Canine CD4+ T-cell lymphoma identified by flow cytometry exhibits a consistent histomorphology and gene expression profile

Lauren J. Harris1 | Kelly L. Hughes1 | E. J. Ehrhart1,2 | Julia D. Labadie1 | Janna Yoshimoto1 | Anne C. Avery1

1Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado
2Ethos Diagnostic Science Division, Ethos Veterinary Health, San Diego, California

Correspondence
Anne C. Avery, Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, 200 West Lake Street, Fort Collins, CO.
Email: anne.avery@colostate.edu

T-cell lymphomas (TCL) are a diverse group of neoplasms with variable diagnostic features, pathophysiologies, therapeutic responses and clinical outcomes. In dogs, TCL includes indolent and aggressive tumours such as T-zone lymphoma (TZL) and peripheral T-cell lymphoma (PTCL), respectively. Delineation of molecular subtypes and investigation into underlying pathophysiology of aggressive TCLs remains inadequate. We investigate the correlations between flow cytometry and histopathology of 73 cases of nodal TCL. The majority of cases (82.2%) were characterized as CD4+ TCL by flow cytometry. Fewer cases were classified as CD8+ TCL (6.8%) or CD4−CD8− TCL (11.0%). All cases, regardless of immunophenotype, exhibited conserved histologic features consistent with the WHO classification of PTCL. Histologic subsets of PTCL corresponding to immunophenotypic features were not identified. Neoplastic cell size determined by flow cytometry correlated significantly with mitotic rate. RNA-seq was performed on a subset of CD4+ PTCL cases (n = 6) and compared with sorted control CD4+ T-cells. The gene expression pattern of CD4+ PTCL was similar between all cases regardless of breed. PTCL was enriched in pathways representing G-coupled protein receptor signalling, extracellular matrix remodelling and vascular development, immune signalling and mitotic activity. Furthermore, global gene expression changes were consistent with downregulation of PTEN signalling and upregulation of the MTOR-P13K-ATK axis. In this study, we evaluated the correlations between flow cytometry, histopathology and gene expression within a large cohort of nodal TCLs. We further demonstrate the ability of flow cytometry to identify a subtype of T-cell lymphoma, CD4+ PTCL, with a uniform histomorphology and gene expression profile.

KEYWORDS
canine, flow cytometry, lymphoma, PTCL, RNAseq, T-cell

INTRODUCTION

Lymphoma is the most common haematopoietic malignancy in dogs and encompasses a broad spectrum of diseases with diverse mechanisms of oncogenesis, diagnostic criteria and biologic behaviours.1,2 Flow cytometry is a powerful non-invasive tool used to differentiate clinically significant subtypes of lymphoma by objectively evaluating cell size, cell complexity and the expression of multiple leukocyte antigens. The utility of flow cytometry is particularly evidenced by T-zone lymphoma (TZL) which is routinely diagnosed by flow cytometry based on an expansion of small to intermediate sized CD3+CD5+ T-cells with characteristic loss of the pan-leukocyte marker CD45.3,4 T-zone lymphoma is an indolent T-cell lymphoma that is clinically important to distinguish from more aggressive types of peripheral T-cell lymphomas.5 Furthermore, diagnosis of T-zone lymphoma by flow cytometry has been shown to reliably predict a distinct histologic pattern and cytormorphology consistent with the disease entity.3,4

CD4+ TCL is the most common subtype of TCL in dogs.5 The clinical presentation and outcome of dogs diagnosed with CD4+ TCLs by flow cytometry have been previously described. In this study, the
most common immunophenotype of neoplastic cells was CD3+CD4+CD45+CD21+. with low levels of MHC class II expression, and variable expression of CD5. This phenotype has been associated with poor clinical outcomes independent of the treatment protocol with a median progressive-free interval (PFI) of 91 to 108 days and overall survival (OS) of 159 to 160 days. Clinically, patients with this immunophenotype often present with a mediastinal mass and/or hypercalcaemia. In the current study we assessed the correlation between flow cytometry and histomorphology of a large group of nodal non-TZL TCL cases and then evaluated the gene expression pattern of the most common immunophenotype, CD4+ TCL. In current veterinary medicine, it is far more common to diagnose lymphoma by cytology and flow cytometry than biopsy, and therefore this study provides the unique opportunity to directly correlate the immunophenotype to histology. Understanding the spectrum of histologic changes associated with specific antigen expression may also provide insight into the disease process and lay the groundwork for future studies that rely on flow cytometry to identify lymphoma subtypes.

Boxer dogs and Golden Retrievers have been shown to be over-represented in the patient population of aggressive T-cell lymphoma. Over 80% of Boxers diagnosed with lymphoma are T-cell origin, the majority of which are CD4+ phenotype. Whole-exome sequencing of Boxer dogs with T-cell lymphoma identified approximately 25% of cases have mutations in phosphatase and tensin homologue (PTEN), a well-established tumour suppressor gene. PTEN negatively regulates phosphoinositide 3-kinase (PI3K)/AKT and mammalian target of rapamycin (mTOR) pathways. The PI3K-AKT-mTOR axis promotes cell growth, survival and motility and therefore uncontrolled activation of this pathway can promote tumour progression. Alterations in the PTEN and PI3K-AKT-mTOR pathways in Boxer dogs with TCL have not been evaluated on a global gene expression level.

The cytomorphology and histomorphology of CD4+ TCLs (excluding T-zone lymphoma) have been classified as peripheral T-cell lymphoma not-otherwise specified (PTCL-NOS), lymphoblastic lymphoma, pleomorphic mixed small, medium and large cell, or pleomorphic large cell type. In these studies a range of classification criteria (World Health Organization [WHO] classification, Kiel classification) and sample preparation (cytology, histology) were used. In veterinary medicine, the term lymphoblastic is vaguely defined, with variable interpretations amongst both anatomic and clinical pathologists. In the WHO classification scheme, T-lymphoblastic leukaemia/lymphoma (T-ALL/LBL), is defined as a neoplasm of precursor lymphocytes and is considered synonymous with acute leukaemia. T-ALL and LBL differ in the primary site of presentation, with T-ALL involving bone marrow and blood and LBL presenting with involvement of the thymus or nodal/extranodal sites. The precursor nature of the T-lymphoblasts is largely identified by expression of terminal deoxynucleotidyl transferase (TdT) and CD34 by the neoplastic cells. In dogs, CD34 expression is routinely evaluated by flow cytometry and is used to identify acute leukaemias. Tumours classified as CD4+ TCLs do not express CD34. Evaluation of TdT expression has not yet been successfully optimized in canine diagnostics. Nodal TCLs with other immunophenotypes, including CD8+ and CD4+CD8+ lymphomas, are less common and not well characterized in dogs.

The goals of this study were to (a) evaluate the heterogeneity of histomorphologies and global gene expression within a group of tumours diagnosed as CD4+ TCL by flow cytometry (b) determine if less common TCL immunophenotypes can be distinguished from CD4+ TCL by histology and (c) identify molecular pathways that are dysregulated in CD4+ PTCL using pathway enrichment analysis.

2 | MATERIALS AND METHODS

2.1 | Case selection for flow cytometry-histopathology correlation

Cases with a T-cell phenotype (expression of the T-cell antigens CD3 or CD5 with variable expression of the T-cell subset antigens, CD4 and CD8) were identified through Aratana Therapeutics T-CHOMP and T-LAB clinical studies. Samples used in this study were collected between 2014 and 2015 from dogs presenting to veterinary specialty clinics throughout the United States with suspicion of lymphoproliferative disease. Dogs were selected for this study in two phases. During the first phase, fine needle aspirates for flow cytometry were taken simultaneously with a biopsy for histopathologic evaluation. All samples were obtained from peripheral lymph nodes. During the second phase of the study, flow cytometry was performed first and dogs with an indolent lymphoma (TZL immunophenotype) and B-cell lymphoma were excluded. TZL lymphoma was identified based on a neoplastic population composed of small to intermediate cells which expressed CD3, CD5 and lost expression of CD45. Dogs with a non-TZL TCL were then entered into the clinical study and a pre-treatment lymph node biopsy was obtained. Thus, the cases in this study represent the diversity of phenotypes and histologies that might be present in nodal TCL not classified as TZL. During the first phase of the trial, 10 TZL cases were evaluated and independently diagnosed by both histomorphology and flow cytometry.

2.2 | Flow cytometry

Routine diagnostic flow cytometry was performed by the Colorado State University Clinical Immunology Laboratory (CSU-CI) on all cases as previously described. Flow cytometry was reviewed for lymph node aspirates of 73 cases of TCL, and compared to lymph node aspirates from 20 healthy dogs utilized for IACUC approved surgical continuing education courses, and 20 cases of CD4+ T-zone lymphoma submitted to the CSU-CI for routine diagnostics. Cases of T-zone lymphoma were selected based on CD45-CD3+CD4+CD5+CD21+ immunophenotype. Although T-zone lymphomas can express CD4, CD8, neither or rarely both subset antigens, for this study CD4-expressing cases were examined. The following features of the neoplastic populations and control CD4+ and CD8+ T-lymphocytes were evaluated: cell size by forward scatter on a linear scale, MHC class II median mean fluorescence intensity (MFI), CD25 median MFI and expression of CD5.

2.3 | Histopathology

Two pathologists (L.J.H., K.L.H.) were blinded to the flow cytometry parameters and independently evaluated whole lymph node or lymph
A canine cutaneous mast cell tumour and normal canine lymph node aspirates or biopsy. Cases were identified based on histomorphology consistent with PTCL and confirmed T-cell origin by flow cytometry, RNA-seq, and immunohistochemistry. 

CD3 and CD79a expressions were evaluated by immunohistochemistry in all biopsies included in the flow cytometry-histopathology correlation study. Three micron sections of formalin-fixed paraffin-embedded tissues were mounted on positively charged slides along with positive control tissue. Slides were deparaffinized and hydrated through two changes of xylene, two changes of 100% alcohol and one change of 95% alcohol to water. Heat induced epitope retrieval (HIER) was performed using citrate buffer, pH = 6, for 30 seconds at 125°C using a Dako Pascal pressure cooker. CD3 (courtesy of Dr. Moore, UC Davis) at 1:50 dilution or CD79a (Biocare, Pacheco, California) at 1:100 were applied for 60 minutes at room temperature. After rinsing, Promark Mouse on Canine Polymer (Biocare) was applied for 60 minutes. After rinsing, Betazoid DAB (Biocare) was applied for 5 minutes. Slides were then rinsed thoroughly and counterstained using Mayer's haematoxylin for 30 seconds. Slides were dehydrated and cleared using graded alcohol and xylene and coverslipped with tape coverslips.

CD30 immunohistochemistry was performed on six cases of PTCL selected from submissions to the CSU Diagnostic Laboratory for necropsy or biopsy. Cases were identified based on histomorphology consistent with PTCL and confirmed T-cell origin by flow cytometry, immunohistochemistry, and/or clonal T-cell receptor rearrangement. A canine cutaneous mast cell tumour and normal canine lymph node were used as positive controls. HIER was performed on a Leica Bond Max or Leica Bond III IHC stainer using Bond Epitope Retrieval Solution 2 (Bond Epitope Retrieval Solution 2, Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne, UK) for 30 minutes. The monoclonal mouse anti-human CD30 antibody (clone Ber-H2, Dako North America Inc., Carpinteria, California) at 1:20 dilution was applied. Labelling was performed on an automated staining platform (Bond-Max, Leica Biosystems). Fast Red (Fast Red Substrate System, Dako North America Inc) was used as a chromogen and slides were counterstained with haematoxylin. Negative controls were incubated in diluent consisting of Tris-buffered saline with carrier protein and homologous nonimmune sera. All sequential steps of the immunostaining procedure were performed on negative controls following incubation.

2.5 | Flow cytometry-histopathology correlation statistical analysis

All statistical analyses were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego California). Nonparametric Mann-Whitney U tests were used to compare flow cytometric parameters amongst samples. Nonparametric correlations between flow cytometry and histologic features were determined using Spearman’s correlation matrix.

2.6 | RNA-seq

Lymph node aspirates from six dogs submitted to the CSU-CI for routine diagnostics and diagnosed with CD4+ PTCL by flow cytometry were selected for RNA-seq. Patients were naïve to treatment at the time of sample submission. The six patient samples selected for RNA-seq were not enrolled in the above mentioned T-LAB and T-CHOMP clinical studies, and were therefore not included in the group of samples evaluated for flow cytometry-histopathology correlation. Patient information is summarized in Table 3. Aspirates from selected cases had a purity of greater than 88% neoplastic cells. Control CD4+ T lymphocytes were harvested from lymph nodes of six healthy dogs utilized for IACUC approved surgical continuing education courses. CD4+ T lymphocytes were sorted using a MoFlo cell sorter (Beckman Coulter, Brea, California). RNA was extracted from the six individual CD4+ PTCL cases. RNA from sorted CD4+ lymphocytes from six total dogs were pooled in groups of two to obtain three control samples. RNA extraction was performed using the PureLink RNA mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts) and quality was measured with an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, California). All samples had a RIN value greater than 8.80. RNA was shipped to Novogene Corporation Inc (Sacramento, California) where the libraries were constructed and sequenced. Briefly, mRNA was enriched using oligo(dT) beads and randomly fragmented. Random hexamer primer and reverse transcriptase were used for first strand cDNA synthesis followed by second strand synthesis via custom synthesis buffer (Illumina, San Diego, California), dNTPs, RNase H, and Escherichia coli polymerase I. Double-stranded cDNA fragments were purified and end-repaired by A-tailing and ligation of sequencing adapters. cDNA inserts were selected and enriched via PCR. Transcriptome sequencing was carried out on an Illumina HiSeq PE150 platform and generated 150 bp paired-end raw reads. Sequencing quality is summarized in Supporting Information Table S1. Sequences were aligned to the CanFam3.1 genome using TopHat2.19 Genome mapping is summarized in Table S2. HTSeq20 and DESeq221

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<tr>
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Mitotic rate Mitoses per 10 ×400 fields

Abbreviation: RBC, diameter of a resident red blood cell.
and CD4 + TZL cells (median = 406; P < 0.0001), control CD8+ lymphocytes (median = 379; P value < 0.0001) and CD4+ TCL cells (median = 406; P value < 0.0001) (Figure 1A).

CD4+ TCL cells exhibited low MHC Class II expression (median MFI = 0.80) compared to control CD4+ lymphocytes (median MFI = 12.15; P value < 0.0001), control CD8+ lymphocytes (median MFI = 17.03, P value < 0.0001) and CD4+ TRL cells (median MFI = 26.48; P value < 0.0001) (Figure 1B). CD25 expression in CD4+ TCL (median MFI = 0.1) was similar to that of control T-cell subsets, while CD25 expression was increased in CD4+ TRL cells (median MFI = 0.56; P value < 0.001) (Figure 1C). Nineteen of the 60 CD4+ TCL cases (31.7%) exhibited complete loss of CD5 expression and six cases (10.0%) exhibited partial downregulation of CD5. Consistent with previous studies,5 dogs diagnosed with CD4+ TCL were a median age of 7 years and mixed breeds (30%), Boxers (27%) and Golden Retrievers (13%) were the most common breeds. Male dogs, the majority of which were neutered, composed 62% of the population (Table 2).

CD8+ TCL cases expressed the T-cell antigen CD3, the T-cell sub-set marker CD8, and lacked expression of CD4 (CD3+CD4−CD8+). These tumours exhibited variable expression of CD5 (three out of five CD5−; two out of five CD5+). CD8+ TCL cells did not differ significantly in size (median = 524), MHC class II expression (median MFI = 1.79) or CD25 expression (median MFI = 0.1) from CD4+ TCL cases. CD8+ TCL cases were significantly older at the time of diagnosis with a median age of 11 years (P value = 0.005) and all five cases were female. Similar to CD4+ TCL, two of the five CD8+ TCL cases were Boxer dogs and one was a Golden Retriever.

CD4−CD8− cases lacked expression of CD4 or CD8 (CD4−CD8−) and the majority expressed CD3. One CD4−CD8− TCL case lacked CD3 expression but exhibited CD5 positivity, confirming T-cell origin. Within this group of TCLs there was variable expression of CD5 (four out of eight CD5−, four out of eight CD5+). CD4−CD8− TCL cells did not differ significantly in cell size (median = 495.51), CD25 expression (median MFI = 0.105), or MHC class II expression (median MFI = 0.605) compared to CD4+ TCL. The median age of patients diagnosed with CD4−CD8− PTCL was 6.5 years and was not significantly different from CD4+ TCL cases. Two of the eight total CD4−CD8− TCL cases were mixed breed dogs, one was a Boxer dog, and two were Golden Retrievers.

Control CD4+ and CD8+ lymphocytes were evaluated in healthy, young hound and mixed-breed dogs (Table 2). This population was uniformly 1 year of age, predominantly female (75%), and all dogs were intact. Cases of TRL were significantly older than CD4+ PTCL (P value < 0.001) and CD4−CD8− PTCL (P value = 0.004). CD8+ PTCL cases did not differ significantly in age from TRL cases (P value = 0.83). Golden Retrievers comprised 40% of the TRL cases. It is possible that differences in patient demographics between control, TRL, and PTCL cases could contribute to differences in measured lymphocyte parameters.

3 | RESULTS

3.1 | Flow cytometry features of canine nodal TCL

The 73 nodal TCL cases fell into three immunophenotypic categories: CD4+ TCL (82.3%), CD8+ TCL (6.9%), and CD4−CD8− TCL (11.0%). The demographics of TCL patients are summarized in Table 2. CD4+ TCL cases had a CD3+CD4+CD8− phenotype consistent with that previously associated with aggressive clinical disease.5 By flow cytometry, cells with the CD4+ TCL phenotype were significantly larger based on forward scatter determined on a linear scale (median = 530) compared to control CD4+ lymphocytes (median = 375; P value < 0.0001), control CD8+ lymphocytes (median = 379; P value < 0.0001) and CD4+ TRL cells (median = 406; P value < 0.0001) (Figure 1A). CD4+ TCL cells exhibited low MHC Class II expression (median MFI = 0.80) compared to control CD4+ lymphocytes (median MFI = 12.15; P value < 0.0001), control CD8+ lymphocytes (median MFI = 17.03, P value < 0.0001) and CD4+ TRL cells (median MFI = 26.48; P value < 0.0001) (Figure 1B). CD25 expression in CD4+ TCL (median MFI = 0.1) was similar to that of control T-cell subsets, while CD25 expression was increased in CD4+ TRL cells (median MFI = 0.56; P value < 0.001) (Figure 1C). Nineteen of the 60 CD4+ TCL cases (31.7%) exhibited complete loss of CD5 expression and six cases (10.0%) exhibited partial downregulation of CD5. Consistent with previous studies,5 dogs diagnosed with CD4+ TCL were a median age of 7 years and mixed breeds (30%), Boxers (27%) and Golden Retrievers (13%) were the most common breeds. Male dogs, the majority of which were neutered, composed 62% of the population (Table 2).

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3.2 | Correlations between flow cytometry phenotype and histomorphology

Of the 73 total cases, 70 tumours had diagnostic quality histologic preparations adequate for subtyping. The three tumours with insufficient sample were diagnosed as CD4−CD8− TCL by flow cytometry. All evaluated cases were classified as PTCL, regardless of immunophenotype. All tumours were characterized by sheets of intermediate to large lymphocytes that diffusely infiltrated and effaced the nodal architecture and often infiltrated the fibrous capsule and perinodal adipose tissue. Neoplastic cells had moderate amounts of eosinophilic wispy cytoplasm. Nuclei were two to three times the size of the diameter of an erythrocyte and round to indented or pleomorphic in shape. The majority of cases had open chromatin with one to three small distinct nucleoli, and a high mitotic index (median 49.42; range 10-118 per 10 x400 fields). Throughout all cases there were few to moderate scattered tingible body macrophages, low levels of tumour necrosis (less than 20% examined cross sectional area) and mild fibroplasia. Neovascularization was generally not a prominent feature of these neoplasms (Figure 2).
Cases of CD8⁺ and CD4⁻CD8⁻ TCL could not be reliably distinguished from CD4⁺ TCL by histology. In the single CD4⁻CD8⁻ PTCL case that lost expression of CD3 based on flow cytometry, approximately 40% of neoplastic cells exhibited positive membranous immunoreactivity to the CD3 antibody with immunohistochemistry. This finding is interpreted as a result of the intracellular binding of the CD3 antibody used in immunohistochemistry compared to the cell-surface binding of the CD3 antibody used for flow cytometry. Two of the five CD8⁺ TCL tumours had unique histologic features. In one case, cells had markedly expanded lacy to vacuolated cytoplasm. Cell size, nuclear characteristics, and tumour pattern of this neoplasm was still consistent with that previously described in other cases of PTCL. Another CD8⁺ PTCL case exhibited pronounced tumour-associated fibroplasia that multifocally dissected between neoplastic lymphocytes. A diagnosis of angioimmunoblastic lymphoma was considered in this case, however, because neoplastic cellular morphology was consistent with other cases of PTCL, a consensus diagnosis of PTCL was agreed upon. Because of the small sample size of CD8⁺ TCLs (n = 5), conclusions of whether these unique histologic features represent distinct entities or variations within the PTCL subgroup cannot be definitively determined.

Based on the consistency of the categorically evaluated histologic features throughout all examined cases, statistical evaluation of the categorical features with flow cytometry parameters was not pursued. Spearman’s rank correlation coefficients did identify a weak to moderate correlation between neoplastic cell size as determined by forward scatter via flow cytometry and mitotic rate per 10 ×400 fields (ρ = 0.418, P value < 0.001).

### 3.3 Flow cytometry identifies a consistent gene expression profile

RNA-seq was performed on lymph node aspirates from six dogs diagnosed with CD4⁺ TCL (CD4⁺CD8⁻CD5⁺/ClassIIlo) based on flow cytometry and compared to sorted CD4⁺ T-cells from lymph nodes of dogs with CD4⁺ TCL (n = 60), CD8⁺ TCL (n = 5), CD4⁻CD8⁻ TCL (n = 8), CNTRL CD4+/CD8+ (n = 20), and TZL (n = 20). Patients were selected based on availability, with the total n for each group varying due to sample and material availability. Patient demographics are provided in Table 2.

### Table 2: Patient demographics of flow cytometry-histopathology study

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<th>Parameter</th>
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<th>CD8⁺ PTCL (n = 5)</th>
<th>CD4⁻CD8⁻ PTCL (n = 8)</th>
<th>CNTRL CD4+/CD8+ (n = 20)</th>
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<td>2 (25%)</td>
<td>10 (50%)</td>
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### Figure 1: Flow cytometry features of PTCL.

(A) CD4⁺ PTCL cells were significantly larger than control CD4⁺ T-cells, control CD8⁺ T-cells and TZL cells. Cell size was determined by forward scatter on a linear scale. CD4⁺ TCL had low levels of MHC class II expression (median fluorescence intensity) compared to control CD4⁺ T-cells, control CD8⁺ T-cells, and CD4⁺ TCL cells (B). CD4⁺ TCL had low levels of CD25 expression (median fluorescence intensity) compared to CD4⁺ TCL cells (C). (Mean ± SD, ** = P < 0.001) [Colour figure can be viewed at wileyonlinelibrary.com]
young, healthy, hound dogs. The selected cases were representative of the CD4+ TCL patient population based on clinical presentation and flow cytometric features. Three of the selected cases were Boxers and three cases were non-Boxer breeds (Cavalier King Charles Spaniel, Rhodesian Ridgeback and a Collie dog). Two of the cases exhibited loss of CD5 expression. One case had a mediastinal mass, one case was hypercalcaemic, and one case had both a mediastinal mass and hypercalcaemia (Table 3).

A total of 5011 genes were significantly differentially expressed ($P_{adj} < 0.05$) when comparing CD4+ PTCL cases to control CD4+ lymphocytes (Figure 4A,B). Surface protein expression characterized by flow cytometry correlated with gene expression including decreased expression of CD5, CD25, and MHC class II molecules (DLA-DRA, DLA-DRB1, DLA-DQA1, DLA-DQB1) as well as the MHC class II transactivator, CIITA (Figure 3A-E). Hypercalcaemia is a common clinical presentation in dogs with CD4+ PTCL and this process is driven by paraneoplastic production of parathyroid hormone-like hormone (PTHLH).30 PTHLH expression was increased in all cases regardless of presence of clinical hypercalcaemia.

Hierarchical clustering of samples resulted in distinct separation of control lymphocytes and PTCL cases with intermixing of PTCL from Boxer and non-Boxer dogs (Figure 4A). In fact, only 82 genes were significantly differentially expressed when comparing tumour samples from Boxers and non-Boxer dogs (Figure 4C). Interestingly, one of the most overexpressed genes in PTCL in all three Boxers compared to PTCL in non-Boxer dogs was ROS protooncogene 1 (ROS1) ($\log[\text{fold change}] = 4.15; P_{adj} \leq 0.001$). ROS1 is a receptor tyrosine kinase oncogene that has been shown to undergo genetic rearrangements in a variety of human cancers.31 Overall, it does not appear that the gene expression profile of CD4+ PTCL in Boxer dogs is markedly different from that of CD4+ PTCL in other breeds, despite overrepresentation of Boxers within the patient population.

## 3.4 | TdT and C30 expression in canine CD4+ PTCL

TdT and C30 are useful diagnostic markers in the classification of human TCLs. TdT is reliably positive by immunohistochemistry in lymphoblastic lymphoma (LBL) in people.15,16 Evaluation of TdT protein expression in canine lymphoma has thus far been limited by the lack of optimized reagents for molecular diagnostics. At the gene expression level, TdT was not significantly overexpressed in this group of canine CD4+ PTCL (Figure 4D). When looking at PTCL cases individually, four of the cases had equal or lower expression levels to the control samples. Two cases had increased expression levels compared to

### TABLE 3 | Summary of patient information for individuals with RNAseq analysis

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Breed</th>
<th>Age</th>
<th>Sex</th>
<th>Mediastinal mass</th>
<th>Hypercalcaemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOXER1</td>
<td>Boxer</td>
<td>6</td>
<td>MC</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>BOXER2</td>
<td>Boxer</td>
<td>8</td>
<td>FS</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
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<td>Boxer</td>
<td>6</td>
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<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>OTHER1</td>
<td>Cavalier King Charles Spaniel</td>
<td>3</td>
<td>FS</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
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<td>4</td>
<td>FS</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
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<td>Collie</td>
<td>11</td>
<td>MC</td>
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<td>No</td>
</tr>
<tr>
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<td>Hound</td>
<td>1</td>
<td>F</td>
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<td>No</td>
</tr>
<tr>
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<td>Hound</td>
<td>1</td>
<td>F</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>CNTRL2a</td>
<td>Hound</td>
<td>1</td>
<td>F</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
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<td>Hound</td>
<td>1</td>
<td>F</td>
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<td>CNTRL3b</td>
<td>Hound</td>
<td>1</td>
<td>F</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; FS, female spayed; MC, male castrated.

RNA extracted from lymph nodes from CNTRL dogs 1a and 1b were combined for a single CNTRL1 sample, CNTRL2a and 2b were combined for a single CNTRL2 sample and CNTRL3a and 3b were combined for a single CNTRL3 sample.
controls, however the diagnostic relevance of this increase is undetermined.

CD30 is a cell membrane protein in the tumour necrosis factor receptor family that is expressed on activated lymphocytes and serves as a marker for diagnosis of a number of neoplasms, including anaplastic large cell lymphoma (ALCL) in humans. CD30 expression was significantly decreased in canine CD4+ PTCL (log2-fold change = −2.75; \( P_{adj} < 0.001 \)) (Figure 4D). Immunohistochemistry was performed to evaluate CD30 protein expression (Figure 5). A canine cutaneous mast cell tumour and normal lymph node were used as positive controls. There was positive cytoplasmic immunoreactivity within the neoplastic mast cells as well as low numbers of scattered

FIGURE 3 Flow cytometry cell-surface protein expression is consistent with gene expression. CD4+ PTCL is characterized by enlarged cell size determined by forward scatter (A), expression of the T-cell subset marker CD4 and frequent loss of T-cell marker CD5 (B). CD4+ PTCL exhibits low levels of MHC class II (C) and CD25 (D). RNAseq derived differential gene expression (log base 2 of the fold change) of PTCL cases compared to control CD4+ T-cells demonstrated that expression of CD5, CD25 and MHC class II molecules (DLA-DRA, DLA-DRB1, DLA-DQA1, DLA-DQB1 and CIITA) corresponded with cell-surface protein expression (E). Parathryoid hormone like hormone (PTHLH) expression was increased in all patients, regardless of presence of clinical hypercalcaemia (Mean ± SD, \(* = P_{adj} < 0.05\)) [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 4 Gene expression profile of CD4+ PTCL. Hierarchical clustering (average Euclidean distance) of differentially expressed genes (\( P_{adj} < 0.05 \)) identified distinct separation of control and PTCL case samples. There was intermixing of PTCL cases from Boxer dogs and other breeds (A). MA-plots showed global differential gene expression of all PTCL cases compared to CD4+ T-cell controls (B) and PTCL cases from Boxer dogs compared to PTCL cases from other dog breeds (C). Significantly differentially expressed genes (\( P_{adj} < 0.05 \)) are highlighted in red. Differential expression (log base 2 of the fold change) showed variable expression of TdT and significant downregulation of CD30 when comparing PTCL cases to CD4+ controls (mean ± SD, \(* = P_{adj} < 0.05\) ) (D) [Colour figure can be viewed at wileyonlinelibrary.com]
lymphocytes within the normal lymph node. Six total PTCL cases were evaluated for CD30 protein expression by immunohistochemistry. Four of the six cases had flow cytometry performed. Two cases had a CD4+ TCL phenotype and two cases had a CD4-CD8- TCL phenotype. The two cases without corresponding flow cytometry were diagnosed as PTCL by histology in conjunction with CD3 positivity by immunohistochemistry or clonal T-cell receptor rearrangement. All PTCL cases were negative for CD30 expression by immunohistochemistry.

3.5 PTEN and SATB1 mutation in canine CD4 + PTCL

Previously performed whole-exome sequencing of TCLs from Boxer dogs identified that Boxer dogs are frequently mutated in the PTEN-mTOR pathway as well as SATB1. In our study, one PTCL case (OTHER3) was heterozygous for a C to T single nucleotide polymorphism (SNP) at position 37 910 068 in the PTEN transcript. This was a silent mutation and unlikely to have biological significance. Another PTCL case (OTHER1) was heterozygous for a T to G SNP at position 24 651 976 in the SATB1 transcript. This mutation resulted in a single amino acid change (glutamine to arginine). The PTCL samples with SNPs corresponding to previously identified mutations in Boxer dogs with T-cell lymphoma were from a Collie and Cavalier King Charles Spaniel and not identified in the three Boxers in the current study.

3.6 Pathway analysis

The Gene Ontology Consortium was used to identify significantly enriched biological pathways in PTCL. The top 50 enriched pathways based on all significantly differentially expressed genes, including both those up and downregulated, are shown in Figure 6. Top enriched pathways are involved in G-coupled protein receptor signalling, extracellular matrix remodelling and vascular development, inflammatory response and immune signalling, and mitotic activity.

Amongst the top upregulated pathways was positive regulation of phosphatidylinositol 3-kinase (PI3K) activity. PI3K works together with AKT and mTOR to regulate the cell cycle. Specifically, activation of PI3K/AKT leads to upregulation of mTOR and cell proliferation and survival. This signalling axis is antagonized by PTEN. In PTCL, PTEN gene expression was downregulated and mTOR expression was upregulated (Figure 7A). To further investigate alterations in PTEN signalling based on global gene expression, GSEA was used to compare the rank-ordered list of differentially expressed genes in canine PTCL to gene sets representative of cellular pathways which are often dysregulated in cancer (C6: oncogenic signatures). A gene set representing genes associated with downregulation by PTEN was significantly enriched (nominal $P$ value = 0.003; FDR q-value = 0.02) (Figure 7B). Furthermore, a gene set comprised of genes associated with upregulation of mTOR was also significantly enriched (nominal $P$ value = 0.016; FDR q value = 0.08) (Figure 7C).

4 DISCUSSION

In this study we first investigated the correlations between immunophenotype by flow cytometry and histomorphology of nodal TCLs and then investigated the gene expression profile of the most common tumour immunophenotype in dogs, CD4+ PTCL. Within this cohort of nodal TCL patients, approximately 80% were categorized as CD4+ PTCL and the remaining cases were classified as CD8+ PTCL or CD4-CD8- PTCL. The study population was representative of dogs with non-TZL TCL lymphoma presenting to specialty veterinary hospitals with enlarged peripheral lymph nodes. The less common T-cell phenotypes (CD8+, CD4-CD8-) could not be reliably differentiated from CD4+ PTCL based on histomorphology alone. All tumours within the evaluated cohort were characterized by a diffuse infiltration of intermediate to large cells with pleomorphic nuclei and high mitotic rates. This finding may suggest that these tumours are part of the same disease entity regardless of immunophenotype. Alternatively, this finding may indicate that histomorphology alone is not a sensitive and reliable test to delineate biologically significant subtypes of nodal TCLs. Flow cytometry may be used to further divide this histologic entity into different immunophenotypic subtypes of PTCL. Future studies investigating the clinical outcomes of dogs diagnosed with differing PTCL immunophenotypes and sequencing experiments focused on a large group of PTCL cases with variable immunophenotypes may shed light on the impact of immunophenotype in delineation of molecular subtypes of PTCL.

A histologic diagnosis of lymphoblastic lymphoma (LBL) was considered in few cases, but ultimately these tumours were classified as PTCL based on cell size, pleomorphic nuclear shape, and intertumoural variation in chromatin staining and nucleolar prominence. Lymphoblastic is a loosely defined term in veterinary medicine with variable interpretations by both clinical and anatomic pathologists.
Histologically, LBL and PTCL exhibit overlapping morphologic features including intermediate cell size, variably round to oval to irregularly indented nuclei and high mitotic rates. Differentiation between the histomorphology of these neoplasms has been reliant on nucleolar prominence and number and conspicuousness of mitotic figures. Because there was moderate to marked variation of these histologic features within an individual tumour sample, the authors felt that these features could not be used to reliably differentiate morphologically distinct subtypes and were consistent with the morphologic features described for PTCL by the WHO classification system in both dogs and humans. Furthermore, microarray analysis has shown that the gene expression profile of tumours diagnosed as PTCL or LBL composed a single molecular group, suggesting that these histologic diagnoses represent a single entity in dogs. According to the WHO classification, LBL is a precursor T-lymphocyte neoplasm which typically expresses TdT and CD34, markers of immature lymphoblasts. Cases of PTCL do not express CD34, a commonly used marker of acute leukemias in dogs. Evaluation of TdT expression has not yet been optimized for canine diagnostics by flow cytometry or immuno-histochemistry. In the current study, we did evaluate TdT gene.
expression in a smaller cohort of CD4⁺ PTCL cases. When comparing all six cases to the control lymphocytes, TdT was not significantly upregulated. In four of the six cases, TdT expression was equal to or less than that of control CD4⁺ lymphocytes. Two cases exhibited increased TdT expression as compared to controls. The significance of increased TdT expression in two of the cases is uncertain as the level of RNA transcript that would constitute TdT-positivity for diagnostic purposes is undetermined.

In the current study, gene expression profiling focused on the most common immunophenotype of PTCL. CD4⁺ PTCL exhibited consistent changes in gene expression between tumour samples from different dogs. Homogeneity within the gene expression profile of PTCL from different patients is promising for investigation into novel therapeutics and clinical trial design. Differential gene expression was determined by comparing CD4⁺ PTCL lymph node aspirates to sorted CD4⁺ T-cells from healthy young hound dogs. The control population was uniformly composed of 1 year old, female intact, hound dogs. The lack of age and breed matched control samples may confound interpretation of changes in the global gene expression. Nevertheless, the identification of upregulation of cancer-associated pathways in the PTCL cases compared to controls suggests that our results are representative of the major disease process of interest.

PTCL in Boxer dogs, who are overrepresented in the patient population, did not exhibit a distinct overall gene expression profile from PTCL in other breeds. However one gene that was significantly differentially expressed in Boxer dogs was ROS1. ROS1 is a receptor tyrosine kinase which is often involved in chromosomal translocations and oncogenic fusion gene formation in human cancers. In a preliminary analysis, ROS1 fusion gene transcripts were not identified in canine CD4⁺ PTCL (data not shown).

The global gene expression pattern was consistent with downregulation of PTEN and upregulation of the PI3K/AKT and mTOR pathways. This finding supports previous studies that have identified mutations in PTEN in canine TCL. Variant analysis of RNA transcripts identified a silent single nucleotide polymorphism in the PTEN transcript in one of the six sequenced PTCL samples. This mutation is not predicted to change the amino acid sequence of the protein and therefore is not expected to be functionally significant. Mutation evaluation using RNAseq, however, fails to capture mutations that result in loss of RNA transcript including both complete deletions or point mutations with the potential to lead to nonsense mediated decay. PTEN gene expression was significantly downregulated and the gene signature associated with PTEN inhibition was significantly enriched. These findings suggest that downregulation of PTEN may be a potential driving pathway of CD4⁺ PTCLs. Pathways that are inhibited by PTEN include PI3K/AKT and mTOR signalling. In PTCL cases there was significant upregulation of mTOR gene expression and significant enrichment for the PI3K/AKT and mTOR induced gene signatures. Together these findings suggest that alterations in PTEN, mTOR, and PI3K/AKT signalling may be the result of either functional mutations in PTEN as suggested by Elvers et al⁹ or other regulatory mechanisms resulting in PTEN downregulation. Functional evaluation is needed to confirm the role of altered PTEN, PI3K/AKT, and mTOR signalling pathways in oncogenesis and tumour progression.

In addition to PTEN, SATB1 was amongst the most commonly mutated genes in previous studies of TCLs in Boxers. This finding was corroborated in our study in which SATB1 was mutated in one of six CD4⁺ PTCLs, resulting in a single amino acid change. Variant analysis was not performed with patient matched non-neoplastic tissue samples, and therefore whether this is a tumour specific or germline SNP cannot be definitively determined. SATB1 is a protein that is predominantly found in thymocytes and plays a key role in T-cell development. SATB1 mutant (SATB1⁻/⁻) peripheral T-cells resemble double-positive thymocytes or cells that have just undergone positive selection, suggesting that SATB1 deficiency allows these cells to move into the periphery without undergoing normal maturation. Interestingly, the clinical and diagnostic features of CD4⁺ PTCL, including frequent presentation of mediastinal mass as well as low expression of CD25 and

**FIGURE 7** CD4⁺ PTCL downregulated PTEN signalling and upregulated mTOR signalling. Differential gene expression of PTEN and mTOR (log base 2 of the fold change) in PTCL cases compared to control CD4⁺ T-cells showed downregulation of PTEN and upregulation of mTOR (Mean ± SD, * = Padj < 0.05) (A). GSEA plots identified significant enrichment of gene sets representing downregulation of PTEN (nominal P value = 0.003; FDR q-value = 0.02) (B) and upregulation of mTOR (nominal P value = 0.016; FDR q value = 0.08) (C) [Colour figure can be viewed at wileyonlinelibrary.com]
REFERENCES


MHC class II (consistent with an inactive T-cell) suggest that CD4+ PTCL may originate from naive CD4+ thymic precursor cells.

A recent study used a Human Cancer Hotspot Panel to identify potential tumour-associated mutations in canine PTCL, KDR, STK11, and BRAF were highly mutated and missense mutations were identified in MYC, TP53, and MET. In our study, a synonymous mutation in the promoter region of MYC and synonymous mutations in exons of KDR were present. No other mutations in the genes identified in this previous work were identified in the DNA transcripts in our study.

We describe the ability of flow cytometry to identify a distinct molecular and histologic entity: CD4+ PTCL. Less common immunophenotypes (CD8+, CD4−CD8−) could not be distinguished histologically from CD4+ PTCL, and may represent variations of the same disease. This study lays the groundwork for future work focused on determining if immunophenotype can predict distinct clinical or disease. This study lays the groundwork for future work focused on determining if immunophenotype can predict distinct clinical or disease. This study lays the groundwork for future work focused on determining if immunophenotype can predict distinct clinical or disease. This study lays the groundwork for future work focused on determining if immunophenotype can predict distinct clinical or disease. This study lays the groundwork for future work focused on determining if immunophenotype can predict distinct clinical or disease. This study lays the groundwork for future work focused on determining if immunophenotype can predict distinct clinical or disease.


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