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Increased frequency of CD45 negative T cells (T zone cells) in older Golden retriever dogs

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T zone lymphoma (TZL) is characterized by the clonal expansion of T cells lacking expression of the pan-leukocyte antigen CD45 (TZ cells). A strong breed predisposition is observed in Golden retrievers. This study aimed to confirm aberrant CD45 mRNA expression and determine if Golden retrievers without clinical lymphoma have an increased frequency of circulating TZ cells. Gene expression analysis on confirmed TZL cases showed a significant decrease in CD45 expression compared to normal dogs. Peripheral blood samples from senior dogs, 242 Golden retrievers and 42 non-Golden retrievers, without evidence of lymphoproliferative disease were assessed for the presence of TZ cells by flow cytometry. Thirty-one percent of Golden retrievers had TZ cells compared to 14% of non-Golden retrievers. Thirty-four percent of Golden retrievers with TZ cells had a clonal T cell receptor gamma (TRG) gene rearrangement. Interestingly, 20% of Golden retrievers without TZ cells also had a clonal TRG rearrangement. Golden retrievers may have an increased risk of TZL due to an increased frequency of TZ cells.

KEYWORDS

CD45, dog, flow cytometry, lymphoma, T zone lymphoma

1 | INTRODUCTION

Canine lymphoproliferative disease characterization has improved with the use of ancillary diagnostics including immunocytochemistry, immunohistochemistry, flow cytometry, PCR for antigen receptor rearrangement (PARR) and, more recently, gene expression profiling. Many of the canine lymphoproliferative diseases mimic human disorders and efforts have been made to utilize the World Health Organization scheme when classifying canine lymphoma.[1,2] As these subtypes become more completely defined, some marked breed-specific predilections for different subtypes have become evident. For example, Boxers[3,4] and Golden retrievers (GRs)[4] are highly represented amongst the group of dogs that develop an aggressive form of T cell lymphoma, histologically characterized as lymphoblastic lymphoma or peripheral T cell lymphoma not otherwise specified (PTCL-NOS).[3] Another example includes a recent study in which small breed dogs were found to be over-represented in the group of dogs that develops B cell chronic lymphocytic leukemia.[5] Breed-specific tendencies to develop particular disorders indicate a high likelihood for genetic risk factors underlying these diseases, and position the dog as a useful model for studying the role of genes in the development of lymphoproliferative disease.

T zone (or clear cell) lymphoma (TZL) is a variant of PTCL-NOS that is described in both humans and dogs.[6,7] TZL is characterized by neoplastic T cells expanding the paracortex and medullary cords and compressing the fading germinal centres.[1] The cells are small to intermediate in size with very rare mitoses.[1,8]

The prevalence of TZL is estimated as 3% to 14% of all canine lymphomas in two different large-scale studies[1,2] and there is a striking breed predilection in dogs, with 40% of all TZL cases being diagnosed in American GRs.[9] European GRs, which represent a discreet genetic group[10] do not appear to be over-represented in TZL.[11] The median age of dogs diagnosed with TZL is 10 years in multiple studies.[9,11] Lymphadenopathy and lymphocytosis are both commonly associated with TZL, and the presence of TZL in the blood does not appear to indicate a more aggressive clinical course.[9,11,12] The overall median survival time is reported to be 760[11] to 1022 days,[12] consistent with an indolent nature. The disease is often detected as an incidental finding of lymphocytosis and/or lymphadenopathy; interestingly 10% to 50% of cases present with or develop demodectic mange,[12,13] suggesting that the TZL may cause immunosuppression in a subset of cases.

TZL exhibits a unique phenotype: the neoplastic T cells do not express the pan-leukocyte antigen CD45, allowing for reliable

diagnosis by immunophenotyping.[9] Recently this was corroborated with immunohistochemistry.[14] Additionally, real-time PCR for CD45 transcript and DNA showed almost complete lack of CD45 gene expression but presence of CD45 DNA.[14] CD45 is a transmembrane protein tyrosine phosphatase that has multiple isoforms that are abundantly expressed on the cell surface of all leukocytes.[15–17] Two major isoforms of CD45 have been recognized in canine T cells, with expression varying based on phenotype and activation status.[18] The T cell receptor (TCR) is closely associated with CD45, and CD45 expression is necessary for T cell response to antigen.[15]

T zone (TZ) cells express increased levels of the complement receptor CD21,[9,19] which is commonly used to identify B cells in dogs. In previous studies, CD21 expression has been identified by flow cytometry[9,19] and with microarray in TZL,[20] however, expression was not compared with normal T cells in these studies. The neoplastic cells can express CD4, CD8, neither subset antigen[9] or, rarely, both.

Our laboratory is conducting a study of environmental and genetic risk factors for TZL in GRs. While screening control GRs (dogs ≥9 years with no clinical evidence of lymphoma of any type), we discovered that many dogs had small but detectable numbers of CD5+ CD45− cells (TZ cells) in their peripheral blood. This observation caused us to ask if TZ cells are equally frequent in all dogs over the age of 9 or if this is a breed-specific phenomenon.

The objectives of this study are 2-fold. First, we wanted to validate in detail the phenotype of TZ cells by examining mRNA levels for the proteins that characterize these cells: CD45 and CD21. Then, we wanted to determine the frequency of TZ cells in non-lymphoma bearing older GRs and other purebred dogs. We hypothesized that TZ cells would be found more frequently in GRs than other breeds reflecting the breed predilection observed for this form of lymphoma. Validation of this hypothesis would suggest that an early event in the development of TZL is loss of CD45 expression, and would pave the way for mechanistic studies of oncogenesis.

2 | MATERIAL AND METHODS

2.1 | Selection of cases and controls for gene expression analysis

To investigate the aberrant antigen expression seen in TZL, we measured the levels of CD45 and CD21 mRNA from case and control samples. Case samples included blood or lymph node aspirates from 34 dogs diagnosed with TZL by flow cytometry through the Colorado State University Clinical Immunology (CSU-CI) laboratory between December 2013 and March 2015. These cases were not breed restricted. A diagnosis of TZL included greater than 60% of the lymphocytes characterized as TZ cells (CD5+ CD45− cells) in lymph node samples or the presence of ≥5000 TZ cells/μL in the peripheral blood of dogs with lymphadenopathy or lymphocytosis, respectively. These criteria were based on previous publications in which the minimum criteria for the diagnosis of lymphoid malignancy was 60% or greater aberrant cells in a lymph node,[21,22] or 5000 cells/μL or greater phenotypically homogeneous expansion of lymphocytes in peripheral blood.[5] The control group consisted of T cells purified from lymph node and thymic tissue collected from 8 young, healthy, hound mix dogs that were being utilized for a surgical continuing education course. All these procedures were approved by the IACUC committee at Colorado State University.

2.2 | Cell sorting for gene expression studies

2.2.1 | TZL cases

TZ cells were purified from blood or lymph node of TZL cases by negatively selecting CD45-expressing cells. Briefly, based on the Miltenyi Biotec protocol, the nucleated cells were re-suspended in MACS buffer (Phosphate-buffered saline [PBS]-0.5% BSA-2 mM Ethylenediaminetetraacetic acid [EDTA] −1 mM) resulting in a total volume of 100 μL after addition of the primary antibodies. All antibodies were purchased from Abd Serotec/Bio-Rad (Kidlington, UK). Anti-CD45 PE (Pan-leukocyte, clone YKIX716.13) at a concentration of 0.01 μg/μL was added and the samples were incubated for 10 minutes in the dark at 4°C, and then washed with MACS buffer. The supernatant was removed and samples were re-suspended in 80–90 μL of MACS buffer and 10 μL of anti-PE beads (Miltenyi Biotec, San Diego, California). The samples were incubated for 10 minutes in the dark at 4°C and washed with MACS buffer. The supernatant was removed and the cells were re-suspended in 500 μL of MACS buffer. For magnetic depletion of CD45+ cells, the 500-μL cell suspension was loaded onto an LD column that had been placed into a MidiMACS separator. CD45− cells that were not bound by beads were collected and tested for purity by flow cytometry. Figure 1 shows an example of sorted CD5+ CD45− (TZ) cells purified by this method. The median purity of the sorted TZ cells was 98%, and only one sample had lower than 90% purity (82%).

2.2.2 | Controls

CD4 and CD8 T cells were purified from the lymph nodes and thymus of 8 healthy control dogs to be used as normal controls in a series of studies of gene expression profiling in T cell lymphoma. Cells were stained with anti-CD18-PE (Human CD18, clone YFC118.3), anti-CD5-FITC (T cells, clone YKIX322.3) and either anti-CD4-Pacific Blue (CD4 T cells, clone YKIZ302.9) or anti-CD8-FITC (CD8 T cells, clone YCATE 55.9) and sorted by fluorescence-activated cell sorting on a MoFlo cell sorter (Beckman Coulter, Brea, California). All samples were analyzed for purity and only those samples with >90% of the desired cells were used for RNA purification.

2.3 | Gene expression using NanoString technology

Expression of CD45 and CD21 was measured using NanoString technology. NanoString technology was employed because it does not require high quantity or quality of RNA, such as RNA derived from convenience clinical samples that have been shipped and then further manipulated once arriving in the laboratory. Only 100 ng or less of total RNA is needed, the detection method has a 0.1 to 0.5 fM detection limit and the assay is reproducible (R^2 = .999 average for replicates).[23] Gene expression is measured by counting the number of transcripts for each gene of interest, rather than using relative expression as is done with microarrays, and there is no PCR amplification step.

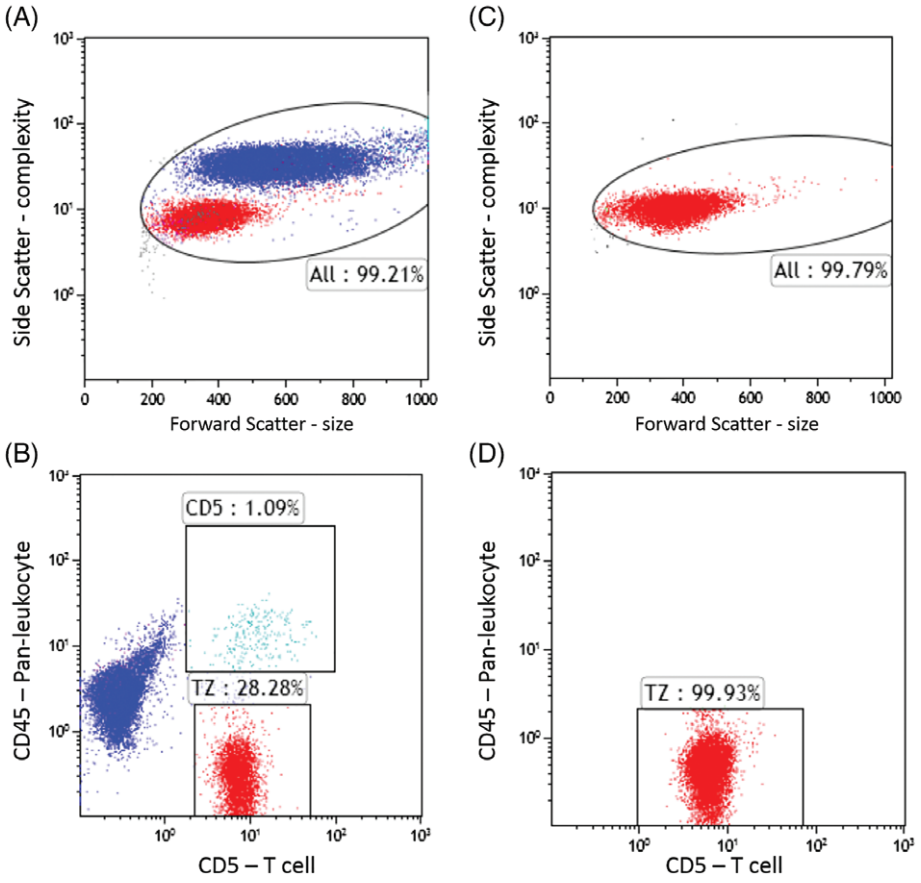

FIGURE 1 Example of T zone (TZ) cells purified from one dog for gene expression. A and B are plots from the peripheral blood before sorting for TZ cells, and C and D are plots from the same sample of peripheral blood after purification of TZ cells. The TZ cells are in the TZ gate, normal T cells are in the CD5 gate. A and C depict light scatter properties. B and D depict expression of CD5 and CD45. After the sort, the sample is composed of 99.93% TZ cells

After sorting, the cell fractions were suspended in RNA lysis buffer prepared according to the Purelink RNA mini kit (Life Technologies, Carlsbad, California) and stored at −80°C until analysis. RNA samples were analyzed for degradation and concentration using Agilent RNA ScreenTape assay (Agilent Technologies, Santa Clara, California). Samples had concentrations ranging from 1250 to 129 000 pg/μL and RNA integrity numbers (RINs) ranging from 2.6 to 7.6. Approximately, 100 ng of purified RNA per sample was hybridized overnight to the mRNA custom-built probes and hybridized reactions were loaded into the nCounter Digital Analyzer (NanoString Technologies, Seattle, Washington) through the University of Arizona Genetic Core.

Probes were designed based on the predicted sequence of Canis lupus familiaris CD45. While multiple CD45 isoforms have been annotated in the dog, only two isoforms have been shown to be expressed in the dog. The difference in these two isoforms is the presence or absence of exon 4, which is included in the larger molecular weight form of CD45.[18] Exon 4 is expressed by naïve T cells, but not expressed in the majority of activated and memory T cells in humans.[15,24] Therefore, two probes were designed to detect CD45 expression (Figure 2, Table 1). The probe designed for PTPRCb was a pan-CD45 probe predicted to bind to both demonstrated canine isoforms[18] as well as all predicted possible canine isoforms by analogy with mouse and human studies.[17] The second probe, PTPRCa, was predicted only to bind to the higher molecular weight isoform of CD45 (Figure 2).

The CD21 probe was designed to detect all three predicted canine isoforms of this protein (Table 1). Gene expression was normalized using 6 housekeeping genes (Table 1) which were chosen from a group of 15 housekeeping genes because they exhibited low variability between dogs.

2.4 | Selection of dogs for quantifying CD45− T cells in peripheral blood

The frequency of TZ cells in the peripheral blood was assessed in 2 populations of dogs: GRs and non-Golden retrievers (non-GRs).

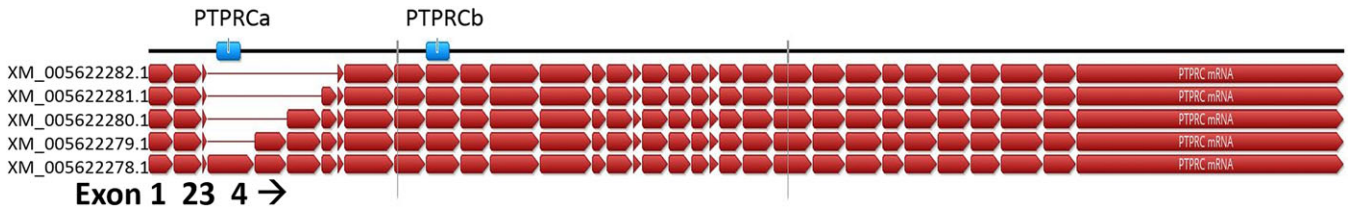

FIGURE 2 CD45 splice variants. Graphical depiction of CD45 mRNA, predicted splice variants and mapping of designed probes for PTPRCa and PTPRCb. Each red arrow represents an exon and the first 4 exons are enumerated

TABLE 1 Probe design for CD45, CD21 and housekeeping genes

Gene symbol	NCBI reference sequence	Target region of sequence
PTPRC (CD45b)	XM_005622278.1	1134-1233
PTPRC (CD45a)	XM_005622278.1	279-378
CR2 (CD21)	XM_005622319.1	2436-2535
Housekeeping genes		
GUSB	NM_001003191.1	1363-1462
SDHA	XM_535807.4	1641-1740
HPRT1	NM_001003357.1	177-276
EEF1G	XM_848484.3	1066-1165
TBP	XM_005627736.1	666-765
POLR2A	XM_852751.1	671-770

TABLE 2 Antibody panel for flow cytometry analysis[a]

Tube	Antibody specificity and flurochrome
1	M IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-750/M IgG1-Pacific Blue
2	CD3-FITC/CD25-PE/CD5-APC/CD8-Alexa 700/CD4-Pacific Blue
3	Class II MHC-FITC/CD22-PE/CD21-Alexa 647
4	Class II MHC-FITC/CD34-PE/CD5-APC/CD14-PE-Alexa 750
5	Class II MHC-FITC/CD18-PE/CD5-APC/CD14-PE-Alexa 750/CD4-Pacific Blue
6	CD5-FITC/CD45-PE/CD21-Alexa 647

[a] Unless otherwise noted, all antibodies were purchased from AbD Serotec/Bio-Rad. Clones are as follows: CD45 = YKIX716.13, CD18 = YFC118.3 (human CD18), CD4 = YKIX302.9, CD8 = YCATE 55.9, CD5 = YKIX322.3, CD21 = CA2.1D6, CD22 = RFB4 (human CD22, purchased from AbCam, Cambridge, UK), CD3 = CA17.2A12, CD14 = TUK4 (human), class II MHC = YKIX334.2, CD34 = 1H6, CD25 = P2A10 (purchased from eBiosciences, San Diego, CA).

GR samples were obtained from the control group of a case–control study of risk factors for TZL being conducted by the CSU-CI laboratory. Samples from dogs collected from June 2013 to April 2015 were utilized. Criteria for controls for this study included GRs, 9 years of age or older, with no suspicion or history of lymphoproliferative disease and no lymphocytosis. Other medical conditions, including cancers other than lymphoma/leukaemia, did not exclude dogs from the study. Clinical signs were reported by veterinarians based on physical examinations and history obtained during sample submission. Non-GR dogs were selected from dogs presenting to the Colorado State University Veterinary Teaching Hospital (CSU-VTH) between April 2014 and January 2015. Shih tzus, which were reported to be the second most frequent breed to develop TZL,[9] were excluded from this study and will be part of a separate investigation. Medical records were reviewed to identify purebred dogs, 9 years and older. Dogs with prior diagnosis or evidence of lymphoma/leukaemia (lymphocytosis, etc.) were excluded from the study. Age for all dogs was calculated from the date of birth provided to the date of sample received and were rounded to the nearest year. Since we were detecting small numbers of TZ cells, we wanted T cell numbers to be adequate. In order to avoid small numbers of T cells leading to false negatives, absolute lymphopenia identified on the complete blood cell count (CBC) resulted in exclusion from the study.

2.5 | Immunophenotyping

Routine CBCs were performed by the Clinical Pathology Laboratory at the CSU-VTH and included both automated cell counts (Advia 120 Hematology Analyzer, Siemans, Tarrytown, New York) and blood smear evaluation for assessment of cell morphology and a manual white blood cell differential count.

Immunophenotyping by flow cytometry was performed on peripheral blood collected in EDTA tubes with a panel of antibodies used for cellular labelling, as previously described[9] and listed in Table 2. Antibodies included CD45 (pan-leukocyte), CD21 (B cells) and CD5 (pan-T cell). CD45 expression was assessed in the same staining reaction as CD5 and CD21. All data analysis for flow cytometry was performed with Kaluza software (Beckman Coulter). Samples were considered positive for TZ cells if the percentage of TZ cells was greater than 1% of the total population of all lymphocytes (T and B cells). If 1% or fewer of the lymphocytes were TZ cells the dog was considered to be negative for TZ cells and was categorized as such in both the GR and non-GR populations.

2.6 | Clonality testing

The presence of a clonally expanded lymphocyte population was detected by PARR for the TRG as previously described[25] with modifications noted in Table S1 and Figure S1 (Appendix S1). The amount of blood used for each reaction was 200 μL and a result was considered clonal if 1 or 2 peaks were seen in the electropherogram that were greater than ×3 the baseline. Equivocal clonal TRG gene rearrangements were identified when the peak height did not quite reach the ×3 threshold, but was nonetheless prominent.

2.7 | Statistical analysis

nCounter software (NanoString Technologies) was used to carry out the normalization of mRNA levels in the gene expression study. To compare gene expression between cases and controls, a Student's *t* test was performed (Prism 6; Graphpad, La Jolla, California). The gene expression value was reported as the absolute number of transcripts, which was transformed to the signal $\log_2$ ratio (a signal $\log_2$ ratio of 1 is equal to a fold change of 2) to determine the *P* value. To compare the prevalence of TZ cells in the peripheral blood of GRs vs non-GRs and the percentage of GRs (with and without TZ cells) with a TRG clonal result, a Pearson's Chi-squared test was performed. The difference in CD21 expression between TZ cells and normal T cells in the same dog was calculated using a paired Wilcoxon test of the log median fluorescence intensity. The scatter size of each population was compared using a paired *t* test. Differences in breed, sex and haematological characteristics were analyzed using Pearson's Chi-squared tests (Prism 6). Median age between groups was compared using the Mann-Whitney test. Statistical significance was defined as $P < .05$.

TABLE 3 Expression of CD45 and CD21 in T zone lymphoma (TZL) cases and controls

	Cases (median[a])	IQR	Controls (median)	IQR	log_2-fold change	*P* value case vs control
CD45a	168.8	129.3-491.3	6113.0	4840-7476	−5.2	<.0001
CD45b	124.3	32.75-1165	43190.0	40 740-48 800	−8.4	<.0001
CD21	814.7	460-1051	83.5	53.7-300.9	3.3	<.0001

Abbreviation: IQR, interquartile range.

[a] Normalized mRNA counts.

3 | RESULTS

3.1 | Gene expression of CD45 and CD21

We first wanted to validate the observation that TZ cells downregulate CD45 and upregulate CD21 by measuring mRNA levels, since expression of these two proteins has been described as unique features of TZL.[9,14,19,20] Forty-eight samples (14 sorted T cell controls and 34 TZL cases) from lymph node, peripheral blood and thymus (controls only), were analyzed for mRNA counts using NanoString technology.

The results showed that the mean log_2-transformed CD45 mRNA counts in TZ cells was 5.2- to 8.4-fold (log_2-fold change) lower than in controls ($P < .0001$, Table 3) depending on the isoform detected. CD21 mRNA counts were more variable among TZL cases, but the median value was 3.3-fold higher than CD21 mRNA counts in controls (Table 3) ($P < .0001$). These findings indicate that mRNA levels reflect protein expression, and verify that the antibodies used for these studies are recognizing the appropriate corresponding proteins. Furthermore, the results support previous findings that no variant of CD45 is expressed by the TZ cells.

3.2 | Frequency of TZ cells in senior GRs and non-GRs purebred dogs

We enumerated TZ cells in 242 peripheral blood samples from GRs. Seventy-four of these dogs (30.6%) had variable numbers of TZ cells in their peripheral blood. This was significantly higher than the percentage of non-GRs with TZ cells in the peripheral blood ($P = .03$). The percentage of TZ cells varied from 2% to 95% of all T cells, median = 18.1% (Figure 3). The median fluorescence intensity (MFI) of CD21 in TZ cells (Median MFI = 7.3) compared to normal T cells in the same dog (Median MFI = 0.37) was significantly higher (median difference = 7.0, $P < .0001$) (Figure 4). In GRs, the size of TZ cells (median size = 361) was also significantly larger than normal T cells (median size = 342) with a median difference of 15.21 ($P = .0003$).

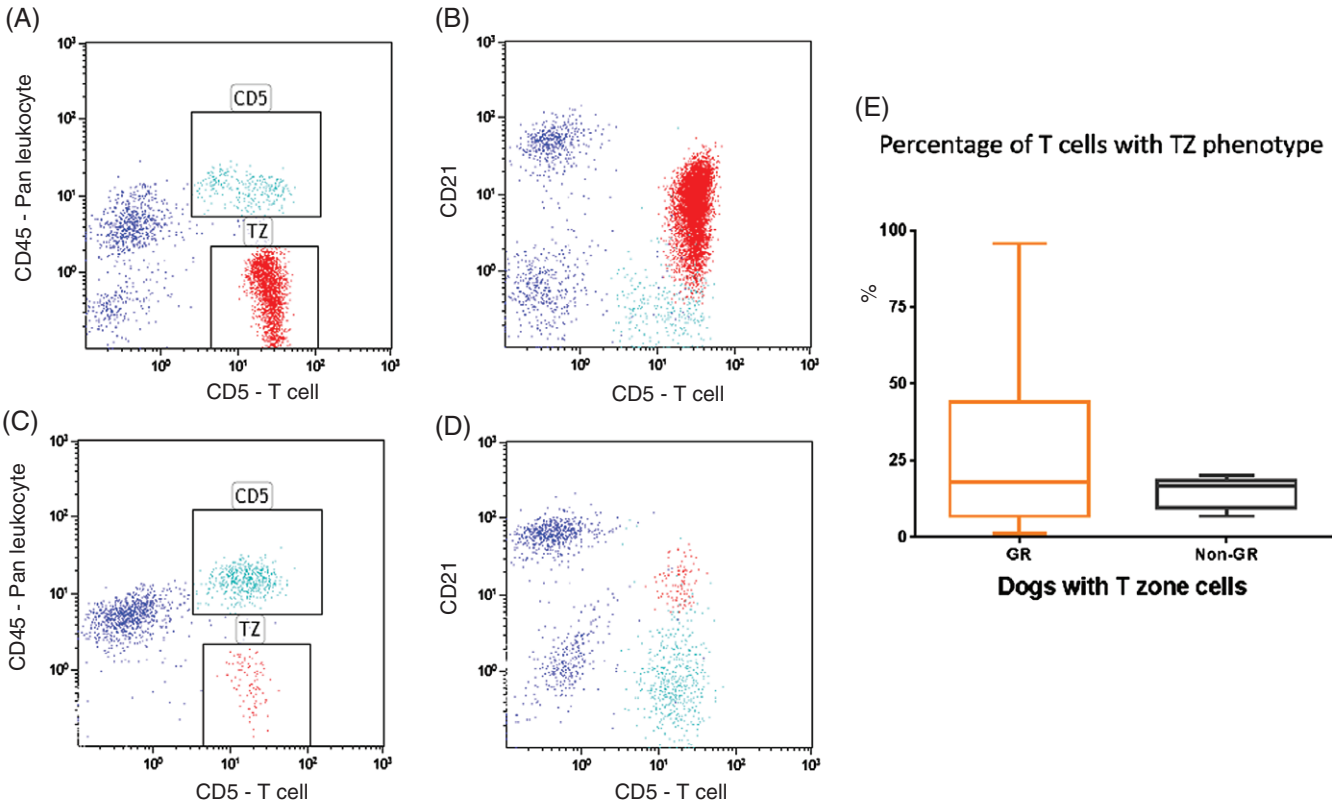

FIGURE 3 A and B show T zone (TZ) cells in a healthy Golden retriever (GR). In this sample, the number of TZ cells represented 95% of the total T cell population, the highest percentage of TZ cells detected in non-lymphoma bearing GRs. C and D show TZ cells in a non-Golden retriever (non-GR), the highest percentage TZ cells detected in the blood of non-GRs. In this sample, the number of TZ cells represented 20% of the total T cell population. E shows the range of TZ cells in GRs ($n = 74$, median = 18.1%) vs non-GRs ($n = 6$, median = 17.4%). The whiskers are set at the minimum and maximum value

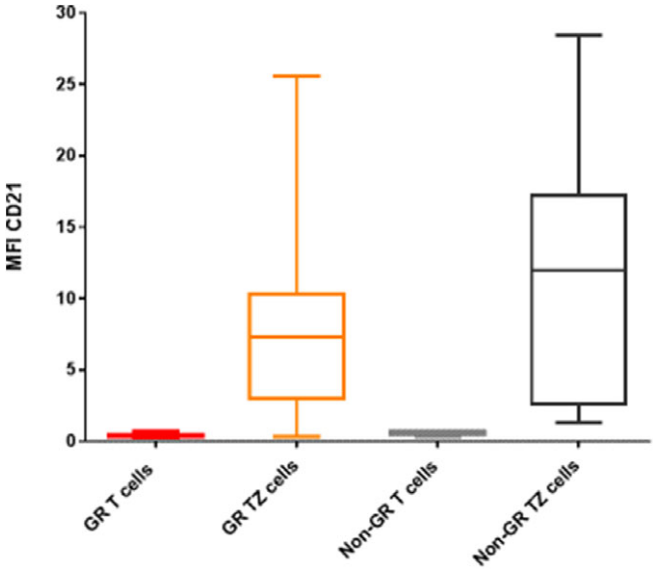

FIGURE 4 The median fluoresce intensity (MFI) of CD21 in CD45+ T cells (normal T cells) vs CD45− T zone (TZ) cells in Golden retrievers (GRs) and non-Golden retrievers (non-GRs). In both the GR and non-GR samples, TZ cells express higher levels of CD21 than the normal T cells. The whiskers are set at the minimum and maximum value

Of the 42 non-GRs, there were 6 (14%) dogs with small numbers of TZ cells identified by flow cytometry and 36 dogs without TZ cells. Among the 6 dogs with TZ cells, the proportion of TZ cells ranged from 6% to 20% of all T cells, median = 17.4% (Figure 3). In dogs with TZ cells, the MFI of CD21 in TZ cells (Median MFI = 0.55) compared with normal T cells (Median MFI = 12) was significantly higher (median difference = 11.5, $P < .03$) (Figure 4). There was no significant difference in the size of TZ cells vs normal T cells in non-GR dogs. There was no significant difference in age, sex, presence of anaemia or presence of thrombocytopenia between the dogs with TZ cells and the dogs without TZ cells in either the GR group or the non-GR group.

3.3 | Clonality assessment

PARR was performed in 67 of the 74 cases of GRs with TZ cells (Table 4). Twenty-three (34.3%) of the cases had clonal TRG gene rearrangements, 1 dog (1.5%) had an equivocal result and 43 (64.2%) were polyclonal. Additionally, 32 (20%) of 157 GRs with no evidence of TZ cells in peripheral blood had a clonal TRG gene rearrangement. The percentage of GRs with TZ cells with a clonal TRG gene rearrangement was significantly higher than the percentage of GRs without TZ cells with a clonal TRG result ($P = .026$).

All 6 non-GRs with TZ cells had PARR performed, and all had polyclonal TRG gene rearrangements, demonstrating no evidence for clonality (Table 4). The PARR assay was performed on 34 of the non-GRs without TZ cells and 2 had a clonal TRG gene rearrangement. The clonal TRG gene rearrangements in these dogs may have represented an emerging T cell lymphoma/leukaemia although this cannot be confirmed because both dogs were euthanized shortly after the sample was obtained due to progressive transitional cell tumour of the bladder in one dog and progressive immune-mediated haemolytic anaemia in the other.

3.4 | Follow-up studies of GRs with TZ cells

Sequential peripheral blood samples were available for 50 dogs within the entire population of GRs. Twenty-two of these dogs had TZ cells in the first sample and all these dogs also had TZ cells in the follow-up sample. Twenty-eight dogs had no TZ cells in the first sample. Of these, 12 had TZ cells in the follow-up sample and the remaining 16 did not. At the time of this manuscript preparation, none of these dogs have developed evidence of clinical disease, in particular lymphocytosis or peripheral lymphadenopathy.

PARR was performed on 45 of these samples and the results did not change for 42 of the dogs. In 3 dogs, however, the PARR assay changed from a polyclonal to clonal result in the follow-up sample. These 3 dogs had TZ cells in their blood on both occasions.

4 | DISCUSSION

In this study, we have found that the loss of CD45 antigen expression and the increased expression of CD21 by TZ cells is reflected in the expression of the relevant genes. These findings indicate that the abnormal antigen expression in TZL is not a function of conformational changes in the antigen, or in the case of CD45, differentially spliced protein that is not detected by the antibody used in the analysis and support recent findings from another group.[14] The expression of CD21 in TZL in our study is in concordance with previously identified CD21 expression by flow cytometry,[9,19] and increased CD21 (CR2) gene expression identified in dogs with TZL using the Affymetrix Canine_2.0 gene chip.[20] In our study, the isolation methods for TZL cases differed from the isolation of normal T cells because of the limitations imposed by the use of samples submitted for diagnostic purposes. However, the purity and quality of RNA was similar for

TABLE 4 PARR results for GR and non-GR with and without TZ cells in the blood

	GR with TZ cells		GR without TZ cells		Non-GR with TZ cells		Non-GR without TZ cells	
PARR results	***n* = 67**	**%**	***n* = 157**	**%**	***n* = 6**	**%**	***n* = 34**	**%**
Clonal TRG gene rearrangement	23	34.3	32	20.4	0	0.0	2	5.9
Equivocal clonal TRG gene rearrangement	1	1.5	1	0.6	0	0.0	0	0.0
Polyclonal TRG gene rearrangement	43	64.2	124	79.0	6	100.0	32	94.1

Abbreviations: GR, Golden retriever; non-GR, non-Golden retriever; PARR, PCR for antigen receptor rearrangement; TRG, T cell receptor gamma; TZ, T zone.

n indicates the number of samples available for the PARR assay. The percentage is the number of samples with that result out of the total samples with a PARR result.

both cell types and our results are supportive of other investigations into phenotypic abnormalities of TZL.[9,14,19,20] These findings further characterize these aberrancies as occurring at both the protein and mRNA level, and support the characterization of this particular subtype of lymphoma in dogs by immunophenotyping. We have demonstrated that 31% of GRs with no evidence of lymphoma/leukaemia have circulating TZ cells. These cells can be identified in 14% of older dogs of other breeds. These proportions reflect the distribution of clinical TZL, where GRs represent almost 40% of all cases.[9] A minority of the TZ cell positive GRs (34%) had clonally rearranged TRG genes. Additionally, 20% of GRs with no evidence of TZ cells in the peripheral blood had a clonal rearrangement of TRG genes. PARR may be a more sensitive test than flow cytometry when there is only a minor clonal population within a sample. The clonal T cells in this study are suspected to be the TZ cells but since they were not sorted we cannot rule-out a separate emerging T cell lymphoma/leukaemia or a reactive clonal population. We favour the hypothesis that the clonal T cells in these dogs may represent a pre-neoplastic state, similar to pre-neoplastic B and T cell disorders in people: monoclonal gammopathy of undetermined significance (MGUS), monoclonal B lymphocytosis (MBL) and T cell clonopathy of unknown significance (TCUS).

MGUS is an asymptomatic plasma cell dyscrasia with low but measurable risk of progressing to multiple myeloma (MM).[26,27] MBL is a monoclonal gammopathy with no evidence of lymphoproliferative disease, but the cells have a chronic lymphocytic leukaemia (CLL) immunophenotype with rare progression to CLL (1%-2% per year).[28] Genetic risk factors have been identified in both of these disorders and they are thought to represent a pre-neoplastic state.[26,28,29] TCUS may represent a similar process for T cells, but it is difficult to equate the process in people with what we have described here in dogs, because T cell clonality in people is not defined by DNA-based clonality assessment but by expansion of a family of T cells that use the same Vb gene, but that do not have identical TCRs.[30]

In GRs, genetic risk factors may also contribute to increased frequency of TZ cells as well as progression to overt lymphoma similar to these human syndromes. If the above hypothesis is correct, we might expect to see progression to TZL in a subset of the dogs that carry these cells. Although we only have follow-up on a small portion of these GRs, we did not see significant expansion of TZ cells in any dog. When multiple samples of peripheral blood were available, TZ cells were generally mildly increased and none of these dogs showed progression of disease, such as lymphocytosis or lymphadenopathy. In MGUS, patients are monitored throughout their life as the risk of transformation is considered to be life-long. The risk for development of MM in MGUS is only 1% per year.[26] MBL with a low count (<500 clonal B cells/μL)[31] can be identified in about 5% of adults over 40 years with rare progression to CLL,[28] and in one study, no individuals with a low-count MBL showed progression to lymphoid neoplasia with a median follow-up of 34 months.[32] If there was a similar risk of development of TZL in dogs with TZ cells, a much larger cohort needs to be identified and followed for a longer period of time in order to determine the risk. It is also possible that there is an environmental factor that contributes to disease development that has yet to be identified. Recently, it was reported that diagnosis of TZL in GRs showed differential geographic distribution suggesting a possible environmental risk factor.[33]

The ability to recognize potential pre-neoplastic states provides a system in which stepwise progression to neoplastic transformation can be investigated. Additionally, identifying diagnostics to recognize pre-neoplastic states in dogs may aid in early diagnosis of more aggressive subtypes of lymphoma. There has been limited research looking at early disease detection markers in dogs with only one study applying clinical screening studies in neoplasia.[34] In this study, blood ferritin levels showed promise as a biomarker for early evidence of disseminated histiocytic sarcoma in Bernese Mountain dogs.[34] Determining the significance of TZ cells in GRs and possible environmental factors or disease states which lead to persistence of T cell infiltration and eventual neoplastic development is ongoing. Further development and identification of novel pre-neoplastic markers would be extremely valuable since many purebred dogs may have genetic components contributing to higher incidence of certain types of cancer.

The presence of clonal T cells in the blood of older GRs, and a smaller number of non-GRs should be considered when interpreting the results of clonality assays. First, a positive T cell clonality test without supporting clinical data should not be taken to indicate neoplasia and the assay should not be used as a screening test. Second, the presence of a clonal TRG gene rearrangement in B cell neoplasms reported by some laboratories[35–37] should not necessarily be interpreted as aberrant rearrangements; they may in fact reflect the presence of TZ cells, which would not be identified without flow cytometry.

In conclusion, TZ cells were identified in greater than 30% of GRs, the breed with the highest number of dogs to develop TZL. These cells are readily identified by flow cytometry and aberrant antigen expression is confirmed at the gene expression level. We have found that a higher percentage of GRs without lymphoma have TZ cells in their blood when compared with other purebreds, and this reflects the frequency with which GRs develop TZL. Additionally, the lack of clinical signs or progression to disease in these dogs suggests that this is still a pre-neoplastic state despite evidence of clonal TRG gene rearrangement. Given these findings, clonal TRG gene rearrangements should be interpreted with caution in non-clinical older dogs, particularly GRs. Continued prospective analysis of GRs to determine the biological significance of TZ cells and possible identification of other genetic and environmental factors involved in the development of TZL is ongoing.

Conflict of interest

The authors declare no potential conflict of interests.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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