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Characterization of a novel canine T-cell line established from a spontaneously occurring aggressive T-cell lymphoma with large granular cell morphology

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ABSTRACT

Dogs with lymphoma are established as good model for human non-Hodgkin lymphoma studies. Canine cell lines derived from lymphomas may be valuable tools for testing new therapeutic drugs. In this context, we established a canine T-cell line, PER-VAS, from a primary aggressive T-cell lymphoma with large granular morphology. Flow cytometric analysis revealed a stable immunophenotype: PER-VAS cells were positively labelled for CD5, CD45, MHC II and TLR3, and were negative for CD3, CD4 and CD8 expression. Although unstable along the culture process, IL-17 and MMP12 proteins were detectable as late as at passages 280 and 325i.e. respectively 24 and 29 months post isolation. At passage 325, PER-VAS cells maintained the expression of IL-17, CD3, CD56, IFNγ and TNFα mRNAs as shown by RT-PCR analysis. Stable rearrangement of the TCRγ gene has been evidenced by PCR. PER-VAS cells have a high proliferation index with a doubling time of 16.5 h and were tumorigenic in Nude mice. Compared to the canine cell lines already reported, PER-VAS cells display an original expression pattern, close to NKT cells, which makes them valuable tools for in vitro comparative research on lymphomas.

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1. Introduction

Non Hodgkin lymphomas represent the most frequent canine hematopoietic tumour with an incidence of 15–30/100 000 similar to human (Marconato et al., 2013; Vail and MacEwen, 2000). Moreover, dogs develop spontaneous lymphomas resembling the human tumour regarding the type, grade, site of involvement and clinical features. The canine NHL classification followed the human classification by grouping lymphomas according to their cytological characteristics (updated Kiel classification (Lennert, 1991)) and more recently based on the revised European–American (Harris et al., 1994) and World Health Organization (WHO) classification (Jaffe, 2001; Pileri et al., 1998; Swerdlow, 2008) taking into account phenotypic, genetic and molecular features (Fournel-Fleury et al., 1997, 2002; Ponce et al., 2010; Turinelli et al., 2005; Valli et al., 2011). Importantly, dogs and humans sharing the same environment, the cartography of human and canine lymphomas related to outdoor carcinogens contributes to the evaluation of environmental risk factors (Pastor et al., 2009).

In human, peripheral T-cell lymphomas (PTCL) represent a group of heterogeneous lymphomas characterized by clonal expansion of T or NK cells with generally poorer prognosis than B-cell lymphomas. Standard therapeutic regimens resulted in insufficient patient outcomes with a rapid relapse and poor 5-years overall survival rates (Coiffier et al., 2014). Since PTCL account for approximatively 10–15% of all non-Hodgkin lymphomas in Western countries, therapeutic strategy of large clinical trials could not be conducted as for B-cell lymphomas counterparts (Gooptu et al., 2015; Jain et al., 2015). Therefore, new therapeutic approaches are required, especially for highly aggressive clinical forms showing multidrug resistance like extranodal NK-T cell lymphomas (Gill et al., 2010). Unfortunately, the lack of valuable preclinical model as well as the difficulties to derive cell lines phenotypically close to the primary tumors restricted the opportunities to test potentially new reagents towards PTCL.

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In dogs, PTCL also represent a heterogeneous group with various prognoses (Fournel-Fleury et al., 2002; McDonough and Moore, 2000). Among them, Large Granular Lymphomas (close to human extranodal NK/T-cell lymphomas) displayed a very high aggressive clinical course, often associated with hemophagocytic and macrophages activation syndromes and very poor overall survival rates (Turinelli et al., 2005). Therefore, the opportunity to develop a preclinical spontaneous canine model or to obtain canine NK/T-cell line to assess novel therapeutic approaches for such lymphomas constitutes a major challenge.

To date, twelve still available canine cell lines have been described and characterized according to their morphology, immunophenotype, pattern of TCR genes status and ability to induce tumours in mice (Kisseberth et al., 2007; Momoi et al., 1997; Nakaichi et al., 1996; Rutgen et al., 2010; Steplewski et al., 1987; Suter et al., 2005; Umeki et al., 2013; Yamazaki et al., 2008). Importantly, cell phenotype may change either from the primary material to the establishment of the cell line (Rutgen et al., 2010) or during in vitro maintenance (Umeki et al., 2013), leading to differences in the pattern of expression of specific markers. In this context, long-term study of ex-vivo derived cells is crucial to adequately establish their phenotype.

In the present study, we followed the morphology as well as the expression of the CD3, CD56, IL-17, TNFα and INFγ markers in the PER-VAS T-cell line, derived from an aggressive large granular T-cell lymphoma, along 325 in vitro passages (over four years). We described a new lymphoma-derived cell line expressing markers similar to the tumour it originated from.

2. Materials and methods

2.1. Case report

A seven year old male Labrador was initially diagnosed with Leishmaniasis (assessed with serological and PCR tests), and was presented to the veterinary hospital for severe dyspnea and asthenia. The dog showed moderate regenerative normochromic normocytic anemia, with the following hemogram values: red cells: 3.53 tera/L; hemoglobin: 7.8 g/dL; hematocrit: 22.3%; VGM: 63 fL; CCMH: 34.8 g/dL; TCMH: 22 pg; reticulocytes: 0.5%. No abnormal lymphoid cells were present on the peripheral blood smear. The ultrasonic scan confirmed a pleural and pericardial extravasation with cardial tamponade related to the main clinical signs (severe dyspnea and asthenia) and the absence of abnormal neoplastic mass. The abdominal ultrasonic examination gave no additional information. Cytological examination showed that pericardial effusion was mainly composed of tumoral cells with laboratory values indicating cell count: $29.5 \times 10^3/mm^3$ and protein: 6.4 g/dL. Pleural effusion displayed a mix of mesothelial, histiocytic, lymphocytic, neutrophilic and rare tumoral cells, with cell count: $2.67 \times 10^3/mm^3$ and protein: 5.8 g/dL. Axillary and popliteal lymph nodes were slightly enlarged at the first clinical examination, but fine needle aspiration of these superficial lymph nodes showed no signs of tumoral cells. An aggressive T-cell lymphoma with large granular cell morphology was diagnosed on cytological examination of pericardial and pleural effusion aspirations. The disease progressed rapidly towards death before any chemotherapy. No histopathological information was available because the autopsy was unauthorized by the dog's owner.

2.2. Establishment of PER-VAS lymphoma cell line

Cells from the pericardial effusion were cultured at 10^6 cells/ml and 5×10^5 cells/ml in complete RPMI medium (RPMI 1640 medium supplemented with 20% fetal calf serum, 2 mM L-glutamine, 1% penicillin/streptomycin) in 75 cm^2 flasks at 37°C in humidified atmosphere with 5% CO_2. Passages were conducted 3 times a week by replacing half of the culture with fresh complete RPMI medium. Primary effusion consisted of a mixed cell population including lymphocytes, histiocytes, mesothelial cells and atypical lymphocytes with large acidophilic cytoplasmic granules. After 3 weeks of culture with 3 passages a week, cells started to grow in suspension forming floating cell clusters. The cells were maintained in continuous culture during 9 months with high proliferative growth, and were then stored in liquid nitrogen at a concentration of 10×10^6 cells/ml in 90% FCS, 10% DMSO at passage 142. In a second period, PER-VAS cell line was thawed 4 months later for culturing from passages 143 to 199 during 7 months, and then was frozen at passage 199. In a third period, the cell line was thawed 2 months later from passages 200 to 280 during 8 months before freezing. The fourth period began one year and a half later from passages 281 to 325 during 5 months and immunophenotype was assessed. Splitting was conducted 15 times per month during the first cycle by replacing half of the culture with fresh complete RPMI medium. SVF was maintained at 20% in the medium up to passage 150 and then decreased to 10% for the following cycles. From the second cycle and further, 5×10^5 cells were seeded in 25 cm^2 flasks and were subcultured 8–10 times per month by replacing at least three quarters of the culture with fresh complete RPMI medium. Finally, PER-VAS cell line was cultured along 325 passages over four years through 4 freezing/thawing cycles.

PER-VAS cells were cloned at passage 200 by seeding at a density of ≤1 cell per well in 96-well flats bottom culture plates (Nunc). Twenty four out of the 32 clones obtained were expanded and analysed for antigen receptor rearrangements and phenotype.

For doubling time calculation, cells were seeded in triplicate at 2×10^4 cells/well in 96-well plate; viable cells were counted every 24 h for 3 days using trypan blue dye exclusion. The logarithmic least squares fitting technique was used for the calculation (Roth, 2006).

2.3. Primary cells and cell lines used as controls

A20 murine B cell lymphoma and Jurkat human T-cell leukemia cell lines were obtained from the CelluloNet facility (SFR BioSciences Gerland-Lyon Sud, UMS3444/US8). They were used as positive controls for western-blot analysis of Per-Vas cells for IL17 and MMP12 expression, respectively and in comparison with canine and human PBMC. Canine PBMC were isolated form healthy adult beagle dogs (Claude Bourgelat Institut of the VetAgro Sup veterinary campus, Lyon, France) in accordance with the institutional guidelines. Human PBMC were obtained from healthy donors under informed consent (Etablissements Français du Sang, Lyon, France).

RT-PCR analysis of PER-VAS cells were done in comparison with canine leukemia T-cells (CD3+/CD5+/CD45+/CD4−/CD8−/CD21−).

Lymphoid hyperplasia T- and B- cells from fine needle aspiration of lymph nodes were obtained from dogs sampled at Vetagro Sup Veterinary campus and were used as positive control for clonality assessment (PARR assay).

2.4. Phenotype analysis

PER-VAS cells were analyzed by flow cytometry from passages 25 to 325. Immunocytochemistry was performed on primary pericardial effusion and on PER-VAS cells at passage 240 using cytospin smears. Cells were labelled with monoclonal antibodies listed in Table 1. For flow cytometry analysis, 1– 5×10^5 cells were incubated with mAb or isotype matched irrelevant Igs for 30 min at 4°C. For indirect staining, after a PBS washing, FITC-labelled anti-mouse secondary antibody was incubated for 30 min

Table 1
Antibodies used in this study.

Specificity	Clone	Isotype	Conjugation	Application used	Source	References for cross reactivity
Human CD1a	NA1/34-HLK	mIgG2a	–[a]	FC	Serotec, Oxford, UK	Galkowska et al. (1996)
Canine CD3	CA17.2A12	mIgG1	FITC	FC	Serotec, Oxford, UK	–
Human CD3ϵ	CD3-12	rIgG1	–[a]	FC, ICC	Serotec, Oxford, UK	Jones et al. (1993)
Human CD3ϵ	polyclonal	rabbit	–	ICC	Dako, Glostrup, Denmark	Jones et al. (1993)
Canine CD4	YKIX302.9	rIgG2a	FITC	FC, ICC	Serotec, Oxford, UK	–
Canine CD5	YKIX322.3	rIgG2a	FITC	FC, ICC	Serotec, Oxford, UK	–
Canine CD8	YCATE55.9	rIgG1	FITC	FC, ICC	Serotec, Oxford, UK	–
Canine CD11c	CA11.6A1	mIgG1	–[a]	FC	Serotec, Oxford, UK	–
Human CD14	TÜK4	mIgG2a	–[a]	FC	Dako, Glostrup, Denmark	Drexler (2001)
Canine CD21	CA2.1D6	mIgG1	PE	FC	Serotec, Oxford, UK	–
Canine CD45	YKIX716.13	rIgG2b	FITC	FC	Serotec, Oxford, UK	–
Canine MHC II	YKIX334.2	rIgG2a	FITC	FC	Serotec, Oxford, UK	–
Canine TCRαβ	CA15.8G7	mIgG1	–	ICC	P.F. Moore, UC Davis	–
Canine TCRγδ	CA20.8H1	mIgG2a	–	ICC	P.F. Moore, UC Davis	–
Human TLR3	722E2	mIgM	–[a]	FC, WB	Gift from Dendritics, Lyon, France	Bonnefont-Rebeix et al. (2007)
Human IL-17	412H6	mIgG1	–[a]	FC, WB	Gift from Dendritics, Lyon, France	This study
Human MMP12	703D10	mIgG1	–[a]	FC, WB	Gift from Dendritics, Lyon, France	This study
Anti-mouse Ig	–	Goat Ig	FITC	FC	Dako, Glostrup, Denmark	–

Abbreviations: m = mouse; rat; FC = flow cytometry; ICC = immunocytochemistry; WB = Western blot analysis.
[a] Required anti-mouse Ig - FITC-labelled secondary antibody for flow cytometry.

at 4°C. Intracellular staining for CD3ϵ (dilution 1:100), TLR3, IL-17 and MMP12 (16 μg/ml) was performed after permeabilization with 0.3% saponin (SIGMA) in PBS. Cells were analyzed on a BD Accuri flow cytometer (BD Biosciences).

Immunocytochemistry was performed on cytospin smears using the UltraTek HRP Anti-Polyvalent kit (ScyTek Laboratories) with Vector® NovaRED™ (Vector Laboratories) as substrate, following the manufacturer's instructions, with hematoxylin counterstaining. Negative control was performed without the primary mAb. TCRαβ and TCRγδ staining were kindly performed by Pr Peter F. Moore, U.C. Davis, California, USA.

2.5. Western Blot

Canine and human PBMC, A20, Jurkat and PER-VAS (at passage 280) cells were lysed as previously described (Bonnefont-Rebeix et al., 2007). Cell lysates were denatured at 95°C for 5 min in the presence of 2% β-mercaptoethanol and 2% SDS. Recombinant human MMP12 produced in COP5 murine cells (rhuMMP12, gift from Dendritics, Lyon, France) was used as positive control for MMP12 detection. Fifteen μg of proteins were separated on 12 or 15% SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were blocked with "Protein-Free T20 Blocking Buffer" (Thermo Scientific Pierce) at room temperature for 10 min. before incubation for 1 h at room temperature and overnight at 4°C with anti-IL-17A mAb (9 μg/ml) and anti-MMP12 mAb (15 μg/ml) (Dendritics, France). Blots were developed as previously described (Bonnefont-Rebeix et al., 2007).

2.6. Polymerase chain reaction for antigen receptor rearrangements (PARR)

For clonality assessment, both immunoglobulin and T-cell receptor γ (TCRγ) genes rearrangements were investigated using PARR as previously described (Burnett et al., 2003). Total genomic DNA was extracted from 5×10^6 frozen primary effusion cells and PER-VAS cells at passages 145, 205 and 325 using the "NucleoSpin® Tissue kit" (Macherey Nagel). Cells from lymphoid T- and B-hyperplasia with polyclonal receptor rearrangement were included in all assays as controls. PCR reactions were carried out at 95°C for 4 min, followed by 40 cycles at 95°C for 45 s, 55°C for 45 s and 72°C for 45 s. PCR products were separated onto 2.5% agarose gel and visualized after staining with GelRed (Thermo scientific).

2.7. mRNA expression

Total RNA was extracted from PER-VAS cells at passages 280 and 325 and from canine leukemia CD3+ T-cell using the "Ambion® PureLink® RNA Mini Kit" (Life technologies). After DNase treatment ("Ambion® TURBO DNA-free DNase Treatment and Removal Reagents"), 100 ng of total RNA were reverse-transcribed using the "iScript™cDNA Synthesis Kit" (Bio-Rad). RT-negative controls have been performed to confirm the absence of contaminating DNA. PCR reactions were performed in 20 μl with 10–200 ng of cDNA using the "Kapa™ Taq DNA Polymerase kit" (Kapa Biosystems). The amplification was performed at 95°C for 3 min, followed by 40 cycles of 95°C for 20 s, 60°C (except 63°C for IL17) for 20 s and 72°C for 20 s using dog-specific primers (0,2 μM) for CD3δ, CD56, INFγ, TNFα, IL-17, MMP12, and ACTB as housekeeping gene (Table 2). PCR products were size-fractionated on a 2% agarose gel.

2.7. Tumorigenicity assay

Xenografted tumor cell assays were conducted in Cancer Research Center, Toulouse, France, in accordance with the Animal Care and Use Committee (approval number 06 563DG-04). Two adult (7 weeks old) athymic nude mice (RjOrl:NMRI-Foxn1nu/Foxn1nu) were inoculated subcutaneously in the right side with 10^7 PER-VAS cells (passage 60) in 0.25 ml PBS per mouse. Mice were observed weekly for tumour growth. Euthanasia was conducted 11 weeks after inoculation. Tumor mass was excised from the inoculation site and fixed in 10% formalin and embedded in paraffin. For tissue morphology, glass slides sections were prepared and stained with hematoxylin and eosin (HE).

3. Results

3.1. Characteristics of the primary effusion cells and establishment of PER-VAS cell line

The primary malignant pericardial effusion of the 7 year-old male Labrador consisted of a mixture of median-sized and large lymphoma cells with nucleus twice larger than an erythrocyte (Fig. 1A) in a hemorrhagic background. These cells displayed a high nuclear/cytoplasmic ratio, irregularly folded nuclei, irregular coarse chromatin, poorly visible pale nucleoli, and a basophilic cytoplasm containing numerous microvacuoles and some azurophilic granules. Expression of CD3ϵ, CD4, CD8, CD79a,

Table 2
Oligonucleotide primers used for RT-PCR of PER-VAS.

Target gene	Accession number	Primer	Nucleotide sequence (5′-3′)
CD3δ	XM_536556	FORREV	GAACAATCCGACAAAGCACCT AGTGGCGATTATGTCAGCGA
CD56	AY860627	FORREV	TTGTCCCCAGCCAAGGAG TAGATGGTGAGCGTGGAGGAAGA
INFγ	NM_001003174	FORREV	GGCTGTAACTGTCAGGCCAT ACGAAAAGAGACCCACTC
TNFα	NM_001003244	FORREV	GCCTGCTGCACTTTGGAGT GTTGGCCAGGAGGGCATT
IL-17	NM_001165878	FORREV	CTGAGCCTGGTGGCTATCAT GGGCCTTCTGGAGTTCGTATT
ACTB	NM_001009784	FORREV	CCAACCGTGAGAAGATGACC CCAGAGGCGTACAGGGACAG

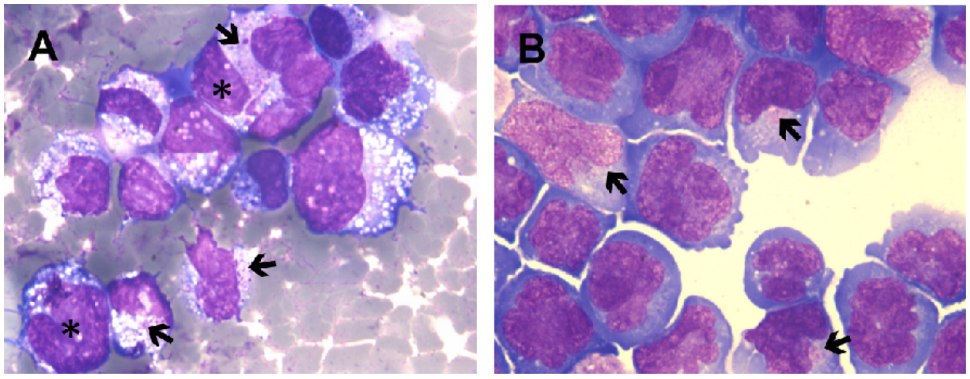


Fig. 1. Comparative features of the primary malignant pericardic effusion and the established PER-VAS cell line.
(A). The primary malignant pericardic effusion displayed an important contingent of medium-sized and large lymphoma cells with a basophilic cytoplasm containing numerous clear vacuoles (*) and azurophilic granules (→) in a hemorrhagic background. (B). PER-VAS cells established from the primary malignant pericardic effusion showed large cells with a high nuclear to cytoplasmic ratio very similar to the primary effusion cells, and a deep basophilic cytoplasm containing rare azurophilic granules (→). Staining with May-Grünwald-Giemsa stain, original magnification ×100.

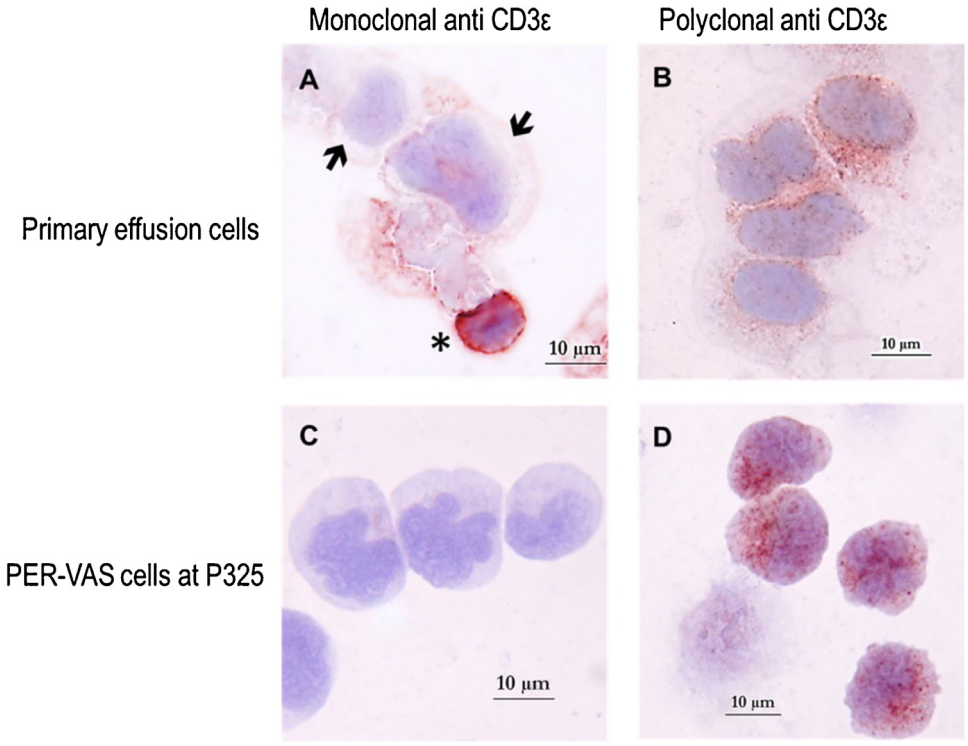


Fig. 2. Immunocytochemistry on primary effusion cells (A, B) and PER-VAS cell line (C, D) smears using monoclonal (A, C) and polyclonal (B, D) anti-CD3ϵ.
(A) Primary effusion cells showing a positive staining for normal lymphocyte (*) whereas atypical medium-sized and large lymphocytes (→) are negative (through background) using CD3ϵ mAb. (B) Positive staining of primary malignant effusion cells using polyclonal rabbit anti-human T-cell CD3ϵ. (C) Negative staining of PER-VAS cell line at passage 325 using CD3ϵ mAb. (D) Positive staining of PER-VAS cell line at passage 325 using polyclonal anti-CD3ϵ.

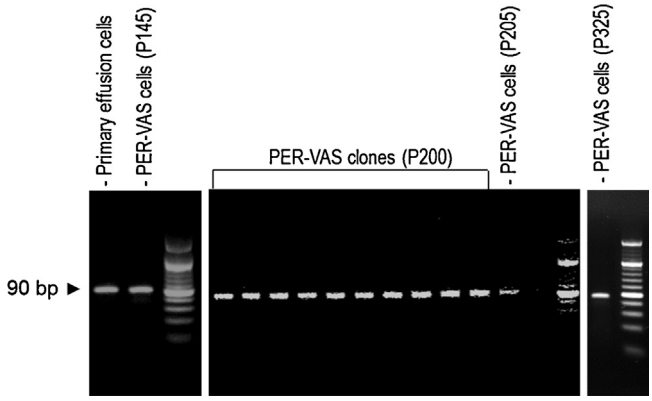


Fig. 3. PCR for TCRγ gene rearrangement.
Stable monoclonal TCRγ rearrangement of the primary lymphoma effusion cells, the established PER-VAS cell line at passages 145, 205 and 325, and 10 clones out of the 24 clones derived from PER-VAS cell line at passage 200. No band was obtained for IgH gene.

as well as of TCRαβ and TCRγδ were undetectable by immunocytochemistry analysis of lymphoma cells. Interestingly, a positive staining was obtained only when a polyclonal anti-human T-cell CD3, also targeting an intra-cytoplasmic peptide epitope on the ϵ-chain of the CD3 is used (Fig. 2A and B).

PER-VAS cell line derived from the primary malignant pericardial effusion was cultured over 4 years during four periods (passages 1–142; passages 143–199, passages 200–280 and passages 281–325), with periodic freezing/thawing. PER-VAS cells showed a doubling time of 16.5 h and grew in suspension with numerous clusters of large round to ovoid non-adherent cells morphologically very similar to the primary effusion cells. May-Grünwald-Giemsa staining revealed a high nuclear to cytoplasmic ratio, a basophilic cytoplasm and a few azurophilic granules (Fig. 1B). Using immunocytochemistry on cytospin smears at passage 325, CD3ϵ staining was found negative using the monoclonal antibody (Fig. 2C), but positive, as were the cells from the primary effusion, when using the polyclonal anti-intra-cytoplasmic (Fig. 2D). TCRαβ and TCRγδ were undetectable both in primary effusion cells and in PER-VAS cell line at passage 280.

PARR analysis revealed a clonal TCRγ gene rearrangement in primary effusion cells and in PER-VAS cells at passages 145, 205 and 325 (Fig. 3). The same TCRγ clonal rearrangement was found in 24 independent clones derived form PER-VAS cell-line. Sequencing analysis conducted on primary effusion cells and a randomly chosen PER-VAS cells clone confirmed the identical clonal rearrangement of the TCRγ gene.

3.2. Tumor cell growth in mice

PER-VAS cells (passage 60) subcutaneously injected in two nude mice produced expanding tumors macroscopically detected 5 weeks after inoculation. Only one mouse survived and was euthanized 11 weeks after inoculation with tumor mass reaching $1 \times 1.5 \times 0.8$ cm at the injection site. HE stained sections of tumor mass showed the location of tumor cells forming dense areas within murine loose subcutaneous connective tissue (Fig. 4A). Microscopic examination (Fig. 4B) revealed that tumor areas consisted exclusively of large cells presenting blastic aspect: narrow hyperacidophilic cytoplasmic rim, large nucleus containing coarse chromatin and prominent multiple nucleoli. Mitotic figures were frequent. This morphology was in accordance with a canine high-grade large-cell lymphoma. A lymphoproliferative reaction to the inoculation would have shown a population of small blastic cells with decondensed chromatin, but such small reactive lymphocytes were not observed, suggesting that tumor derived from the injected PER-VAS cells.

3.3. Immunophenotype of PER-VAS cell-line

The immunophenotype of PER-VAS cells was investigated at passages 25, 50, 145, 150, 205, 220, 260, 280 and 325 (Table 3). Cells showed stable surface expression of CD5, CD45, MHCII, stable cytoplasmic expression of TLR3, and were negative for the expression of CD4, CD8, CD21, CD14 (Fig. 5A). PER-VAS cells were negative for CD1a and CD11c at 2 passages 25, 220 and 260. CD3ϵ staining showed 60% of weakly positive cells at passage 25, remaining stable by passage 50, and then rapidly decreasing from only 14% by passage 145 (five days after thawing) to no positive cells at passage 150 two weeks later (Fig. 5B); the loss of CD3ϵ staining was confirmed in all further flow cytometry analysis. Surface expression of CD3 was first investigated at passage 205 and showed constant negative result along further passages (Table 3).

Expression of IL-17 and MMP12 analysed by flow cytometry was unstable over time. Initially weak at passage 50, 16 weeks after cell derivation, expression of IL-17 and MMP12 were respectively undetectable and weak by passage 220, and then positive at passages 260, 280 and 325 (data not shown). IL-17 and MMP12 expression detected by flow cytometry at passage 280 (Fig. 6A and C) was confirmed by Western blot analysis (Fig. 6B and D). RT-PCR analysis confirmed the presence of IL-17 mRNA at passages 280 and 325 in comparison with canine leukemia CD3+ T cells, as well as CD3δ, CD56, IFNγ and TNFα mRNA (Fig. 7).

4. Discussion

In the present study, we describe the establishment and characterization of PER-VAS cell line, a novel canine lymphoma cell line.

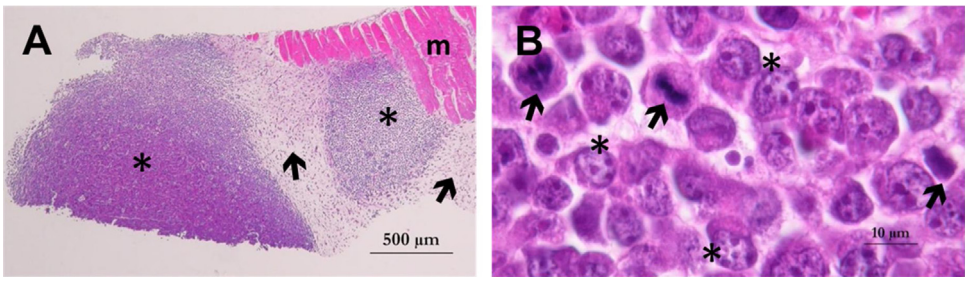


Fig. 4. PER-VAS cell-growth in mice.
(A) Histological section shows the location of tumor cells forming dense areas (*) within murine loose subcutaneous connective tissue (→) and infiltrating skeletal striated muscle (m) at the site of injection (subcutaneously). (B) Tumor consists of large cells with narrow hyperacidophilic cytoplasmic rim, large nucleus containing coarse chromatin, prominent multiple nucleoli (*), and frequent mitotic figures (→). HE stain.

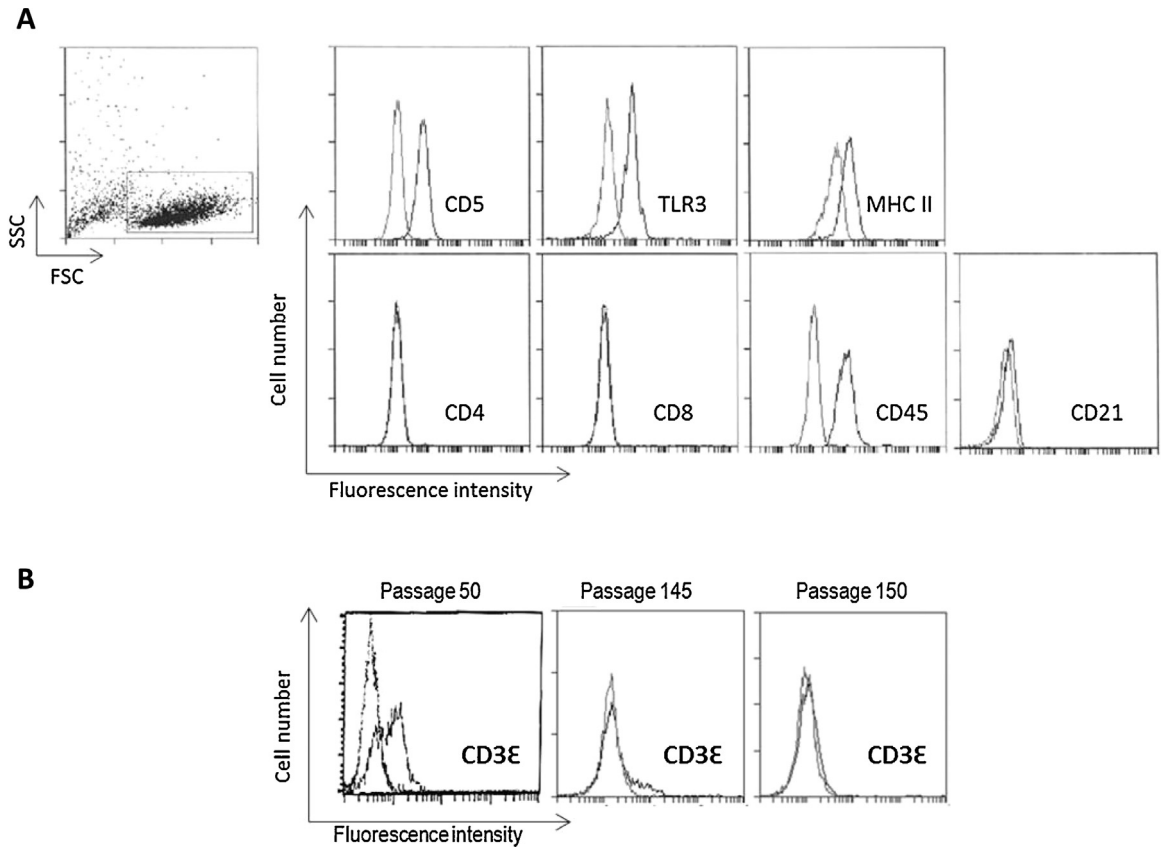


Fig. 5. Flow cytometry analysis of PER-VAS cell line.
(A) Stable expression of CD5, TLR3 and MHCII, and constant negativity for CD21, CD4, CD8 and CD45 at passage 150, representative of 9 analyses along 325 passages. Dot plot depicting forward/side scatter (FSC/SSC) is shown to illustrate population gating. (B) Decrease of CD3ϵ staining over passages 50, 145, and 150 (this last passage is representative of further passages up to 280).

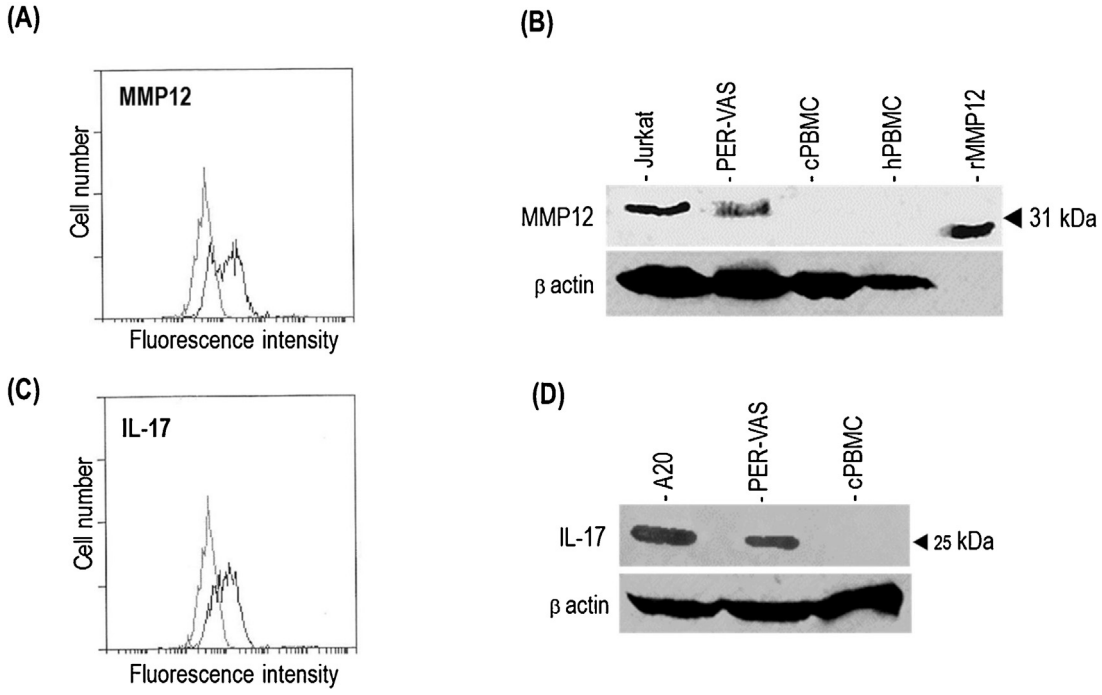


Fig. 6. Expression of IL-17 and MMP12 in PER-VAS cells at passage 280.
Flow cytometry analysis of permeabilized cells using anti-MMP12 mAb (A) and anti-IL-17 mAb (C), overlaid with FITC-labelled secondary Ab. Western blot analysis performed under reducing conditions using anti-MMP12 mAb (B) and anti-IL-17 mAb (D). Expression of actin has been used as a loading control. Jurkat and A20 cells have been used as positive controls respectively for MMP12 and IL-17. Recombinant human MMP12 (rhuMMP12) produced in COP5 murine cells was used as positive control for MMP12 detection.

Table 3
Immunophenotype of PER-VAS cells from primary effusion cells to established cell line through 325 passages.

Passage number	Flow cytometry (or ICC*) immunophenotype																	
	rCD3ϵ	CD3ϵ	CD3	CD79a	CD21	CD5	CD4	CD8	CD45	TCRαβ	TCRδγ	MHCII	CD14	CD1a	CD11c	TLR3	IL-17	MMP12
Primary cells	+*	–*	nd	–*	nd	nd	–*	–*	nd	–*	–*	nd	nd	nd	nd	nd	nd	nd
P25	nd	60%	nd	nd	–	+	–	–	+	nd	nd	+	–	–	–	+	nd	nd
P50	nd	60%	nd	nd	–	+	–	–	+	nd	nd	+	–	nd	nd	+	$+^{low}$	$+^{low}$
P142: 4 months in liquid nitrogen																		
P145	nd	14%	nd	nd	–	+	–	–	+	nd	nd	+	–	nd	nd	+	nd	nd
P150	nd	–	nd	nd	–	+	–	–	+	nd	nd	+	–	nd	nd	+	nd	nd
P199: 2 months in liquid nitrogen																		
P205	nd	–	–	nd	–	+	–	–	+	nd	nd	+	–	nd	nd	+	nd	nd
P220	nd	–	–	nd	–	+	–	–	+	nd	nd	+	–	–	–	+	–	$+^{low}$
P260	nd	–	–	nd	–	+	–	–	+	nd	nd	+	–	nd	nd	+	+	+
P280	nd	–	–	nd	–	+	–	–	+	–*	–*	+	–	nd	nd	+	+	+
P280 : 1 year and an half in liquid nitrogen																		
P325	+*	–/–*	–	nd	–	+	–	–	+	nd	nd	+	–	nd	nd	+	+	+
P325: storage in liquid nitrogen																		

Abbreviations: rCD3ϵ = rabbit polyclonal anti-CD3ϵ; nd = not determined.

This cell line derived from an aggressive large granular T-cell lymphoma with a CD3ϵ+ CD4- CD8- phenotype, resembling to human large granular lymphocytes (LGL) aggressive malignancies characterized by (NK) or T-cell phenotype and frequent extra-nodal involvement i.e. extra nodal NK/T cell lymphoma nasal type, aggressive NK-cell leukemia and hepatosplenic T-cell lymphoma (Chan et al., 1996; Turinelli et al., 2005). PER-VAS cells show a 16.5 h doubling time which is a high proliferative rate when compared to other published canine lymphoma cell lines (Table 4). PER-VAS cells have a stable TCRγ gene rearrangement, and express CD5, CD45, MHC II surface antigens as well as cytoplasmic TLR3. Expression of CD3δ, CD56, IL-17, IFNγ and TNFα mRNAs was confirmed as late as at passage 325. When compared to the other canine non B cell lines published and still available (Table 4), phenotype differences are observed with CL-1 which is CD5-, OSW which is CD5- and shows oligoclonal TCRγ gene rearrangement, CLGL which is CD3+ and TCRαβ+, Ema and Nody-1 which express surface CD3, UL-1 and CLK which are MHCII-, and CLC which shows no TCRγ gene rearrangement.

As previously reported for CLC and CLK cell lines (Umeki et al., 2013), PER-VAS cells progressively lost CD3ϵ staining with monoclonal antibody by flow cytometry over time, while CD3δ mRNA expression was detectable as late as at passage 325. The rapid loss of normal T-cell antigens expression has already been reported in human and canine T-cell leukemias (Avery and Avery, 2004; Jennings and Foon, 1997), and loss of markers is often observed during the transition from primary cells to cell lines. Nevertheless, taking into account the positive result of polyclonal anti-CD3ϵ by immunocytochemistry on primary effusion cells and PER-VAS cells at passage 325 along with CD3δ mRNA expression in PER-VAS cells, we could confirm intra-cytoplasmic CD3 expression in PER-VAS cells, and consider that monoclonal anti-CD3ϵ antibody recognizes an epitope formed by interaction of the various chains of the complete CD3 molecule, as it was proposed in human for the reactivity of monoclonal Leu4 anti-CD3 in nasal T/NK cell lymphomas (Chan et al., 1996).

Thus, it is noteworthy that the PER-VAS cells retained their CD5+/CD45+/MHCII+/TLR3+/CD4–/CD8–/CD21–/CD14– phenotype as well as their intra-cytoplasmic expression of CD3 over the 4 years period.

T-cell identity of PER-VAS cell line was witnessed by the monoclonal TCRɣ gene rearrangement, which was revealed by PARR. Intriguingly, protein expression of TCR (–αβ and –γδ) was undetectable by immunocytochemistry both in primary effusion cells and in PER-VAS cell line.

Alternatively, CD56, IFNγ and TNFα mRNA expression would support the belonging of PER-VAS cells to natural killer (NK) or natural killer T (NKT) lineage. To date, NK cells are defined as non-T, non-B lymphoid cells, while in dogs, NK and T cells shared phenotypic characteristics such as CD3 and CD8 expression (Lin et al., 2010; Otani et al., 2002; Shin et al., 2013). In human, CD56 is considered as a marker discriminating NK cells from other leukocytes (Cooper et al., 2001). Due to the inability of the cross reactive mAb to recognize all forms of CD56 (Otani et al., 2002), canine NK lineage was assessed through either CD56 mRNA expression or cytotoxic activity against canine thyroid adenocarcinoma cell line (Bonkobara et al., 2005; Helfand et al., 1995; Shin et al., 2013). Interestingly, we showed that PER-VAS cells expressed CD56, arguing for a NK or NKT phenotype. In addition, a further characterization of PER-VAS cells will be of interest with a cross reacting anti bovine NCR1 antibody after the recent report of NCR1+ cells in dogs that shared phenotypic characteristics with natural killer cells (Grondahl-Rosado et al., 2015).

In human, TCR genes are in germ-line configuration in NK-cells while rearranged in NKT cells. The cytotoxic activity against tumor cells was reportedly mediated by NK cells rather than NKT cells (Matsuo and Drexler, 2003; Voskens et al., 2010). Moreover, human NKT cells can be broadly divided into $CD4^+$ and $CD4^-$ subsets, with $CD4^-$ NKT cells primarily producing the Th1 cytokines IFNγ and TNFα (Coquet et al., 2008). Thus, the rearrangement of TCR genes, together with the expression of CD56, IFNγ and TNFα mRNA support the belonging of PER-VAS cells to the NKT lineage.

Other studies suggested that CD56 may not be a NK specific marker. In human, CD56 expression has been reported in NKT-cell lymphoma, in few cases of B-cell lymphomas (Gu and Ha, 2013; Isobe et al., 2007), and in acute myeloid leukemia where it correlates with proliferation and decreased apoptosis (Gattenloehner et al., 2007). In dogs, a study indicated that CD56 mRNA was only detected in a lymphoid leukemia case resembling human blastic NK cell leukemia but not in malignant T and B cells collected for controls (Bonkobara et al., 2007, 2005).

Cytoplasmic expression of IL-17 and MMP12 was first detected by flow cytometry in PER-VAS cells, and then analyzed by Western blot. Given the predicted molecular weight of canine IL-17 (17 kDa), the observed 26kDa band may correspond to a N-glycosylated form of IL-17, since recombinant human IL-17 migrated as two bands of 15 and 22 kD on SDS-Page under reducing conditions, with the higher molecular weight species representing N-glycosylated form (Fossiez et al., 1996). In the same way, the anti-MMP12 mAb revealed a band of 31 kDa for PER-VAS cells and 26 kDa for recombinant human MMP12, in comparison with the 22 and 45 kDa predicted forms corresponding to the cleavage of the 54 kDa MMP12 inactive enzyme (Shapiro et al., 1993). MMP12 is conserved among several species and may play a role in cancer cell invasion

Table 4
Canine lymphoma/leukemia cell lines reported.

Cell line	Derived from	Diagnosis	Flow cytometry (or ICC[a]) immunophenotype													PARR		PDT	Publication or published in
			CD3	CD4	CD5	CD8	CD8α	TCRαβ	TCRδγ	CD20	CD21	CD79a	CD45	CD45RA	MHCII	TCRγ	IgH		
17-71	Lymph node	Acute B cell lymphoma	–	nd	nd	nd	nd	–	–	nd	–	+	+	nd	nd	nd	nd	nd	Steplewski et al. (1987); Suter et al. (2005)
GL-1	Peripheral blood	B cell leukemia	–	50%	nd	–	nd	nd	nd	nd	–	+	+	+	–	+	–	27.3 h	Nakaichi et al. (1996); Rutgen et al. (2010)
CL-1	Pl. fluid	Thymic lymphoma	–	–	–	–	nd	nd	–	nd	33%	80%	+	–	+	+	–	39.6 h	Momoi et al. (1997); Rutgen et al. (2010)
CLL-1390	ni	Leukocytic neoplasia	–	nd	nd	nd	nd	nd	nd	nd	–	–	+	nd	nd	nd	nd	nd	Suter et al. (2005)
CLGL-90	ni	CLGL T cell leukemia	+	nd	nd	nd	+ var	+	nd	nd	nd	–	nd	nd	nd	nd	nd	nd	Suter et al. (2005)
OSW	Pl. fluid	T-cell lymphoma	–	–	–	–	–	–	–	+	–	–	+	–	–	oligo	–	24 h	Kisseberth et al. (2007)
CLBL-1	Lymph node	Diffuse large cell lymphoma	–	–	–	–	nd	nd	–	nd	–	+	+	+	+	–	+	31 h	Rutgen et al. (2010)
Ema	Pl. fluid and ascite	Gastrointestinal lymphoma	+	–	nd	nd	–	–	+	nd	–	nd	+	+	–	+	–	26.6 h	Umeki et al. (2013)
CLC	Pl. fluid and ascite	Gastrointestinal lymphoma	–	–	nd	nd	–	–	–	nd	–	nd	+	+	+	–	–	14.5 h	Umeki et al. (2013)
CLK	Ascite	Gastrointestinal lymphoma	–	–	nd	nd	+	–	–	nd	–	nd	+	+	–	+	–	36.2 h	Umeki et al. (2013)
Nody-1	Ascite	Gastrointestinal lymphoma	+	–	nd	nd	–	–	–	nd	–	nd	+	+	+	+	–	21.5 h	Umeki et al. (2013)
UL-1	Ascite	Renal lymphoma	–	–	nd	nd	+	–	–	nd	–	nd	+	+	–	+	–	20.9 h	Umeki et al. (2013); Yamazaki et al. (2008)
PER-VAS	Pericardic fluid	Aggressive T-cell lymphoma	–	–	+	–	nd	–	–	nd	–	–	+	nd	+	+	–	16.5 h	This study

Abbreviations: CLGL = chronic large granular lymphocytic; Pl. = Pleural; oligo = oligoclonal; var = variable; PDT = population doubling time; nd = not determined; ni = not indicated.
[a] ICC for CD20 and CD79a in (Kisseberth et al., 2007), and for CD79a in this study.

by the activation of other MMPs, leading to a cascade of proteolytic processes (Gronski et al., 1997). MMP-12 was mainly found to be expressed in tumor-associated macrophages but also in tumoral cells from aggressive B cell lymphomas (Ford et al., 2015) and nasal NK/T cell lymphomas (Kishibe et al., 2009). While MMPs inhibitors may be efficiently used in mice (Kondraganti et al., 2000; Yonemura et al., 2001), several clinical trials in humans were not as efficient suggesting the need for better targeted inhibitors (Moore et al., 2003; Zucker et al., 2000). MMP-12, among other MMPs, was shown to be the primary protease responsible for proteolytic release of angiostatin from plasminogen, leading to the selective inhibition of endothelial cell proliferation, and thus retarding lung tumor growth (Houghton et al., 2006). While MMP-2 and -9 were able to induce the degradation of plasminogen in vitro, they had a minor or no effect on the production of angiostatin in mice (Dong et al., 1997).

The IL-17 expression was detectable both at the transcript and protein level, at passages as late as 285 and 325. In human, IL-17 is typically viewed as a product of Th17 CD4+ helper T cells which have a key role in autoimmune pathology, whereas NKT-17 cells are a subset of NKT cells family with CD4- NK1.1- phenotype (Coquet et al., 2008). IL-17 is mainly a pro-inflammatory cytokine that can induce secretion of chemokines, matrix metalloproteinases and pro-inflammatory cytokines from stromal cells, leading to recruitment and activation of inflammatory cells. The role of IL-17 in carcinogenesis is complex, since IL-17 promotes angiogenesis and tumor growth in mice (Numasaki et al., 2003) and human (Matsuyama et al., 2011). IL-17 has also been shown to inhibit tumorigenesis, in part by enhancing cytotoxic antitumor responses (Benchetrit et al., 2002; Murugaiyan and Saha, 2009). The first demonstration of IL-17 mRNA expression in human lymphoma cells arose from cutaneous T-cell lymphomas (CTCL) (Ciree et al., 2004). IL-17 is also found in some cases of human anaplastic large cell lymphoma (ALCL). Canine ALCL is not so well characterized.

Stable TLR3 expression in PER-VAS cells could be not surprising since lymphocytes are known to express all TLRs (Sandor and Buc, 2005). In T cells, TLR2, TLR3, TLR5 and TLR9 act as co-stimulators to the T-cell receptor and enhance proliferation and/or cytokine production (Liu et al., 2006). NK cells were also found to express TLR3 which stimulation leads to TNFα and IFNγ production and up-regulation of cytolytic activity (Sivori et al., 2004). Among human lymphoma subtypes, TLR expression is highly variable and represent potent therapeutic target (Smith et al., 2010). Sivori et al., 2007, found a correlation between the amount of TLR3 mRNA transcript, expressed in the NK cell clones, and their capability to respond to poly (I:C) treatment.

In conclusion, PER-VAS is a novel canine lymphoma cell line derived from an aggressive T-cell lymphoma with large granular cell morphology, and presents a constant high proliferative growth in culture over a 4 year period with freezing/thawing cycles. PER-VAS cells maintain a clonal TCRγ rearrangement, and present a unique immunophenotype among canine cell-lines described up to now, with mRNA expression of CD56, IL-17, IFNγ and TNFα detected as lately as at passage 325, suggesting a NKT lineage. Due to its stability in culture and its rapid proliferation rate, PER-VAS cell line will be of importance to decipher the molecular pathways leading to lymphomas in dogs as well as a valuable tool to test new therapeutic tools.

Authors' contributions

CBR, CFF, FP and TM designed the research study; CFF contributed to the clinical and cytological diagnosis; CBR performed most of the experiments; SEB contributed to cell culture; SR, TM and DW performed the immunocytochemical analysis; SB designed the PARR analysis; CL developed the Western blot analysis and designed the primers used for the mRNA expression; CBR, TM, CL, DRD, JJP analyzed and interpreted the data; CBR, CL and DRD wrote the paper and generated the figures; all authors read and approved the final manuscript.

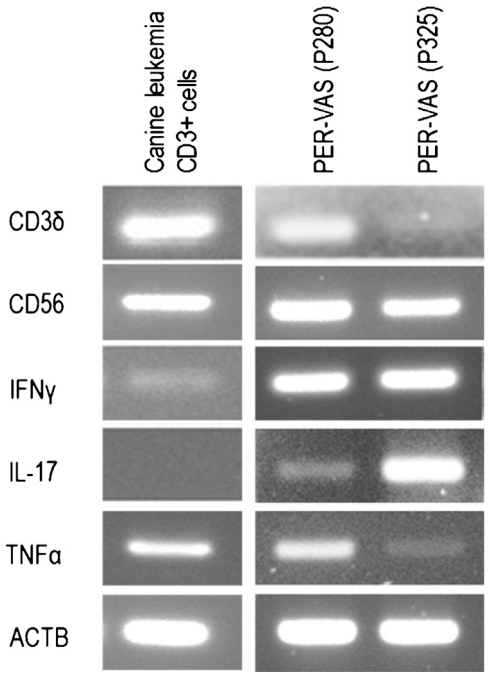


Fig. 7. mRNA expression pattern in PER-VAS cells.
Comparison of mRNA expression pattern of PER-VAS cells at passages 280 and 325 with canine leukemia CD3+ T cells by RT-PCR. Band intensities cannot be compared since we did not perform real time RT PCR.

Conflict of interest

The authors declare no conflict of interest.

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