of canine leukemias and lymphomas, as well as in other diseases where the expression of the DLA-DR antigen in increased.

One aspect of the invention are two murine monoclonal antibodies B5 and E11, and hybridoma cells secreting these antibodies. Both antibodies have IgG2a heavy chain isotype and recognize conformational epitopes on the DLA-DR molecule.

The DNA and amino acid sequences of the variable regions of each of the antibodies are shown in Figures 1 and 2. Example 1 discloses the sequence of variable regions, and Example 4 demonstrates the antibodies' antigen specificity to DLA-DR.

Antibodies obtained by the invention may be labeled with fluorescent dyes, biotin, radionuclides, paramagnetic compounds or enzymes, and used for immunological assays. Example 2 shows staining with biotin-conjugated antibodies, while other types of antibody labeling can easily be obtained by routine prior art methods.

The disclosed antibodies may be used in an ELISA immunoenzyme assay, one of which is used to coat ELISA plates and the other is labeled with biotin or peroxidase as a detection agent. Example 5 describes an example of such an assay.

A further aspect of the invention is antibody variable regions, recognizing the antigen, which can be attached to heavy (Fc) and light chain constant regions of canine immunoglobulins to obtain a chimeric murine-canine antibody of reduced immunogenicity for the purpose of canine lymphoma therapy. Example 7 illustrates the method of constructing a murine-canine chimeric antibody based on sequences of variable regions of mouse E11 antibody (disclosed in Fig. 2) and constant regions of canine immunoglobulins. Figure 6 shows the specificity analysis of the obtained chimeric antibodies using flow cytometry.

A further aspect of the invention is antibody fragments (Fab) obtained by enzymatic proteolysis which may be further conjugated to cytotoxic and/or cytostatic substances (e.g. doxorubicin, betulin, methotrexate) and used in therapy of (canine/human) leukemias and lymphomas or autoimmune diseases.

The B5 and E11 monoclonal antibodies disclosed in this application are the only antibodies that are not based on cross-reactivity between DLA-DR and HLA-DR molecules, but are produced by immunizing mice with canine antigens, which guarantees optimum affinity to the target antigen. Example 2 demonstrates that the fluorescence level obtained after binding the same amount of B5 and E11 antibodies to the DLA-DR antigens present on dog-derived lines is significantly higher than on human lines expressing HLA-DR antigen, indicating optimal affinity to DLA-DR.

IgG2a heavy chain isotype of both antibodies, due to the optimal binding of complement components and Fc receptors on immune cells, allows for antibody-dependent cytotoxic effects. There are also the only available anti-DLA-DR murine antibodies with IgG2a isotype.

Example 6 illustrates the cytotoxicity level in an *in vitro* assay with rabbit complement and indicates that both antibodies exhibit activity in this assay.

The diagnostic potential in canine lymphomas and B-cell and mixed B/T-cell leukemias for the combined use of B5 and E11 antibodies exceeds 94% and is significantly higher than for other available antibodies. Example 3 indicates that the use of B5 and E11 antibodies allows a specific diagnosis of more than 94% of B-cell or mixed B/T neoplasms in lymph nodes using fine needle aspiration biopsy (which is currently the accepted standard of diagnostic procedure). It has been shown, however, that biopsies from enlarged lymph nodes of dogs not suffering from lymphoma or B-type leukemia or of dogs with Lyme disease do not yield a positive reaction with B5 and E11 antibodies.

B5 and E11 MAbs recognize soluble forms of DLA-DR antigens in the ELISA, which is not characteristic of all antibodies reacting with cell membrane-associated HLA-DR molecules. Example 5 confirms the potential of B5 and E11 antibodies to bind DLA-DR antigens present in body fluids or tumor cell lysates.

Example 1. Hybridoma generation and DNA sequencing of genes encoding light and heavy chain variable regions of the secreted antibodies, specifically interacting with canine B-cell lymphoma cells.

A standard, commonly described procedure of mice immunization and splenocyte fusion was used to generate monoclonal antibody-producing hybridomas. Briefly, 6 x 10^6 cells of canine B-cell lymphoma (CLB70) were suspended in 300 microliters of saline and emulsified in 300 microliters of incomplete Freund's adjuvant. Such prepared antigen was administered in three intraperitoneal injections of 200 microliters/injection to CD-1 mice at two-week intervals. Four days after the last injection, splenocytes of the immunized mice were fused in the presence of polyethylene glycol (PEG 1500) with myeloma SP2.0 line and cultured in the presence of selection medium containing hypoxanthine, aminopterin and thymidine at 37°C in atmosphere containing 5% CO_2. Supernatants from hybridoma culture (500 clones) were screened for reactivity with CLB70 cells using flow cytometry.

In the pool of the analyzed hybridomas, unexpectedly, two lines were identified that produced monoclonal antibodies with very high affinity to canine B-cell lymphomas (CLB70). These antibodies were named B5 and E11, and hybridomas producing them – IITD PAN B5 and IITD PAN E11, respectively. These hybridomas were deposited in accordance with the

Budapest Treaty in the DSMZ (German Collection of Microorganisms and Cell Cultures) under the following access numbers: DSM ACC3287 (line IITD PAN B5) and DSM ACC3288 (line IITD PAN E11).

mRNAs were isolated from selected hybridomas producing the antibodies of interest, which, following transcription into cDNA using standard methods of molecular biology, were amplified with oligonucleotide primers of sequences complementary to the regions:

V_k (5'-CCAGTTCCGAGCTCGTGCTCACCCAGTATACA)

and V_H (5'-AGGTCCAGCTGCTCGAGTCTGG) and

C_H 5'-GCGTCTAGAAYCTCCACACACAGGRRCCAGTGGATAGAC

and C_k 5'-GCGTCTAGAACTGGATGGTGGGAAGATGG

of murine immunoglobulin. The obtained cDNA fragments were cloned and sequenced using Sanger method. Fig. 1 and 2 show DNA sequences encoding the heavy chain VH and light chain Vk variable regions of B5 and E11 immunoglobulin. Kabat nomenclature was used in the positioning of amino acids corresponding to variable regions of immunoglobulins. Hypervariable regions in the amino acid sequence are given in bold type and CDR-1, -2, -3 were described accordingly.

Example 2. Reactivity analysis of B5 and E11 antibodies with surface antigens present on selected canine and human cell lines by flow cytofluorimetry.

Biotinylated B5 and E11 antibodies or biotinylated mouse anti-DNP control antibody in amount of 1.5 micrograms per 100 microliters of buffered saline (PBS) supplemented with 2% fetal bovine serum (FBS) were incubated with suspensions of 10^5 cells indicated in Fig. 3 on ice for 30 minutes. After washing of unbound antibodies with PBS + 2% FBS, the cells were incubated in 100 microliters per sample with a fluorescent conjugate of streptavidin with phycoerythrin (Streptavidin PE, eBioscience) diluted 1:1000. Cytofluorimetry was performed using BD Calibur device. Results are shown as histograms (gray fill) referring to fluorescence of cells stained with control antibody (no fill). These analyzes have shown that antigens recognized by B5 and E11 antibodies are present on the surface of the canine (CLBL1 and CLB70) and human (Raji) lines of B-cell lymphomas, which have, documented in other publications, expression of MHC II haplotype DRB antigens; however, the antigens are absent on canine (GL-1) and human (Jurkat) T-cell lines and canine mastocytoma line (NL-1). Canine GL-1 line with B and T lymphocyte markers, but lacking MHC class II antigens, interacts poorly with B5 antibody and does not interact with E11 at all.

Example 3. Reactivity analysis of B5 and E11 antibodies with blood samples and dog lymph node biopsies.

The obtained suspensions of mononuclear cells from peripheral blood (PBMCs) (Table 1) or from lymph node biopsies (Table 2) of dogs with diagnosed lymphomas or enlarged as a result of confirmed *Borrelia burgdorferi* infection were stained as in Example 2 and subjected to FACS cytofluorimetry. Percentage of PBMCs with B5 and E11 mAb reactivity was the highest for B-cell lymphoma cases and was three out of three analyzed for B5 mAb and two out of three for E1 mAb, respectively (Table 1). However, no reactivity of either antibody was reported for samples from healthy dogs, dogs infected with *Borrelia burgdorferi* or diagnosed with T-cell lymphomas. Percentage of lymph node cells with specific fluorescence, above the isotype control fluorescence (> 15% positive cells), are summarized in Table 2, separately for each dog. For 13 patients with confirmed B-cell lymphomas, 11 (84.6%) and 12 (92.3%) showed reactivity with B5 and E11 antibodies, respectively. Among dogs with T-cell lymphomas these values were respectively 1 in 5 with B5 mAb (20%) and 0 in 5 with E11 mAb. For 5 analyzed lymphomas of mixed B/T-cell phenotype, 5 (100%) were positive for B5 mAb and 4 (80%) for E11 mAb. Table 3 evaluates the expression level of antigens recognized by B5 and E11 mAbs. It was demonstrated that the highest mean fluorescence intensity (MFI) of 539 and 294 was shown by mixed B/T-cell and B-cell lymphomas stained with B5 antibody, respectively. For E11 antibody, these values were respectively 308 and 162. The MFI for T-cell lymphomas was 23 and 15 for B5 and E11 antibodies, respectively. The MFI values for PBMCs from healthy dogs or patients with Lyme disease did not exceed 10.5.

Example 4. Identification of antigens recognized by B5 and E11 antibodies.

Based on the sequencing of immunoprecipitated proteins from CLBL1 line lysates by mass spectrometry, histocompatibility class II antigen dimers, DLA-DRs, were provisionally pre-selected as specifically bound by B5 and E11 antibodies. In order to confirm this identification, human fetal carcinoma line (HEK293) was transfected with gene constructs encoding canine DLA-DRα and DLA-DRβ histocompatibility antigen chains in pcDNA3 expression vector or control empty expression vector. Briefly, 3 μg of purified plasmid DNA were incubated with Metafecten Pro reagent (Biontex) according to the manufacturer's protocol and then DNA/Metafecten Pro mixture was introduced into 2 x 10^5 HEK293 cells cultured on a 12-well plate in 1 ml of OptiMEM medium (Life Technologies). 24 hours after transfection, the cells were analyzed with FACS cytofluorimetry using B5 and E11 antibodies

as described in Example 2. Fig. 4a shows an exemplary, representative FACS analysis of cells after transfection. Cells transfected with single control constructs (pcDNA3) or encoding either DLA-DRα or DLA-DRβ chains did not stain with fluorescent conjugates of B5 and E11 antibodies, while staining was demonstrated for both antibodies in case of co-transfection of HEK293 cells with constructs coding for DLA-DRα and DLA-DRβ chains.
Using Western blotting technique on protein lysates from cells transfected with the gene constructs described above, that B5 and E11 antibodies have been shown to recognize the DLA-DRαβ dimer with a molecular weight of 55 kD, but do not recognize single DLA-DRα or DLA-DRβ chains (Fig. 4b). Furthermore, reactivity of both antibodies in Western blotting with lysates from B-cell lymphoma lines (CLBL1 and CLB70) was demonstrated, but not with lysates from B-cell line (GL-1) lacking DLA-DR antigens, as well as with lysates from peripheral blood mononuclear cells PBMCs, Fig. 4b. Moreover, it has been established that the epitopes recognized by B5 and E11 antibodies are conformational, as the treatment of protein lysates with 6 M urea solution or boiling them at temperatures above 70°C abolished the antibody-antigen interaction.

Example 5. DLA-DR and s-DLA-DR ELISA on cell lysates and body fluids based on the use of B5 and E11 antibodies.

ELISA plates (Maxisorp, Nunc) were coated with a solution of E11 antibody at 2 µg/ml PBS at 4°C for 12 hours. After blocking the plate with 1% solution of casein in TBS buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) with 0.05% Tween-20 detergent for 1 hour at 37°C, plates were incubated with test solutions (cell lysate, blood serum) for 1 hour at 37°C, then washed 3 times with TBS+0.05% Tween-20 and incubated with biotinylated B5 antibody (1.5 µg/ml) for 1 hour at 37°C. After washing three times, as described above, plates were incubated with streptavidin-horseradish peroxidase conjugate (1:1000, eBioscience) for 1 hour at 37°C, followed by a colored reaction by adding TMB substrate. Table 4 shows that lysates from cells expressing DLA-DR histocompatibility antigens demonstrate approximately 40 times higher specific ELISA absorbance versus control cells, and that blood sera from dogs with lymphoma show on average twice higher specific absorbance in this assay in relation to the controls. Fig. 5 shows results of the ELISA test with lysates from lymph node biopsies from healthy dogs and patients with B-cell, T-cell and B/T lymphomas. More than 85% of the lysates from diseased dogs give a positive result.

Example 6. Complement-dependent cytotoxicity analysis for B5 and E11 antibodies

against canine lymphoma CLBL1.

Canine CLBL-1 lymphoma cells (2 x 10^5) were incubated with B5 or E11 antibodies at a concentration of 1 µg/100 µl for 1 hour on ice in RPMI medium without serum. After centrifugation of the cells (300 x g for 5 minutes) and antibody washing (5 ml of RPMI medium), the cells were suspended in 1 ml of RPMI medium supplemented with 50 µl of non-toxic rabbit complement (Sigma). Cells with complement were incubated for 40 minutes in 37°C water bath with periodic mixing of the sample every 10 minutes. After incubation, the cells were centrifuged (300 x g for 5 minutes), washed with PBS+2% FBS and the viability was assayed by incubation with propidium iodide (50 ng/ml) in a FACS cytofluorimetic assay. Dead and live cells were also counted in Burker's chamber using trypan blue staining. Table 5 shows that B5 and E11 antibodies, but not a murine control antibody of the same isotype as B5 and E11, exhibit a complement-dependent cytotoxic effect.

Example 7. Construction method and specificity analysis of murine-canine chimeric antibody (cE11) based on sequences using variable regions of murine E11 antibody.

mRNA molecules encoding heavy and light chain variable regions were isolated from the E11 hybridoma using Trizol reagent (Thermofisher Scientific). Rapid reaction of mRNA transcription into cDNA was performed using MMLV reverse transcriptase enzyme using oligonucleotide primers complementary to 3' portions of heavy chain V_H 5'-GCGTCTAGAAYCTCCACACACAGGRRCCAGTGGATAGAC or light chain V_L 5'-GCGTCTAGAACTGGATGGTGGGAAGATGG variable regions of murine immunoglobulins. The resulting PCR products were cloned by TA method into pGEM T-easy vector (Promega) and sequenced.

Fragments of mRNA molecules encoding constant regions of canine immunoglobulins were obtained by mRNA isolation from dog peripheral blood leukocytes with Trizol reagent (Thermofisher Scientific), transcription into cDNA and amplification by PCR using the following oligonucleotide primers:

Heavy chain constant region of canine Ig (cIgH)

HCANISF 5'-CTCAGCCTCCACCACG

HCANISR 5'-CAGGATCCTCATTTACCCGGAGAATGG

Light chain constant region of canine Ig (cIgL)

LCANISF 5'-CTTGTTCCAACCATCTCCAG

LCANISR 5'-CACTTGCTAGCTTAGTCCACTCTCTGACACTCG

Using the PCR described below, murine and canine cDNA regions encoding immunoglobulin variable and constant chains were amplified. Murine and canine amplicons contained complementary regions which allowed to produce chimeric molecules in subsequent amplification cycles using the following oligonucleotide primers:

Heavy chain variable region of murine Ig (mIgH)

HE11F CTTCCGGAATGGGATGGAGCTGGATC

HE11R *CGTGGTGGAGGCTGAG*GAGACGGTGACTGAGGTTC (the fragment complementary to HCANISF primer is given in italic font)

Light chain variable region of murine Ig (mIgL)

LE11F GCCAGATCTATGAGTGTGCCCACTC

LE11R *CTGGAGATGGTTGGAACAAG*GATACAGTTGGTGCAGC (the fragment complementary to LCANISF primer is given in italic font)

The PCR reaction using high-fidelity KapaTaq HiFi polymerase (Kapa Biosystems) was performed under the following temperature conditions:

98°C 3 min
95°C 20 sec.
52°C 15 sec.
72°C 20 sec.
72°C 1 minute
10°C ∞

Amplified DNA fragments were cloned into pVitro neo vector (Invivogen) using BspEI and BamHI restriction sites for the heavy chain and BglII and NheI for the light chain and taking advantage of the presence of suitable restriction sites in amplified cDNA fragments for murine-canine immunoglobulins.

The amino acid sequences of both chains of the resulting chimeric cE11 antibody (i.e., the caninized E11 antibody) and the nucleotide sequences encoding them are shown in Fig. 6.

Moreover, the sequence listing shows the heavy chain constant region of canine immunoglobulin and the light chain constant region of canine immunoglobulin, which were used to obtain the chimeric cE11 antibody.

The generated constructs were transfected into cells of CHO line by lipofection with Lipofectamine 2000 (Life Technologies). After 48 hours, supernatants from the cultures were screened for antibodies interacting with canine lymphoma lines (Fig. 7).

Claims

1. A polypeptide that is a heavy chain variable region of an antibody specifically interacting with canine lymphoma and leukemia cells, comprising CDR region having a sequence selected from SEQ ID Nos. 1-6, preferably comprising CDR regions designated as SEQ ID Nos. 1-3 or comprising CDR regions designated as SEQ ID Nos. 4-6, especially having the amino acid sequence designated as SEQ ID No. 7 or 8.

2. A polypeptide that is a light chain variable region of an antibody specifically interacting with canine lymphoma and leukemia cells, comprising CDR region having a sequence selected from SEQ ID Nos. 9-14, preferably comprising CDR regions designated as SEQ ID Nos. 9-11 or containing CDR regions designated as SEQ ID Nos. 12-14, especially having the amino acid sequence designated as SEQ ID No. 15 or 16.

3. An antibody specifically interacting with canine lymphoma and leukemia cells having:
A) antibody heavy chain containing CDR regions designated as SEQ ID Nos. 1-3, especially comprising heavy chain variable region having an amino acid sequence designated as SEQ ID No. 7 and
antibody light chain containing CDR regions designated as SEQ ID Nos. 9-11, especially comprising light chain variable region having an amino acid sequence designated as SEQ ID No. 15
or
B) antibody heavy chain containing CDR regions designated as SEQ ID Nos. 4-6, especially comprising heavy chain variable region having an amino acid sequence designated as SEQ ID No. 8 and
antibody light chain containing CDR regions designated as SEQ ID Nos. 12-14, especially comprising light chain variable region having an amino acid sequence designated as SEQ ID No. 16.

4. The antibody of claim 3, characterized by being a chimeric murine-canine antibody having: antibody heavy chain containing heavy chain constant region derived from a canine immunoglobulin, especially from a canine antibody, and antibody light chain containing light chain constant region derived from a canine immunoglobulin, especially from a canine antibody.

5. The antibody of claim 3, characterized by being produced by a hybridoma selected from cell lines deposited in DSM under access numbers DSM ACC3287 and DSM ACC3288.

6. The antibody of claims 3-5 for use in the treatment or prevention of leukemia, especially canine, or lymphoma, especially canine.

7. The antibody of claims 3-5 for use in the diagnosis of leukemia, especially canine, or lymphoma, especially canine.

8. Hybridoma selected from cell lines deposited in DSM under access numbers DSM ACC3287 and DSM ACC3288.

Sequences of murine immunoglobulin B5 variable regions

B5VH:

CAGGTCCAACTGCAACAGTCTGGACCTGAACTGATGAAGCCTGGGACTTCAGTGAAGATTTCCTGCAA
GGCTTCTGGCTACACTTTCACAAGCTACTTTATACACTGGATGAAGCAGAGGCCTGGACAGGGACTTG
AGTGGATTGGATGGATTTTTCCTGGAAATATTAATGCTAAATATAATGAGAACTTCAGGGGCAAGGCC
ACACTGACGGCAGACACATCCTCCACCACCGCCTACATACAGCTCAGCAGCCTAACATCTGAGGACTC
TGCGGTCTATTACTGTGCAAGAGCCCCTTTACTGGGGAACTACTTTGACTACTGGGGCCAAGGCACCA
CTCTCACAGTCTCCTCA

Q V Q L Q Q S G P E L M K P G T S V K I S C K A S G Y T F T **S Y F I H** W M K
1 10 20 30 CDR-1

Q R P G Q G L E W I G **W I F P G N I N A K Y N E N F R G** K A T L T A D T S S
40 50 52 a CDR-2 60 70

T T A Y I Q L S S L T S E D S A V Y Y C A R **A P L L G N Y F D Y** W G Q G T T
80 82 a b c 90 CDR-3 100a b

L T V S S
110

B5Vk:

GAGCTCGTGCTCACCCAGTCTCCAGCTTCACTGTCTGCATCTGTGGGAGAAACTGTCACCATCACATG
TGGAGCAAGTGAGAATATTTACGGTGCTTTAAGTTGGTATCAGCGGAAACAGGGCAAGTCTCCTCAGC
TCCTGATCTATGGTGCAACCAACTTGGCAGATGGCTTGTCATCGAGGTTCAGTGGCAGTGGATCTGGT
AGACAGTATTCTCTCAAGATCAGTAGCCTGCATCCTGACGATGTTGCAACGTATTACTGTCAAAATTT
TTTTATTACTCCGTACACGTTCGGAGGGGGGACCAAGCTGGAAATGAAACGG

E L V L T Q S P A S L S A S V G E T V T I T C **G A S E N I Y G A L S** W Y Q R
1 10 20 CDR-1 30

K Q G K S P Q L L I Y **G A T N L A D** G L S S R F S G S G S G R Q Y S L K I S
40 50 CDR-2 60 70

S L H P D D V A T Y Y C **Q N F F I T P Y T** F G G G T K L E M K R
80 90 CDR-3 100

Fig. 1

Sequences of murine immunoglobulin E11variable regions

E11VH:

CAGGTCCAGCTGCAGCAGTCTGGACCTGAACTGGTGAAGCCTGGGACTTCAGTGAAGATATCCTGCAA
GGCTTCTGGCTACAGGTTCACAAGCTACTATATACACTGGGTGAAGCAGAGGCCTGGACAGGGACTTG
AGTGGATTGGATGGATTTATCCTGGAAGTGGCAATAGTAAGTACAATGAGAAGTTCAAGGGCAAGGCC
ACACTGACGGCAGACACATCCTCCAGCACTGCCTACATGCAGCTCAGCAGCCTAACATCTGAGGACTC
TGCGGTCTTTTACTGTGCAAGAGGTGGCTCAGGCTACGTAGGGGCTATGAACTGCTGGGGTCAAGGAA
CCTCAGTCACCGTCTCCTCA

```
Q V Q L Q Q S G P E L V K P G T S V K I S C K A S G Y R F T S Y Y I H W V K
1               10                  20                  30   CDR-1

Q R P G Q G L E W I G W I Y P G S G N S K Y N E K F K G K A T L T A D T S S
  40                50  52 a      CDR-2   60                  70

S T A Y M Q L S S L T S E D S A V F Y C A R G G S G Y V G A M N C W G Q G T
        80  82 a b c             90         CDR-3   100a b c

S V T V S S
   110
```

E11Vk:

GACATCCAGATGACTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATG
TCGAACAAGTGAGAATATTTACAGTTTTTTAGCATGGTCTCAGCAGAAACAGGGAAAATCTCCTCAGC
TCCTGGTCTATAATGCAAAAACCTTAGTAGAAGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGC
ACACAGTTTTCTCTGAGGATCAACAGCCTGCAGCCTGAAGATTTTGGGAGTTATTACTGTCAACATCA
TTATGGTACTCCTCTCACGTTCGGCGCTGGGACCAAGCTGGAGGTGAAACGG

```
D I Q M T Q S P A S L S A S V G E T V T I T C R T S E N I Y S F L A W S Q Q
1               10                  20          CDR-1  30

K Q G K S P Q L L V Y N A K T L V E G V P S R F S G S G S G T Q F S L R I N
  40                50 CDR-2          60                  70

S L Q P E D F G S Y Y C Q H H Y G T P L T F G A G T K L E V K R
      80                90   CDR-3
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Fig. 2

FACS analysis of canine and human cell lines with B5 and E11 antibodies

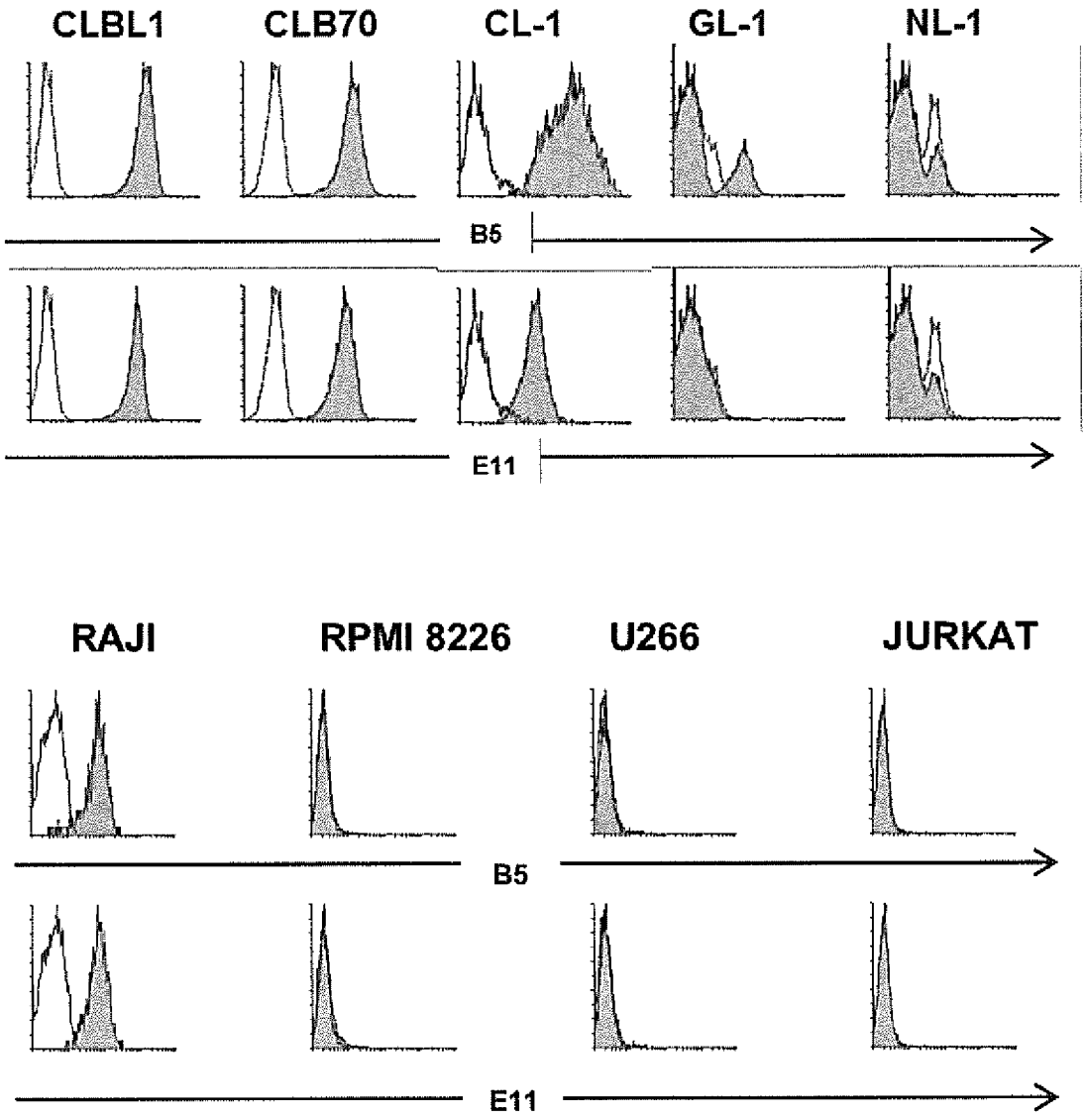
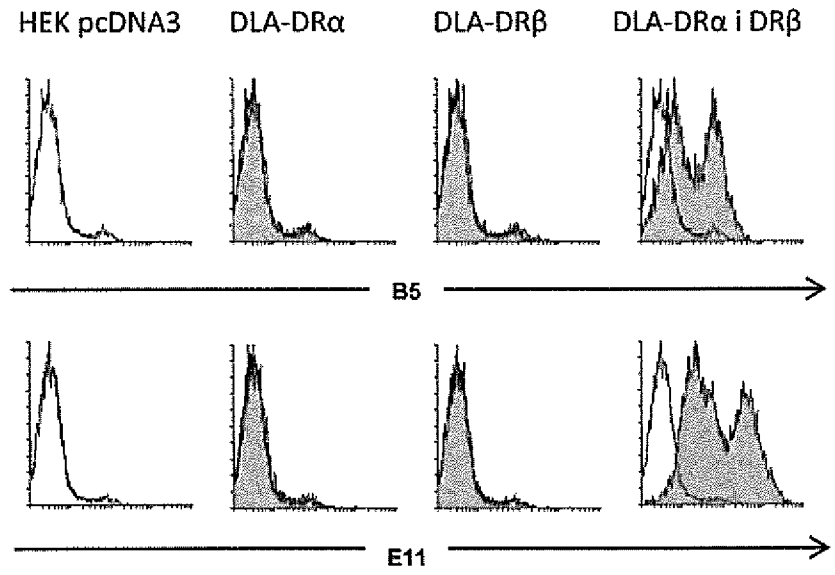


Fig. 3

Reactivity of B5 and E11 antibodies to HEK293 cells transfected with gene constructs coding for DLA-DR chains by a) FACS and b) Western blotting

a)



b)

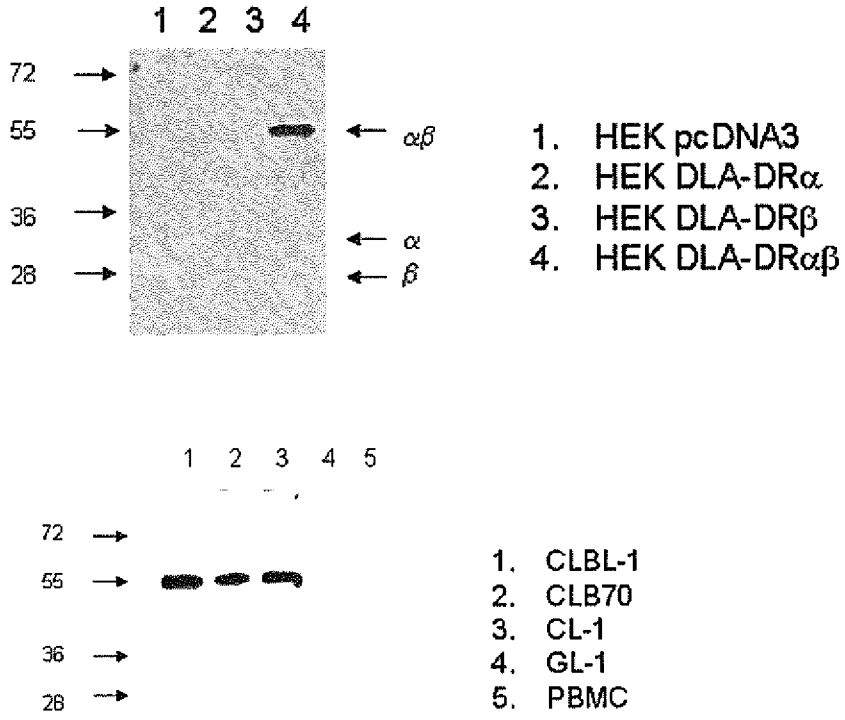


Fig. 4

Detection of DLA-DR molecules in lysates from lymph node biopsies of healthy dogs (C) and dogs with B-cell (B), T-cell (T) and B/T (B/T) lymphomas

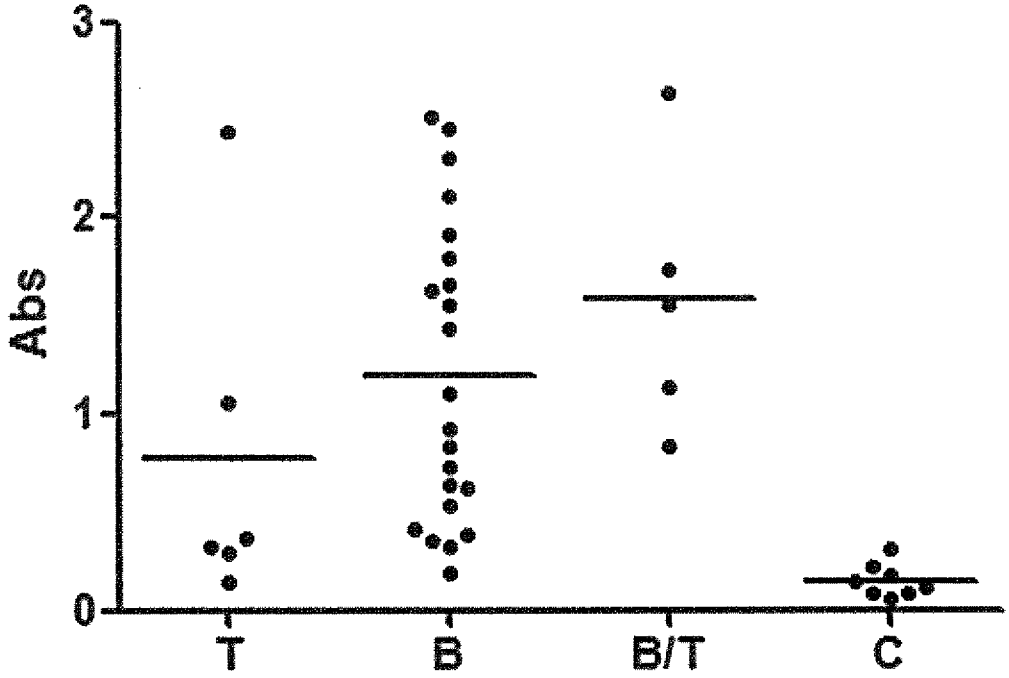


Fig. 5

cE11

Heavy chain

ATGGGATGGAGCTGGATCTTTCTCTTCCTCCTGTCAGGAACTGCAGGTGTCCATTGCCAGGTCCAGCTGCAGCAG
TCTGGACCTGAACTGGTGAAGCCTGGGACTTCAGTGAAGATATCCTGCAAGGCTTCTGGCTACAGGTTCACAAGC
TACTATATACACTGGGTGAAGCAGAGGCCTGGACAGGGACTTGAGTGGATTGGATGGATTTATCCTGGAAGTGGC
AATAGTAAGTACAATGAGAAGTTCAAGGGCAAGGCCACACTGACGGCAGACACATCCTCCAGCACTGCCTACATG
CAGCTCAGCAGCCTAACATCTGAGGACTCTGCGGTCTTTTACTGTGCAAGAGGTGGCTCAGGCTACGTAGGGGCT
ATGAACTGCTGGGGTCAAGGAAC**CTCAGTCACCGTCTCCTCAGCCTCCACCACGGCCCCCTC**GGTTT
TCCCACTGGCCCCCAGCTGCGGGTCCACTTCCGGCTCCACGGTGGCCCTGGCCTGCCTGGTGTCAGGCTACTTCC
CCGAGCCTGTAACTGTGTCCTGGAATTCCGGCTCCTTGACCAGCGGTGTGCACACCTTCCCGTCCGTCCTGCAGT
CCTCAGGGCTCTACTCCCTCAGCAGCATGGTGACAGTGCCCTCCAGCAGGTGGCCCAGCGAGACCTTCACCTGCA
ACGTGGCCCACCCGGCCAGCAAAACTAAAGTAGACAAGCCAGTGCCCAAAAGAGAAAATGGAAGAGTTCCTCGCC
CACCTGATTGTCCCAAATGCCCAGCCCCTGAAATGCTGGGAGGGCCTTCGGTCTTCATCTTTCCCCCGAA
ACCCAAGGACACCCTCTTGATTGCCCGAACACCTGAGGTCACATGTGTGGTGGTGGATCTGGACCCAGAA
GACCCTGAGGTGCAGATCAGCTGGTTCGTGGACGGTAAGCAGATGCAAACAGCCAAGACTCAGCCTCGTG
AGGAGCAGTTCAATGGCACCTACCGTGTGGTCAGTGTCCTCCCCATTGGGCACCAGGACTGGCTCAAGGG
GAAGCAGTTCACGTGCAAAGTCAACAACAAAGCCCTCCCATCCCCGATCGAGAGGACCATCTCCAAGGCC
AGAGGGCAAGCCCATCAGCCCAGTGTGTATGTCCTGCCGCCATCCCGGGAGGAGTTGAGCAAGAACACAG
TCAGCTTGACATGCCTGATCAAAGACTTCTTCCCACCTGACATTGATGTGGAGTGGCAGAGCAATGGACA
GCAGGAGCCTGAGAGCAAGTACCGCACGACCCCGCCCCAGCTGGACGAGGACGGGTCCTACTTCCTGTAC
AGCAAGCTCTCTGTGGACAAGAGCCGCTGGCAGCGGGGAGACACCTTCATATGTGCGGTGATGCATGAAG
CTCTACACAACCACTACACACAGGAATCCCTCTCCCATTCTCCGGGTAAATGA

M G W S W I F L F L L S G T A G V H C Q V Q L Q Q S G P E L V K P G T S V K
I S C K A S G Y R F T S Y Y I H W V K Q R P G Q G L E W I G W I Y P G S G N
S K Y N E K F K G K A T L T A D T S S S T A Y M Q L S S L T S E D S A V F Y
C A R G G S G Y V G A M N C W G Q G T S V T V S S A S T T A P S V F P L A P
S C G S T S G S T V A L A C L V S G Y F P E P V T V S W N S G S L T S G V H
T F P S V L Q S S G L Y S L S S M V T V P S S R W P S E T F T C N V A H P A
S K T K V D K P V P K R E N G R V P R P P D C P K C P A P E M L G G P S V F
I F P P K P K D T L L I A R T P E V T C V V V D L D P E D P E V Q I S W F V
D G K Q M Q T A K T Q P R E E Q F N G T Y R V V S V L P I G H Q D W L K G K
Q F T C K V N N K A L P S P I E R T I S K A R G Q A H Q P S V Y V L P P S R
E E L S K N T V S L T C L I K D F F P P D I D V E W Q S N G Q Q E P E S K Y
R T T P P Q L D E D G S Y F L Y S K L S V D K S R W Q R G D T F I C A V M H
E A L H N H Y T Q E S L S H S P G K Stop

Light chain

ATGAGTGTGCCCACTCAGGTCCTGGGGTTGCTGCTGCTGTGGCTTACAGGTGCCAGATGTGACATCCAGATGACT
CAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATGTCGAACAAGTGAGAATATTTAC
AGTTTTTTAGCATGGTCTCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATAATGCAAAAACCTTAGTA
GAAGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCAGGCACACAGTTTTCTCTGAGGATCAACAGCCTGCAGCCT
GAAGATTTTGGGAGTTATTACTGTCAACATCATTATGGTACTCCTCTCACGTTCGGCGCTGGGACCAAGCTGGAG
GTGAAACGG**GCTGATGCTGCACCAACTGTATCCTTGTTCCAACCATCTC**CAGACCAGTTACACACAG
GAAGTGCCTCTGTTGTGTGTTTGCTGAATAGCTTCTACCCCAAAGACATCAATGTCAAGTGGAAAGTGGATGGTG
TCATCCAAGACACAGGCATCCAGGAAAGTGTCACAGAGCAGGACAAGGACAGTACCTACAGCCTCAGCAGCACCC
TGACGATGTCCAGTACTGAGTACCTAAGTCATGAGTTGTACTCCTGTGAGATCACTCACAAGAGCCTGCCCT
CCACCCTCATCAAGAGCTTCCAAAGGAG**CGAGTGTCAGAGAGTGGACTAA**

M S V P T Q V L G L L L L W L T G A R C D I Q M T Q S P A S L S A S V G E T
V T I T C R T S E N I Y S F L A W S Q Q K Q G K S P Q L L V Y N A K T L V E
G V P S R F S G S G S G T Q F S L R I N S L Q P E D F G S Y Y C Q H H Y G T
P L T F G A G T K L E V K R A D A A P T V S L F Q P S P D Q L H T G S A S V
V C L L N S F Y P K D I N V K W K V D G V I Q D T G I Q E S V T E Q D K D S
T Y S L S S T L T M S S T E Y L S H E L Y S C E I T H K S L P S T L I K S F
Q R S E C Q R V D Stop

Fig. 6

Reactivity of chimeric murine-canine cE11 antibodies with canine and human lymphoma lines

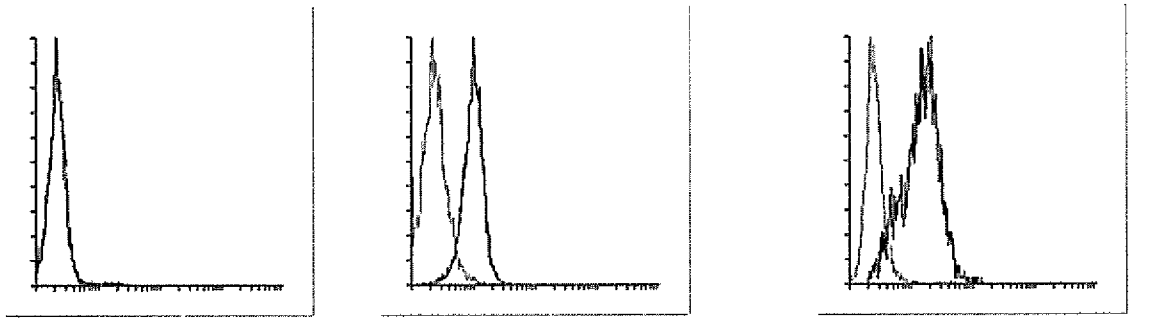


Fig. 7

Table 1

FACS analysis of peripheral blood mononuclear cells of dogs with B5 and E11 antibodies

MAb	Healthy		Lyme disease	B-cell lymphomas			T-cell lymphoma
	1	2	1	1	2	3	1
B5	5.5*	0	6	42	80	47	2
E11	7	0	7	33	5	97	6.5

* Percentage of positive cells (>15% positive cells relative to isotype control)

Table 2

FACS analysis of cells from dog lymph node biopsies

mAb	B-cell lymphomas													%LP	T-cell lymphomas					%LP	mixed (T/B) lymphomas					%LP	Lyme disease
	1	2	3	4	5	6	7	8	9	10	11	12	13		1	2	3	4	5		1	2	3	4	5		
B5	88*	15	99	98	94	80	30	52	62	8	82	75	50	**84.6**	2.5	15	5	30	14	**20**	77	65	83	98	97	**100**	8
E11	89	12	99	96	86	74	24	38	52	76	63	79	48	**92.3**	1.5	2	4.5	14	3	**0**	71	3	95	99	95	**80**	14

* - percentage of positive cells (>15% positive cells relative to isotype control)
%LP - percentage of positive lines

Table 3

Evaluation of expression level of antigens recognized by B5 and E11 mAbs on cells from enlarged lymph nodes and canine peripheral blood mononuclear cells by flow cytofluorimetry

	B5	E11	n
Lymph nodes			
Lymphomas:			
B-cell	294*	162	9
T-cell	23	15	4
B/T-mixed	539	308	5
Lyme disease	2.5	10.5	1
PBMCs			
healthy	7	8	2
Lyme disease	3	7	1

* Mean Fluorescence Intensity (MFI)
(n) number of independent biological samples
PBMCs - peripheral blood mononuclear cells

Table 4

Detection of DLA-DR molecules in protein lysates from cell lines and blood sera of dogs by ELISA method

	mean abs.*	SD**
Lysates		
CLBL1	2.56	
CLB70	2.55	
HEK + DLA-DRαβ	1.15	
HEK	0.06	
Blood serum		
healthy dogs (n = 3)	0.26	± 0.03
dogs with lymphomas (n = 4)	0.53	± 0.15
Background (n = 4)	0.05	± 0.01

* mean absorbance at λ = 450 nm
** standard deviation
n = number of independent biological samples

Table 5

Complement-dependent cytotoxicity (CDC) analysis for B5 and E11 antibodies towards canine B-cell lymphoma CLBL1 line *in vitro*

mAb	% of living*
B5	7
E11	4.5
mIgG2a	98

* Percentage of living cells after incubation with rabbit complement

INTERNATIONAL SEARCH REPORT

International application No.
PCT/PL2017/050014

A. CLASSIFICATION OF SUBJECT MATTER
C07K16/30 (2006.01); A61K39/395 (2006.01); A61P35/02 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPODOC, WPI, STN (SEQUENCE SEARCH, CAplus, EMBASE, BIOSIS, MEDLINE, DWPI)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0289053 A2 (WISTAR INST. US) 1988-11-02 claims 1-14 examples 1, 5, 6 & STEPLEWSKI, Z. ET AL.: "Canine lymphoma-associated antigens defined by murine monoclonal antibodies", CANCER IMMUNOLOGY, IMMUNOTHERAPY, (1987), 24: 197-201, doi:10.1007/BF00205629 abstract, p.197, right-hand col. par. 1-2 pp. 199, right-hand col. par. 3 - 200, left-hand col. par. 1-2 p. 200, left hand col. par. 3	1-8

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 2017	24 July 2017

Name and mailing address of the ISA/	Authorized officer
Visegrad Patent Institute / Branch Office PL Al. Niepodległości 188, 00-950 Warsaw, Poland Facsimile No. +48 22 579 00 01	Małgorzata Kozłowska Telephone No. +48 22 579 06 07

INTERNATIONAL SEARCH REPORT

International application No.
PCT/PL2017/050014

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This International Searching Authority found two groups of inventions in this international application, as follows:

1. claims 1-8 (partially)
An antibody designated as B5, and its fragments, having a heavy chain containing CDR regions of SEQ ID No. 1-3 and/or a light chain containing CDR regions of SEQ ID No. 9-11, especially comprising a heavy chain variable region (VH) of SEQ ID No. 7 and a light chain variable region (VL) of SEQ ID No. 15 and hybridoma deposited in DSM under the accession No. ACC3287 producing it.
2. claims 1-8 (partially)
An antibody designated as E11, and its fragments, having a heavy chain containing CDR regions of SEQ ID No. 4-6 and/or a light chain containing CDR regions of SEQ ID No. 12-14, especially comprising a heavy chain variable region (VH) of SEQ ID No. 8 and a light chain variable region (VL) of SEQ ID No. 16. and hybridoma deposited in DSM under the accession No. ACC3288 producing it.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SARMIENTO ET AL.: "A canine lymphocyte surface antigen detectable by a monoclonal antibody (DT200)", CANADIAN JOURNAL OF VETERINARY RESEARCH, (1987), 51(1): 110-116 the whole document	1-8
X	WO 2014114801 A1 (AMGEN INC. US) 2014-07-31 SEQ ID No. 136, claim 3, p. 105 SEQ ID No. 166, claim 3, p. 106	1
X	US 9079949 B1, (ALEXION PHARM INC. US) 2015-07-14 SEQ ID No. 5, claim 1, col. 12, l. 25-27, col. 95	2
X	EP 2949672 A1 (CHUGAI SEIYAKU KABUSHIKI KAISHA. JP) 2015-12-02 SEQ ID No. 13, ex. 7 (p. 36), p. 56	2
X	CN 102690789 A (UNIV PLA THIRD MILITARY MEDICAL ET AL. CN) 2012-09-26 SEQ ID No. 30, claim 5, p. 27	2
X	WO 2014111704 A1 (CANCER RES TECHNOLOGY LTD. GB) 2014-07-24 SEQ ID No. 21, claim 10, p. 67	2

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		WO2015134894 A1	2015-09-11

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		RU2010126078 A	2013-01-10
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WO2014111704 A1	2014-07-24	US2016108128 A1	2016-04-21
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