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An aggressive CD4^{-}CD8^{-} T-cell neoplasm in young English bulldogs

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Abstract

T-cell leukemia/lymphoma accounts for roughly 30% of all types of lymphoproliferative neoplasia in dogs. Two forms of T-cell lymphoma (T-zone and peripheral T-cell lymphoma) exhibit breed-specific predilections. During the course of routine immunophenotyping, we observed a breed-specific presentation of a unique form of T-cell leukaemia in young English bulldogs. To describe the clinical presentation and outcome of a novel T-cell leukaemia in English bulldogs and determine the frequency of this neoplasm in other breeds. The Clinical Hematopathology database, containing immunophenotyping data from peripheral blood of nearly 11 900 dogs, was queried for the phenotype observed in young English bulldogs: CD45^{+}CD4^{-}CD8^{-}CD5^{+} CD3^{+} class II major histocompatibility complex (MHC)-low T-cell leukaemia. Clinical presentation, treatment, and survival data were collected for a subset of cases. Fifty-five English bulldog cases and 64 cases of other breeds were identified. No other breed was represented by >5 cases. Complete medical records were obtained for 50 bulldogs. Median age at diagnosis was 3 years and 76% of cases were male. Median lymphocyte count was 44 286 lymphocytes/μl (range, 1800–317 684/μl) and lymphocytes were described as small to intermediate-sized. Many dogs were thrombocytopenic and had liver and spleen involvement, but not lymphadenopathy. Bulldogs that received multi-agent chemotherapy had longer median survival times (83 days) compared to dogs that received no treatment (6 days) or less aggressive therapy (15 days) ($p = .001$). Non-bulldogs had similar outcomes. CD4^{-}CD8^{-} class II MHC-low T-cell leukaemia has an aggressive clinical course and predilection for young English bulldogs. Breed-specific presentation suggests an underlying genetic cause.

KEYWORDS
canine, clinical pathology, flow cytometry, immunophenotyping, leukaemia, T-cell

1 | INTRODUCTION

Non-Hodgkin lymphoma and leukaemia (NHLs) are heterogeneous neoplasms broadly subdivided into T- and B-cell phenotypes, with numerous subtypes. Classification of canine NHLs have been largely based on the human World Health Organization (WHO) criteria and the use of flow cytometry to accurately diagnose specific immunophenotypes of lymphoproliferative neoplasia has become a widely used objective application in both human and animal clinical oncology. Flow cytometry can be particularly useful in distinguishing between reactive and neoplastic lymphocytosis in the peripheral blood and several studies have demonstrated the value

of defining a tumour's immunophenotypic signature by flow cytometry to predict prognosis in different subtypes of leukaemia/lymphoma.[1–7]

An estimated 30%–40% of leukaemia/lymphomas known to occur in dogs are of the T-cell phenotype.[8,9] Currently, the most commonly described types of canine T-cell lymphoproliferative neoplasia in the blood are $CD8^+$ T-cell leukaemia and T-zone disease, which are readily diagnosed by flow cytometry,[2,3,6,7] though little has been described with regard to other subtypes in dogs. Through immunophenotypic analysis of canine blood samples, we identified a specific phenotype of T-cell leukaemia/lymphoma that was most common in the English bulldog. This study aims to describe the clinical presentation and outcome of a distinct T-cell neoplasm with a specific immunophenotype ($CD45^+CD4^-CD8^-CD5^+CD3^+$ class II major histocompatibility complex [MHC]-low), characterized by an expansion of lymphocytes described cytologically as small to intermediate in size with condensed chromatin. In addition to describing the disease in English bulldogs, we examined the frequency, clinical presentation, and outcome of this disease in non-bulldog breeds.

2 | METHODS

2.1 | Case selection

Peripheral blood samples from 11 889 dogs were submitted for routine immunophenotyping through the Clinical Hematopathology diagnostic service between August 2012 and December 2018. Blood samples from English bulldogs were identified by performing a query of the laboratory database. Cases selected for the study had a unique phenotype of T-cell leukaemia/lymphoma as determined by flow cytometry: $CD45^+CD4^-CD8^-CD5^+CD3^+$, class II MHC-low. Clinical information was obtained retrospectively from veterinary hospitals throughout the United States.

For the purpose of this study, cases met the following criteria: (1) the sample demonstrated a lymphocytosis above the reference interval for the submitting laboratory or clinic, or atypical lymphocytes were noted during cytologic review of the blood, (2) flow cytometry demonstrated an expansion of T-cells that expressed the pan T-cell surface antigens CD3 and CD5, but not subset antigens CD4 or CD8, and low levels of class II MHC, and (3) neoplastic lymphocytes were defined as small in size by linear forward light scatter (FSC) of <500. As reference, the median FSC of neutrophils in canine blood is 620 on the flow cytometer used for this study.

2.2 | Clinical evaluation and outcome assessment

Clinical outcome data including treatment and survival were obtained from hospitals that submitted samples. Patient signalment, physical examination findings at the time of diagnosis, laboratory results including complete blood counts (CBCs), biochemical profiles, and additional diagnostics relevant to diagnosis, staging, or treatment were assessed. Evaluation of the mediastinum and thoracic, peripheral and visceral lymph nodes, spleen, liver, and intestine were considered abnormal if enlargement, masses or thickening were identified by palpation, ultrasound examination or radiology. Cases with missing or unknown data were excluded from analysis.

Anaemia was defined by a haematocrit below the reference interval for the laboratory performing the CBC. Anaemia was considered regenerative when the reticulocyte count exceeded the laboratory's reference interval. Dogs were considered thrombocytopenic when platelet counts were below the laboratory's reference interval and review of a blood film by a clinical pathologist confirmed thrombocytopenia. Neutropenia was defined as a neutrophil count below the laboratory's reference interval. Abnormal biochemistry results were identified using reference intervals on available biochemistry panels.

The type of treatment implemented in each case was evaluated. Treatment was variable among cases and protocols were grouped into three general categories: (1) dogs that received no treatment, (2) dogs that received less aggressive treatment, and (3) dogs that received aggressive treatment. The less aggressive treatment group included use of oral or injectable glucocorticoids and combination therapy with chlorambucil and prednisone. Aggressive treatment included multi-modal chemotherapy protocols such as cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP), mechlorethamine, vincristine, procarbazine and prednisone (MOPP), and oral alkylating agent lomustine (CCNU). One dog received palliative radiation therapy and was excluded from analysis. Dogs that started treatment with less aggressive therapy and were subsequently moved to an aggressive protocol ($n = 6$) were grouped into the aggressive treatment category.

2.3 | Cytology and histology

All cytologic descriptions of blood films, cytology reports from other sites, and histology reports were reviewed. A subset of cases had CBC, cytology, or histology performed at the Veterinary Clinical Pathology Laboratory or Diagnostic Laboratory at Colorado State University. All available blood films ($n = 26$) and cytology samples, as well as one bone marrow histology sample were reviewed by three board-certified veterinary pathologists.

2.4 | Flow cytometry

Blood samples were submitted, processed, and stained as previously described[2] using the antibody panel in Table 1. Live cells were gated based on FSC on a linear scale (cell size) and side scatter (SSC) on a log scale (cell complexity) after dead cells had been excluded by propidium iodide fluorescence. All samples were analyzed with a 3 laser Gallios (Beckman Coulter Inc., Brea, CA). Relative cell size was determined by measuring median FSC. Forward and side light scatter properties and antigen expression of neoplastic cells in the included cases were

TABLE 1 Antibody panels used for immunophenotyping

Tube	Antibody specificity	Species produced in	Species recognized	Fluorochrome	Clone
Antibody panel[a]					
1	Isotype control	Mouse		FITC	W3/25
	Isotype control	Mouse		PE	W3/25
	Isotype control	Mouse		APC	W3/25
	Isotype control	Mouse		Alexa Fluor 647	W3/25
	Isotype control	Mouse		Alexa Fluor 700	W3/25
	Isotype control	Mouse		Pacific Blue	W3/25
2	CD3	Mouse	Canine	FITC	CA17.2A12
	CD25	Mouse	Canine	PE	P4A10
	CD5	Rat	Canine	APC	YKIX322.3
	CD8	Rat	Canine	Alexa Fluor 700	YCATE 55.9
	CD4	Rat	Canine	Pacific Blue	YKIX302.9
				Propidium Iodide	
3	Class II MHC	Rat	Canine	FITC	YKIX334.2
	CD34	Mouse	Canine	PE	1H6
	CD21	Mouse	Canine	Alexa Fluor 647	CA2.1D6
				Propidium Iodide	
4	Class II MHC	Rat	Canine	FITC	YKIX334.2
	CD18	Rat	Human	PE	YFC118.3
	CD5	Rat	Canine	APC	YKIX322.3
	CD14	Mouse	Human	Alexa Fluor 700	TUK4
	CD4	Rat	Canine	Pacific Blue	YKIX302.9
				Propidium Iodide	
5	CD5	Rat	Canine	FITC	YKIX322.3
	CD45	Rat	Canine	PE	YKIX716.13
	CD21	Mouse	Canine	Alexa Fluor 647	CA2.1D6
CD11d panel[b]					
1	FMO[c]				
2	CD5	Rat	Canine	FITC	YKIX322.3
	CD18	Rat	Human	PE	YFC118.3
	CD11d	Mouse	Dog	Alexa Fluor 647	CA11.8H2
	CD8	Rat	Dog	Alexa Fluor 700	YCATE 55.9
	CD4	Rat	Dog	Pacific Blue	YKIX302.9
				Propidium Iodide	

Note: All antibodies were purchased from Bio-Rad Inc, Hercules, CA with the exception of CD25 (eBioscience, San Diego, CA), class II MHC (eBioscience) and CD34 (BD Biosciences, San Jose, CA).

[a]Standard antibody panel for immunophenotyping performed in all cases.

[b]CD11d panel performed on 4 bulldog cases and 1 non-bulldog case.

[c]FMO, Fluorescence Minus One; CD5-FITC, CD18-PE, CD8-Alexa Fluor 700, CD4-Pacific Blue.

compared to lymphocytes and neutrophils in blood samples from 29 dogs without lymphoproliferative neoplasia (used as controls), during the same time period the study was conducted, under the same conditions.

Class II MHC expression was measured as the absolute median fluorescence intensity (MFI), consistent with previous T-cell studies from this laboratory.[2,10] Previous B-cell studies have reported class II MHC expression as the absolute MFI $\times 10$.[11–13] CD25 expression was determined as the percentage of neoplastic cells expressing CD25. Percent CD11d expression was also evaluated in five cases. Samples analyzed for CD11d expression were processed as described above and stained with the panel of antibodies listed in Table 1. To determine the positive boundary for CD11d expression in neoplastic cells, a fluorescence-minus-one (FMO) control was performed. All data analyses were carried out using Kaluza software (Beckman Coulter Inc., Brea, CA).

TABLE 2 Relevant signalment, presentation, physical examination, imaging and laboratory findings for English bulldog and non-bulldog cases with $CD4^{-}CD8^{-}$ class II MHC-low° T-cell leukaemia

	All bulldog cases ($n = 55$)		All non-bulldog cases ($n = 64$)	
	Dogs with available data (*n*)	Number affected (%) or median (IQR; range)	Dogs with available data (*n*)	Number affected (%) or median (IQR; range)
Signalment				
Male	55	42 (76%)	64	48 (75%)
Age at diagnosis (years)	55	3.0 (2.4–3.9; 1.0–7.1)	64	4.0 (2.5–6.9; 1.0–14.2)
Presentation				
Lethargy	53	53 (100%)	40	35 (88%)
Hypo-/anorexia	53	50 (94%)	41	36 (88%)
Weight loss	51	9 (18%)	32	17 (53%)
PU/PD	52	14 (27%)	30	7 (23%)
Gastrointestinal signs	51	45 (88%)	40	32 (80%)
Vomiting and diarrhoea		19 (37%)		8 (20%)
Vomiting		14 (27%)		16 (40%)
Diarrhoea		3 (6%)		6 (15%)
Hematochezia		3 (6%)		0 (0%)
Melena		6 (12%)		2 (5%)
Physical examination and imaging				
Peripheral LAD	53	12 (23%)	48	15 (31%)
Visceral LAD	34	17 (50%)	38	13 (34%)
Mediastinal involvement	33	10 (30%)	33	6 (18%)
Hepatomegaly	48	35 (73%)	41	29 (71%)
Splenomegaly	48	38 (79%)	39	22 (56%)
Peritoneal effusion	38	11 (29%)	35	4 (11%)
Pleural effusion	37	7 (19%)	35	3 (9%)
Skin disease	48	16 (33%)	30	8 (27%)
Fever	51	9 (18%)	29	1 (3%)
Lameness	51	1 (2%)	30	0 (0%)
Neurologic signs	52	10 (19%)	30	5 (17%)
Laboratory findings				
Lymphocyte total/μl	55	44 286/μl (21 620–80 700; 1800–317 684)	64	25 381/μl (15 186–66 150; 800–437 940)
Neutrophil total/μl	55	9200/μl (5800–16 583; 300–26 425)	64	8910/μl (5605–14 168; 1040–39 600)
Anaemia	55	24 (44%)	64	28 (44%)
Thrombocytopenia	55	41 (75%)	64	35 (55%)
Hypercalcemia	52	4 (8%)	46	6 (13%)
Hyperglobulinemia	51	6 (12%)	50	3 (6%)
Hypoalbuminemia	48	26 (54%)	30	17 (55%)
Hypocholesterolemia	44	36 (82%)	25	13 (52%)
Prolonged clotting times	11	8 (73%)	6	2 (33%)
Elevated ALT	53	41 (77%)	34	23 (68%)
Hyperbilirubinemia/elevated GGT	53	31 (59%)	29	14 (48%)

Abbreviations: ALT, alanine aminotransferase; GGT, gamma glutamyl transferase; IQR, interquartile range; LAD, lymphadenopathy; *n*, number; PU/PD, polyuria polydipsia.

2.5 | PCR for antigen receptor gene rearrangement (PARR)

Clonality testing for T-cell receptor gamma gene rearrangements and immunoglobulin heavy chain rearrangements was performed as previously described on all English bulldogs with available sample ($n = 40$).[14]

2.6 | Statistical analysis

English bulldogs were over-represented in the study population and all statistical analyses were performed separately for English bulldog cases and non-English bulldog cases. Continuous and categorical variables were summarized for all cases and descriptive statistics calculated. For continuous variables, a Wilcoxon rank sum test was performed. For categorical variables, Chi-square or Fisher's exact test were used as appropriate.

Overall survival time in days was calculated from time of diagnosis to time of death, euthanasia, or last follow-up. Dogs were censored if they were alive at the time of analysis or lost to follow-up. For survival analysis, Kaplan–Meier curves and log rank tests were used to evaluate whether signalment, hematologic variables, clinical signs, or treatment affected survival.

Graphical analysis was performed using Prism Graph Pad 7. All statistical analyses were performed with R version 3.5.2 and p-values <.05 were considered statistically significant.

3 | RESULTS

3.1 | Cohort characteristics

A total of 119 cases of $CD45^+CD4^-CD8^-CD5^+CD3^+$ class II MHC-low T-cell leukaemia/lymphoma were diagnosed across all breeds between 2012 and 2018. The frequency of this phenotype within all samples diagnosed with leukaemia/lymphoma in the blood ($n = 10\,928$) through the Hematopathology Laboratory between 2012 and 2018 was 1.1% ($n = 119$). The frequency of this specific disease entity in all blood samples diagnosed with T-cell leukaemia/lymphoma ($n = 5524$) was 2.2%. English bulldogs were over-represented, comprising 46% (55/119) of all dogs with this specific T-cell leukaemia/lymphoma. Clinical presentation and survival were assessed in English bulldogs and non-bulldogs separately.

3.2 | Clinical presentation in English bulldogs

Fifty-five English bulldog cases were identified, and medical records were obtained for 50 bulldogs. Hematologic findings, biochemical data and clinical signs are summarized in Table 2. Median age at presentation was 3 years old (range, 1–7 years) and 76% of cases were male. Median lymphocyte count was 44 286/μl (range, 1800-317 684/μl). Thrombocytopenia was common (41/55; 75%), anaemia affected 44% (24/55) of cases, and 9% (5/55) of cases were neutropenic. Among anaemic cases with reticulocyte counts available, ~65% had non-regenerative anaemia (13/20).

Hepatic abnormalities detected by imaging (35/48; 73%) were common and 77% of cases (41/53) had elevated levels of alanine aminotransferase (ALT) in the serum. 59% of cases had concurrent hyperbilirubinemia and increased cholestatic liver enzymes. Splenic abnormalities (38/48; 79%) affected most cases with available data. Peripheral lymphadenopathy was less common (12/53; 23%). Mediastinal abnormalities were detected in 30% of cases (10/33), but it was unknown in all cases whether enlargement was due to a thymic mass or mediastinal lymphadenopathy. Gastrointestinal abnormalities detected by abdominal ultrasound were infrequent (6/36; 17%). Three dogs had non-specific intestinal thickening, 2 dogs had gastric wall thickening with normal layering, and 1 dog had a focal gastric wall mass. 88% of cases (45/51) had gastrointestinal signs, including vomiting, diarrhoea, hematochezia, and melena. Neurological signs were observed in 10/52 cases (19%) and included head tilt, circling, ataxia, head pressing, tetraparesis and seizure activity. One case had brain magnetic resonance imaging, which showed infarction and infiltrative disease in the thalamus suspicious for neoplasia.

3.3 | Cytomorphologic and histologic characteristics

Forty-three cases had a blood smear examined by a board-certified veterinary clinical pathologist and reports were reviewed. By cytology, lymphocytes were often characterized as a monomorphic population

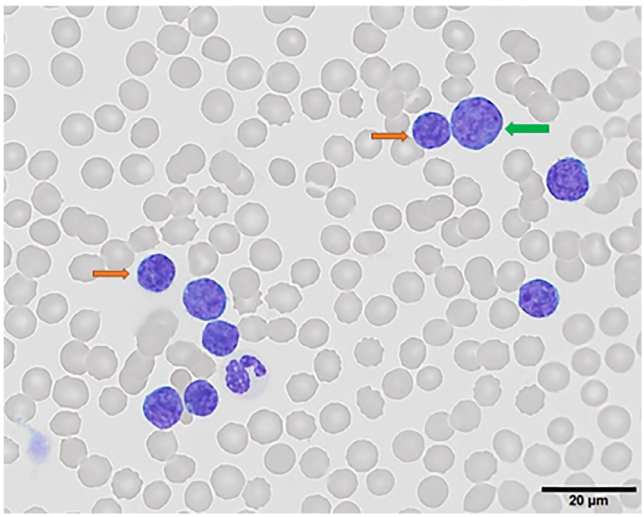

FIGURE 1 Blood film from a bulldog case with $CD4^-CD8^-$ class II MHC-low T-cell leukaemia. Wright-Giemsa, ×100 objective. The majority of lymphocytes were small with a scant amount of basophilic cytoplasm, condensed chromatin and no nucleoli (orange arrow). Occasional though variable intermediate lymphocytes with faint nucleoli were observed (green arrow)

of cells smaller to approximating the diameter of a neutrophil. Lymphocytes contained a scant amount of light to medium basophilic cytoplasm, round to slightly lobulated or cleaved nuclei, with clumped to slightly grainy chromatin and absent to occasional indistinct nucleoli (Figure 1). Eight of 43 bulldogs with a pathologist review of the blood were interpreted as chronic lymphocytic leukaemia and 10 of 43 had lymphocytes described as larger in size, with immature nuclear chromatin patterns and a single nucleolus.

Fifteen cases had tissue cytology evaluated by a board-certified veterinary clinical pathologist. One bone marrow core biopsy was available for histopathologic evaluation and 1 liver histology report was available for review. Bone marrow aspirates were obtained from 5 English bulldogs and a core biopsy from 1 bulldog. All bone marrow fine needle aspirates and single core marrow biopsy demonstrated infiltration of neoplastic lymphocytes, with lymphocytes accounting for 30%–90% of nucleated cells. Four of the 5 marrow aspirates described lymphocytes as small to intermediate sized with coarsely stippled to finely granular chromatin containing a single or occasional nucleolus, and 3 of 5 cases were interpreted as chronic lymphocytic leukaemia. One aspirate described neoplastic lymphocytes as predominantly intermediate to large in size with blast features, and large immature cells were also described in the paired peripheral blood sample from that case.

The single core marrow biopsy was evaluated by a board-certified veterinary anatomic pathologist which showed evidence of myelophthisis with increased numbers of primarily intermediate sized (size of a neutrophil to slightly larger, nuclei = 1.5 × diameter of a red blood cell) round cells characterized by scant eosinophilic cytoplasm. Nuclei were round to oval with a finely stippled chromatin and indistinct nucleoli. There were scattered mitotic figures within this population (0–1 per single HPF). Myeloid, erythroid and megakaryocytic lineages were present with appropriate maturation. One case had a liver biopsy and the histopathology report revealed diffuse periportal, perivascular and bridging infiltration by a monotonous population of

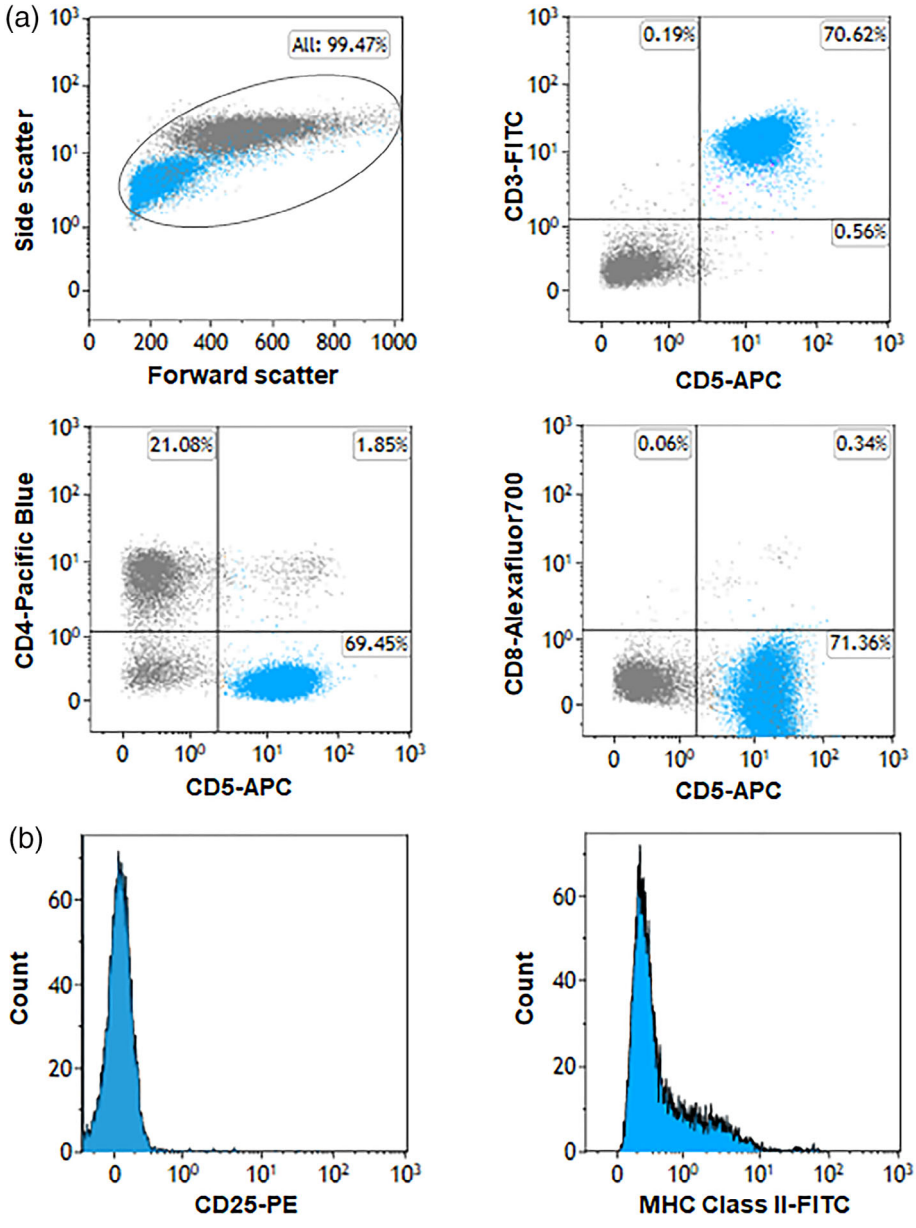

FIGURE 2 Flow cytometric immunophenotyping of neoplastic T-cells in the peripheral blood of a young, male English bulldog. (A) Size plot with linear forward light scatter on the horizontal axis and log side light scatter on the vertical axis. Neoplastic lymphocytes highlighted in blue are small as determined by forward scatter. Granulocytes (monocytes and neutrophils) are depicted in cement grey (top left). Neoplastic lymphocytes express T-cell antigens CD3 and CD5 (top right), but do not express either subset antigen CD4 or CD8 (middle row dot plots). (B) Neoplastic cells lack surface CD25 and class II MHC expression, shown as a percentage of expression on the neoplastic population

intermediate to large sized lymphocytes. Lymphocytes appeared to distend sinusoids and infiltrate hepatic parenchyma. Pathologist review of the paired peripheral blood sample from this case described lymphocytes as intermediate in size.

Additional sites that were aspirated for cytologic evaluation were liver ($n = 4$), spleen ($n = 3$), peripheral lymph node ($n = 2$), mesenteric lymph node ($n = 1$), sternal lymph node ($n = 1$) mediastinal mass ($n = 1$). Hepatic and splenic samples had increased numbers of lymphocytes with morphology similar to that seen in the blood. Two hepatic and 2 splenic cytology samples were interpreted as infiltrative lymphoma, and in the remaining hepatic and splenic samples, it could not be determined whether increased lymphocytes were attributed to lymphocytosis in the blood background or infiltrative disease. All lymph node cytologic aspirates (peripheral, mesenteric and sternal) had a predominance of small lymphocytes, interpreted as lymphoid hyperplasia or possibly an emerging lymphoproliferative disorder or lymphoproliferative disorder of small lymphocytes. The mediastinal mass aspirate revealed large sized immature lymphocytes consistent with lymphoma. In total, 5 of 17 cases with tissue cytology or histology had lymphocytes described as intermediate to large sized and these cases also had intermediate-large sized lymphocytes described in the blood.

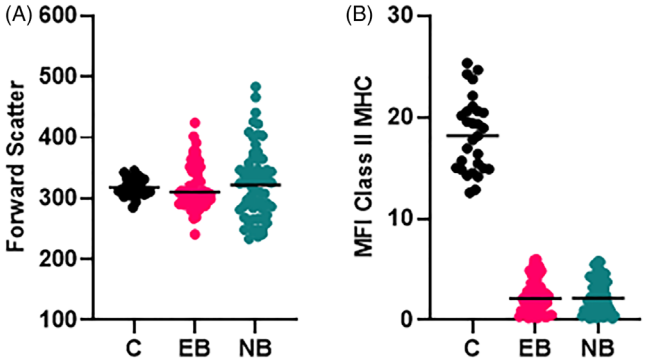

FIGURE 3 (A) Comparison of T-cell size as determined by forward light scatter between control cases (C), English bulldog cases (EB) and non-bulldog cases (NB). Neoplastic $CD4^{-}CD8^{-}$ T-cells in both cohorts were small in size. Median size of neoplastic T-cells was 310 in the English bulldog cohort and 322 in the non-bulldog cohort, while median size of $CD5^{+}$ T-cells in control cases was 318. (B) Comparison of median fluorescence intensity (MFI) of class II MHC surface expression in control versus study cases. Neoplastic $CD4^{-}CD8^{-}$ T-cells in both EB and NB cases expressed low levels of class II MHC (median MFI; 2.14 and 2.17, respectively) compared to control $CD5^{+}$ T-cells (median MFI; 18.2)

3.4 | Immunophenotyping and molecular clonality

All 119 cases of all breeds were characterized by uniform expression of the pan-leukocyte antigen CD45, T-cell surface antigens CD3 and CD5, and lack of surface expression of T-cell subset antigens CD4 and CD8 (Figure 2). Neoplastic T-cells in all cases with exception of 6 bulldogs and 7 other breeds had less than 1% CD25 surface expression (median, 0.13%) (Figure 2). For reference, control dogs had a median of 0.79% CD25-expressing T-cells (range, 0.47%–1.13%). Neoplastic T-cells were small in size by FSC (Figure 3). Median FSC ratio of neoplastic T-cells to $CD5^{+}$ T-cells in control cases was 1:1. All cases had low expression of class II MHC (<6.0; median, 2.17) compared to normal peripheral blood T-cells (median, 18.2) (Figure 3). CD11d surface expression was assessed in 4 bulldog samples and 1 non-bulldog sample. Neoplastic cells did not express CD11d in any case (not shown). No cases expressed CD34 (not shown).

PARR was performed on 40 out of 55 English bulldogs. Clonally rearranged T-cell receptor gene and polyclonal immunoglobulin gene products were seen in all cases (data not shown).

3.5 | Treatment and outcome in English bulldogs

Forty-nine English bulldog cases had survival data available. Irrespective of treatment type, English bulldogs had an overall median survival time (MST) of 26 days from the time of diagnosis (range, 1–490 days) (Figure 4A). Twenty-five patients died within the first month after diagnosis. Two dogs survived over 1 year after diagnosis. One dog was alive at completion of the study and 2 dogs were lost to follow-up and were censored from survival analysis. We evaluated signalment, clinical signs, physical exam findings, and laboratory data for association with survival and only age was associated with prognosis. When assessed above and below the median, cases <3.3 years of age had shorter survival (MST, 17 days) than cases >3.3 years of age (MST, 66 days) ($p = .04$).

Dogs received a variety of treatments and were divided into the three treatment categories described above. Bulldogs that received aggressive multi-agent chemotherapy had longer median survival times (MST, 83 days; $n = 28$) compared to dogs that received no treatment (MST, 6 days; $n = 4$) or less aggressive therapy (MST, 15 days; $n = 17$) ($p = .001$) (Figure 4B).

3.6 | Clinical presentation and outcome in non-bulldog breeds

An expanded search within the laboratory database identified 64 cases of this specific phenotype diagnosed in breeds other than English bulldogs during the same time period (2012–2018). Other dog breeds documented more than once included Golden Retrievers (7%), Labrador Retrievers (5%), and Australian Shepherds (3%). Clinical presentation was similar to English bulldogs. Median age of non-bulldogs was 4 years (range, 1–14 years) and males comprised 75% of cases. Thirty-four dogs had liver values available of which 23/34 (68%) had elevated liver enzymes at presentation. Five non-bulldog cases (17%) had neurological signs either at presentation or developed neurological signs associated with disease progression. Signalment, clinical signs, physical examination findings, and relevant hematologic and biochemical data for non-bulldogs are summarized in Table 2.

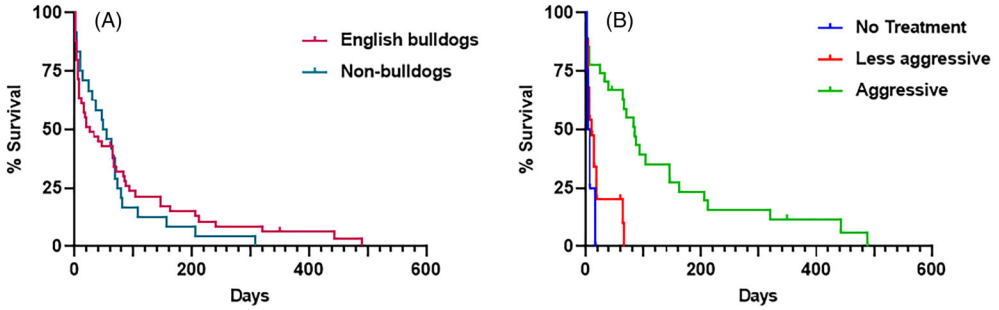

FIGURE 4 (A) Kaplan–Meier curves of the overall survival times of 50 English bulldogs (pink) and 24 non-bulldog breeds (teal). Median overall survival time was poor in both cohorts; English bulldogs; 26 days, Non-bulldogs; 52 days. (B) Kaplan–Meier curves depicting overall survival of 49 English bulldogs divided into three treatment categories. Bulldogs treated with an aggressive multi-modal chemotherapy treatment regimen had a more prolonged survival time (green line, MST 83 days, $n = 28$) compared no treatment (blue line, MST 6 days, $n = 4$) or a less aggressive treatment (steroids with or without chlorambucil) protocol (red line, MST 15 days, $n = 17$) ($p = .001$). Tick marks represent patients censored from survival analysis

Cellular morphology of peripheral blood neoplastic cells in non-bulldog cases was comparable to English bulldogs with 13 cases having neoplastic cells described as small to intermediate in size with similar cytomorphologic features. Neoplastic lymphocytes in four cases were described as large in size with immature nuclear features.

Survival data was available for 24 dogs in this cohort. Prognosis was poor with a median overall survival of 52 days (range, 1–309 days) (Figure 4A).

Similar to the English bulldog group, dogs that received aggressive multi-agent chemotherapy had longer overall survival times (MST, 62 days; $n = 9$) when compared to dogs that received less aggressive therapy (MST, 56 days; $n = 12$) or no treatment (MST, 9 days; $n = 3$) ($p = .013$).

4 | DISCUSSION

This study describes a novel form of leukaemia/lymphoma characterized by an aggressive clinical course that often presents in young, male dogs. The frequency in the English bulldog breed is noteworthy and suggests a genetic predisposition, although this disorder is not restricted to a single breed. As all of our cases originated in the United States, future studies will be necessary to determine if, similar to the frequency of T-zone lymphoma in Golden retrievers, the breed predisposition is geographically restricted.[15] While the shared clinical, morphologic and phenotypic features allowed the identification of this disorder, the overall frequency relative to more common forms of canine T-cell neoplasia with circulating cells is low.

A striking feature of this disease is the mismatch between the morphologic appearance of the circulating cells and the aggressive biologic behaviour. In fact, many bulldogs were started on prednisone and chlorambucil with a presumptive diagnosis of chronic lymphocytic leukaemia based on blood film review. In humans and dogs, B-cell chronic lymphocytic leukaemia (B-CLL) is considered an indolent disease, in which many patients with early-stage disease can be monitored without therapy.[1,16–19] Polyclonal B-cell lymphocytosis of English bulldogs (PBLEB), a recently described non-neoplastic disease of predominantly young, male English bulldogs is also indolent and characterized by an expansion of small mature B lymphocytes without peripheral lymph node involvement.[13] The cytomorphologic distinction between the phenotype described here and that of indolent disease may be challenging because the characteristics of the neoplastic T-cells in this disease can be remarkably similar to that of B-CLL or PBLEB. On the other hand, the immunophenotype as determined by flow cytometry is diagnostic for this disease. This study supports the idea that small cell size as determined by cytology alone is not equivalent to indolent disease and is not predictive of tumour biological behaviour.

In addition to signalment, there are consistent clinical features that can help increase the clinical suspicion for this disease. Thrombocytopenia was documented in 41 bulldogs, a finding that is relatively uncommon in canine B-CLL.[12] Although it was performed infrequently in these dogs, all five English bulldogs with bone marrow aspirates had diffuse infiltration of the marrow space. This may have contributed to the thrombocytopenia. A hypercoagulable state such as disseminated intravascular coagulation is also a possible contributing factor for the selective thrombocytopenia as clotting times were frequently prolonged and melena and hematochezia were reported. Most cases had elevation of leakage and cholestatic enzymes of the liver and, when aspirated, the liver displayed significant lymphocytic infiltration in a subset of cases. A degree of infiltration leading to functional impairment was suggested by the fact that many dogs had decreases in circulating cholesterol and/or albumin and clotting times were prolonged in 8 of the 11 dogs tested. Liver dysfunction and subsequent deficient clotting factor production could also have contributed to prolonged clotting times. The frequency with which dogs presented with gastrointestinal signs such as vomiting and/or diarrhoea was notable. Direct neoplastic extension to the stomach or small intestine appears unlikely as the ultrasonographic appearance of the stomach and small intestine were reportedly normal in all but few dogs. Furthermore, the hypoalbuminemia was selective without the expected pan-hypoproteinemia seen with diffuse gastrointestinal

disease. Negative acute phase response or sequestration in cases with cavity effusions may have contributed to the hypoabuminemia.

A subset of bulldog cases and, to a lesser extent, non-bulldog cases had neurologic signs associated with their disease which included acute blindness, ataxia, and head pressing. These were typically described as progressive, terminal events although 1 bulldog and 2 non-bulldogs presented with neurologic signs. Notably, 3 bulldogs and 2 non-bulldogs had a right-sided head-tilt. Fewer dogs were described as having seizure-like activity. It is difficult to distinguish extension of neoplasia into the central nervous system (CNS) from the potential influence of metabolic factors given the indication of diffuse hepatic involvement in many dogs. None of the dogs with neurologic signs had blood ammonia or bile acids analysis performed to assess for hepatic encephalopathy. However, it is worth noting that some dogs had normal biochemical results at the time of neurologic progression. One bulldog had an MRI that revealed hemorrhagic infarcts within the right thalamic region of the brain, suggesting that direct extension of lymphoproliferative neoplasia into the right forebrain or thalamic region is plausible.

In humans and dogs, T-cell leukaemia is divided into subtypes within two general categories: leukaemia that arises from a mature T-cell and that which arises from precursor T-cells. In humans, mature T-cell leukaemias are a heterogeneous group of neoplasms with a broad variety of outcomes ranging from indolent disease to more aggressive neoplasms such as T-cell prolymphocytic leukaemia (T-PLL).[20,21] T-PLL is a rare aggressive mature T-cell leukaemia, with presenting features of splenomegaly, generalized lymphadenopathy, skin disease, serous pleural effusions, and hepatomegaly.[22–24]

Hepatosplenic T-cell lymphoma is an aggressive subtype of extranodal lymphoma without lymphadenopathy but with infiltration of the liver, spleen and frequently the bone marrow. As currently described in the veterinary literature, hepatosplenic lymphoma typically occurs in older dogs and is comprised of large lymphocytes frequently containing cytoplasmic granules which would be distinct from our cases.[25] Additionally, canine hepatosplenic lymphoma is thought to arise from $CD11d^{+}$ gamma delta T-cells, and where evaluated, neoplastic cells in our study did not express CD11d. Two cases of hepatocytotropic T-cell lymphoma described by Keller et al. had thrombocytopenia, prolonged clotting times, and elevated liver enzymes and were CD11d negative; however, lymphocytes invaded hepatic cords (which was not apparent in the 1 case described here with histology), the dogs were older and lymphocyte morphology differed, suggesting a different entity than the T-cell leukaemia/lymphoma described in this study.[25]

There are features of this neoplasm that are similar to human T-cell acute lymphoblastic leukaemia (T-ALL), a tumour of precursor T-cell origin that affects both children and adults.[26,27] T-ALL is aggressive, has a male predominance and, in addition to diffuse bone marrow infiltration, can involve a variety of extranodal sites.[27–29] Morphology of the circulating cells has been described as variable, although numerous cases have described cells as small with densely packed chromatin, inconspicuous nucleoli and high nucleus: cytoplasm ratios.[30,31] The phenotype of the abnormal T-cells varies in human T-ALL, but subsets lack surface expression of both CD4 and CD8 similar to the dogs described in this report.[21,32,33] Another similarity in clinical presentation between this disease and human T-ALL is the apparent presence of CNS involvement. Approximately 7–15% of human ALL patients will have disease relapse or progression within the CNS.[29,34] This canine leukaemia/lymphoma may arise from a precursor T-cell and share features of T-ALL, but have a distinct and different biological process from that of CD34+ T-ALL. Gene expression profiling will help further define this neoplasm and establish the cell of origin.

Due to the retrospective nature of this study, evaluation of the impact of treatment on overall survival is limited. Within the patient group, a wide range of chemotherapy protocols were used with variable drug combinations, dosages, and frequency of administration. Consequently, patients were divided into broad treatment categories (no treatment, less aggressive therapy, or aggressive therapy) to evaluate impact of treatment on survival. Additionally, complicating the treatment evaluation is inherent bias in treatment decisions based on disease severity at the time of presentation to the veterinarian, response to initial treatment, and/or financial and personal considerations of clients. The two bulldogs with survival times >1 year completed an L-CHOP protocol and achieved clinical remission of 5 to 6 months duration. Various rescue protocols were initiated throughout the course of treatment in both cases with an eventual shift to a MOPP protocol in advanced-stage disease. The relatively small sample size of the study cohort is a limitation to fully assess whether different treatment protocols affect survival times. Progression free interval (PFI) was not assessed due to the retrospective nature of the study, varied treatment protocols, and small sample size.

This previously undescribed T-cell leukaemia/lymphoma with an aggressive clinical course often observed in young, male, English bulldogs adds to the list of canine T-cell malignancies that show a strong, breed-specific predilection. Golden retrievers comprise 40% of T-zone lymphoma cases and Boxers are the most common breed in cohorts of peripheral T-cell lymphoma, with Golden retrievers and Australian shepherds also highly represented.[2,4,10,15,35] While this T-cell leukaemia/lymphoma is relatively rare, English bulldogs are notably overrepresented and have the strongest breed association described to date, although English bulldogs have not been observed as overrepresented in other studies of T-cell neoplasia.

The prevalence of English bulldogs in this entity indicates a strong genetic risk factor. That risk factor does not appear to be shared by breeds in the same genetic clade: French bulldog, Boxer, and Boston terrier.[36] Only two cases were observed in Boxers, a breed which is highly represented within the population of dogs tested for haematopoietic neoplasia, and one case each in a French bulldog and Boston terrier. The study of leukaemia/lymphoma in populations with restricted genetic heterogeneity, such as the English bulldog,[37] may prove fruitful in identifying genetic factors that contribute to the pathogenesis of lymphoproliferative neoplasia in both humans and dogs.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Williams MJ, Avery AC, Lana SE, Hillers KR, Bachand AM, Avery PR. Canine lymphoproliferative disease characterized by lymphocytosis: immunophenotypic markers of prognosis. *J Vet Intern Med*. 2008; 22(3):596-601.
2. Seelig DM, Avery P, Webb T, et al. Canine T-zone lymphoma: unique immunophenotypic features, outcome, and population characteristics. *J Vet Intern Med*. 2014;28(3):878-886.
3. Mcdonough SP, Moore RF. Clinical, hematologic, and immunophenotypic characterization of canine large granular lymphocytosis. *Vet Pathol*. 2000; 37(6):637-646.
4. Avery PR, Burton J, Bromberek JL, et al. Flow cytometric characterization and clinical outcome of CD4+ T-cell lymphoma in dogs: 67 cases. *J Vet Intern Med*. 2014;28(2):538-546.
5. Harris LJ, Hughes KL, Ehrhart EJ, Labadie JD, Yoshimoto J, Avery AC. Canine CD4+ T-cell lymphoma identified by flow cytometry exhibits a consistent histomorphology and gene expression profile. *Vet Comp Oncol*. 2019;17(3):253-264.
6. Comazzi S, Gelain ME, Martini V, et al. Immunophenotype predicts survival time in dogs with chronic lymphocytic leukemia. *J Vet Intern Med*. 2011;25(1):100-106.
7. Martini V, Marconato L, Poggi A, et al. Canine small clear cell/T-zone lymphoma: clinical presentation and outcome in a retrospective case series. *Vet Comp Oncol*. 2016;14:117-126.
8. Modiano JF, Breen M, Burnett RC, et al. Distinct B-cell and T-cell lymphoproliferative disease prevalence among dog breeds indicates heritable risk. *Cancer Res*. 2005;65(13):5654-5661.
9. Seelig DM, Avery AC, Ehrhart EJ, Linden MA. The comparative diagnostic features of canine and human lymphoma. *Vet Sci*. 2016; 3(2):11.
10. Harris LJ, Rout ED, Labadie JD, et al. Clinical features of canine nodal T-cell lymphomas classified as CD8+ or CD4−CD8− by flow cytometry. *Vet Comp Oncol*. 2020;18(3):416-427.
11. Rao S, Lana S, Eickhoff J, et al. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *J Vet Intern Med*. 2011;25(5):1097-1105.
12. Bromberek JL, Rout ED, Agnew MR, Yoshimoto J, Morley PS, Avery AC. Breed distribution and clinical characteristics of B-cell chronic lymphocytic leukemia in dogs. *J Vet Intern Med*. 2016;30(1): 215-222.
13. Rout ED, Moore AR, Burnett RC, et al. Polyclonal B-cell lymphocytosis in English bulldogs. *J Vet Intern Med*. 2020;34(6): 2622-2635.
14. Colopy LJ, Shiu KB, Snyder LA, Avery AC, Rout ED, Moore AR. Immunoglobulin G4-related disease in a dog. *J Vet Intern Med*. 2019;33(6): 2732-2738.
15. Comazzi S, Marelli S, Cozzi M, et al. Breed-associated risks for developing canine lymphoma differ among countries: an European canine lymphoma network study. *BMC Vet Res*. 2018;14(1):1-7.
16. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008;111(12):5446-5456.
17. Milne K, Sturrock B, Chevassut T. Chronic lymphocytic leukaemia in 2020: the future has arrived. *Curr Oncol Rep*. 2020;22(4):1-9.
18. Kipps TJ, Stevenson FK, Wu CJ, et al. Chronic lymphocytic leukaemia. *Nat Rev Dis Prim*. 2017;3(1):17008.
19. Rout ED, Labadie JD, Yoshimoto JA, Avery PR, Curran KM, Avery AC. Clinical outcome and prognostic factors in dogs with B-cell chronic lymphocytic leukemia: a retrospective study. *J Vet Intern Med*. 2021; 35(4):1918-1928.
20. Swerdlow SH, Campo E, Pileri SA, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood*. 2016;127(20):2375-2390.
21. Arber DA, Orazi A, Hasserjian R, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood*. 2016;127(20):2391-2405.
22. Dearden CE. T-cell prolymphocytic leukemia. *Med Oncol*. 2006;23(1): 17-22.
23. Jain P, Aoki E, Keating M, et al. Characteristics, outcomes, prognostic factors and treatment of patients with T-cell prolymphocytic leukemia (T-PLL). *Ann Oncol*. 2017;28(7):1554-1559.
24. Staber PB, Herling M, Bellido M, et al. Consensus criteria for diagnosis, staging, and treatment response assessment of T-cell prolymphocytic leukemia. *Blood*. 2019;134(14):1132-1143.
25. Keller SM, Vernau W, Hodges J, et al. Hepatosplenic and hepatocytotropic T-cell lymphoma: two distinct types of T-cell lymphoma in dogs. *Vet Pathol*. 2013;50(2):281-290.
26. Inaba H, Pui C-HH. Advances in the diagnosis and treatment of pediatric acute lymphoblastic leukemia. *J Clin Med*. 2021;10(9): 1926.
27. Swerdlow SH, Campo E, Lee Harris N, et al. T-lymphoblastic leukaemia/lymphoma. *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. Revised 4t. World Health Organization; 2017: 209-212.
28. Chiaretti S, Vitale A, Cazzaniga G, et al. Clinico-biological features of 5202 patients with acute lymphoblastic leukemia enrolled in the Italian AIEOP and GIMEMA protocols and stratified in age cohorts. *Haematologica*. 2013;98(11):1702-1710.
29. Lustosa de Sousa DW, de Almeida Ferreira FV, Cavalcante Félix FH, de Oliveira Lopes MV. Acute lymphoblastic leukemia in children and adolescents: prognostic factors and analysis of survival. *Rev Bras Hematol Hemoter*. 2015;37(4):223-229.
30. Chiaretti S, Zini G, Bassan R. Diagnosis and subclassification of acute lymphoblastic leukemia. *Mediterr J Hematol Infect Dis*. 2014;6(1): e2014073.
31. Gassmann W, Löffler H, Thiel E, et al. Morphological and cytochemical findings in 150 cases of T-lineage acute lymphoblastic leukaemia in adults. *Br J Haematol*. 1997;97(2):372-382.
32. Noronha EP, Codeço Marques LV, Andrade FG, Santos Thuler LC, Terra-Granado E, Pombo-De-Oliveira MS. The profile of immunophenotype and genotype aberrations in subsets of pediatric T-cell acute lymphoblastic leukemia. *Front Oncol*. 2019;9:1-10.
33. Lin TH, Wu HC, Hsieh YC, Tseng CE, Ichinohasama R, Chuang SS. CD4 and CD8 double-negative adult T-cell leukemia/lymphoma with monomorphic cells expressing CD99: a diagnostic challenge in a country non-endemic for human T-cell leukemia virus. *Pathol Int*. 2013;63(2):132-137.
34. Gaudichon J, Jakobczyk H, Debaize L, et al. Mechanisms of extramedullary relapse in acute lymphoblastic leukemia: reconciling biological concepts and clinical issues. *Blood Rev*. 2019;36:40-56.
35. Ponce F, Marchal T, Magnol JP, et al. A morphological study of 608 cases of canine malignant lymphoma in France with a focus on

comparative similarities between canine and human lymphoma morphology. *Vet Pathol*. 2010;47(3):414-433.
36. Parker HG, Dreger DL, Rimbault M, et al. Genomic analyses reveal the influence of geographic origin, migration, and hybridization on modern dog breed development. *Cell Rep*. 2017;19(4):697-708.
37. Pedersen NC, Pooch AS, Liu H. A genetic assessment of the English bulldog. *Canine Genet Epidemiol*. 2016;3(1):1-16.

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