**Canine T zone lymphoma: unique immunophenotypic features, outcome and population characteristics**

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Canine T-zone lymphoma: unique immunophenotypic features, outcome and population characteristics

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Abbreviations: TZL, T-zone lymphoma; PTCL-NOS, peripheral T-cell lymphoma not otherwise specified; TCL, T cell lymphoma; FNAC, fine needle aspiration cytology; FC, flow cytometry;
**Work performed:** Manuscript preparation performed at Colorado State University and University of Minnesota. Clinical cases acquired at clinics throughout the United States.
Abstract

Background: Canine T cell lymphoma (TCL) is clinically and histologically heterogeneous with some forms, such as T-zone lymphoma (TZL), having an indolent course. Immunophenotyping is an important tool in the classification of TCL in people, and can be equally useful in dogs. Hypothesis/Objectives: We hypothesized that loss of expression of the CD45 antigen is a specific diagnostic feature of TZL. Animals: 20 dogs with concurrent histology and immunophenotyping by flow cytometry were studied in depth. An additional 494 dogs diagnosed by immunophenotyping were used to characterize the population of dogs with this disease. Methods: Lymph node biopsies from 35 dogs with TCL were classified by 2 pathologists using WHO criteria. 20 lymph nodes were from dogs with CD45- TCL and 15 were from CD45+ TCL. The pathologists were blinded to the flow cytometry findings. Outcome information was sought for the 20 dogs with CD45- lymphoma, and population characteristics of the additional 494 dogs were described. Results: All 20 CD45- cases were classified as TZL. The 15 CD45+ cases were classified as aggressive TCL and are described in an accompanying paper. TZL cases had a median survival of 637 days. Examination of 494 additional dogs diagnosed with TZL by immunophenotyping demonstrated that 40% of cases are in Golden Retrievers, are diagnosed at a median age of 10 years, and the majority have lymphadenopathy and lymphocytosis. Conclusions: TZL has unique immunophenotypic features that can be used for diagnosis.
Human lymphoproliferative diseases are a heterogeneous group of disorders comprised of over 50 subtypes. It is essential to distinguish among the different forms because each sub-type has discrete risk factors, epidemiologic characteristics and outcomes. For example, there are several types of mature T cell lymphomas. One form, angioimmunoblastic T cell lymphoma (AITL), is uniquely associated with hyperglobulinemia and immunological dysregulation. This basis for this association recently was explained by the discovery that this tumor arises from follicular helper T cells – T cells whose function is to provide help for B cell proliferation, isotype switching and somatic hypermutation. Survival with this disease is less than 5 years. In contrast with AITL, lymphoblastic T cell lymphoma/leukemia (T-LBL), is derived from immature T cells, and although it is clinically aggressive, unlike AITL it is curable. AITL is seen almost exclusively in adults, whereas T-LBL is seen in both children and adults. A large number of other subtypes of T cell lymphoma, including peripheral T cell lymphoma, adult T cell leukemia/lymphoma and anaplastic large cell lymphoma all have unique diagnostic features, outcomes and treatments. Contemporary classification of human lymphoma utilizes the World Health Organization scheme, which includes morphologic, genetic and immunophenotypic characteristics.

A recent study validated the reproducibility of this system for the successful classification of lymphoma in dogs, demonstrating that a large group of veterinary pathologists without specific expertise in hematopathology could reach consensus on a series of over 200 lymphomas. This study described the histologic features of 3 aggressive lymphomas in dogs as well as 3 subtypes of indolent lymphomas. The indolent lymphomas were marginal zone lymphoma and follicular lymphoma (both B cell diseases) and a single T-cell disease called T-zone lymphoma (TZL). Human TZL is a morphologic variant of peripheral T-cell lymphoma not otherwise specified (PTCL-NOS) characterized by clonal expansion of T-zone lymphocytes that manifest a unique architectural and cytomorphic pattern. Although the true
incidence of canine TZL is unknown, 2 publications suggest it is relatively common, comprising between 15.5 and 62% of all canine indolent lymphomas.\(^7,8\)

Although there are very few studies addressing the clinical outcomes of histologically-defined subsets of canine lymphoproliferative diseases, those that are available illustrate the clinical utility of classification.\(^9,10\) Ponce et. al.\(^10\), demonstrated that a small clear cell variant of TCL (n=5), which was most analogous to TZL, has a prolonged survival (median overall survival, 21 months), whereas other histologic forms of TCL had a significantly shorter survival, with the lowest being plasmacytoid TCL (median survival, 3 months).\(^10,11\) Similarly in a recent, larger study \(^8\), Flood-Knapik et. al. demonstrated a 33 month overall survival for 37 cases of TZL. These descriptions of a biologically indolent variant of canine TCL stand in stark contrast to the more commonly reported 6 month survival time for grouped canine TCL and serve as a powerful reminder of the importance of lymphoma classification.\(^12,13\)

Many, and perhaps most, cases of lymphoma in dogs are diagnosed by fine needle aspiration cytology (FNAC), in large part due to the greater expense and invasiveness of biopsy and histopathology. Unfortunately, there is no data evaluating canine FNAC samples for their utility in subclassification by the current WHO algorithm. However, FNAC samples are amenable to immunophenotyping, either by flow cytometry (FC) or immunocytochemistry. Thus, if histologic subtypes of lymphoma could be accurately identified by FC immunophenotyping using a constellation of surface markers, vital diagnostic and prognostic information could be obtained without the need for a surgical procedure. Moreover, in light of recent reports indicating that dogs with indolent lymphoma, including TZL, are likely to undergo multiple lymph node aspirates yielding either inconclusive or erroneous results, demonstration of the use of FC in the diagnosis of TZL could result in a more rapid, less invasive primary diagnostic tool.\(^5,8\)
Multiparameter FC immunophenotyping is used routinely in the diagnosis of human lymphoproliferative disease and is increasingly being used in veterinary medicine to provide important diagnostic and prognostic information. Most of the entities in the WHO classification scheme require the detection of multiple cell surface proteins. For example, chronic lymphocytic leukemia/small cell lymphoma can be distinguished from mantle cell lymphoma (both B cell diseases) by the levels of expression of a series of proteins including CD5, CD10, CD20, CD22 and CD23.\textsuperscript{14}

The current study was initiated because of observations made during an investigation of CD4 TCL. This investigation is described in the accompanying paper\textsuperscript{15}, in which we found that although most CD4+ TCL have poor outcome, a subset of these dogs has an indolent clinical course. All 6 indolent lymphomas were CD45+, whereas the aggressive cases were all CD45+. Because of the indolent clinical course we hypothesized that these cases were TZL. The objective of the study described here was to determine if this novel immunophenotypic characteristic could be used as a tool for the diagnosis of canine TZL.

Methods

Case Selection

The Colorado State University Clinical Immunology (CSU-CI) database contained records for 5508 unique dogs which had been immunophenotyped (using blood or lymph node samples) because of suspicion for lymphoma or leukemia between 1-1-06 and 12-1-12. Signalment, clinical signs, imaging studies and cytology and histology results for every dog are entered into a searchable database using information provided by the submitting clinic. Immunophenotyping results are entered into the database in both quantitative and qualitative forms. Quantitative data includes percentages of different subsets and percentages of cells with aberrant phenotypes, and for peripheral blood, all CBC information. Qualitative data is descriptors of the summary flow cytometry diagnosis.
For this study, cases were selected by searching the CSU-CI database for dogs that met the following criteria: 1) blood or lymph node aspirate analyzed by FC, 2) presence of a CD45− T cell population (of any subtype, CD4, CD8 or negative for both antigens) which comprised >30% of all T cells present. For a population to be characterized as CD45−, there had to be a comparator population present that was clearly CD45+. Cases were not included if CD45 expression represented a continuum from CD45 low to high (an example is shown in Figure 1).

This search yielded 514 unique dogs over the 7-year period. For the majority of cases, only cytology results were available, but there were 20 cases with concurrent histology. We obtained slides or blocks from 13 of these cases for review by pathologists (EJE, DMS). For the remaining 7 cases, a histology report explicitly stating the diagnosis according to the WHO classification was available. Although these cases were not reviewed by either EJE or DMS, they were evaluated by a board-certified veterinary pathologist. Given its unique immunophenotypic and architectural pattern, the diagnosis of TZL on excisional lymph node samples generally is considered to be straightforward and well-accepted among pathologists who use the WHO classification scheme.

The flow cytometry and histology controls for this study consisted of 15 cases of CD45+ TCL for which histology also was available. These dogs are described in the accompanying manuscript.

**Diagnosis of TZL**

Histology from cases CD45− TCL (13 cases) and all cases of CD45+ TCL (15 cases), were reviewed by 2 board-certified veterinary pathologists (DMS and EJE). At the time of review, the pathologists were blinded to FC classification, but were aware that all samples were T cell phenotype. Histology and
immunohistochemistry were available for all cases. To make their diagnosis, the pathologists used
previously described histologic and immunohistologic criteria and arrived at a consensus diagnosis.

Flow cytometry

Blood collected in EDTA and lymph node samples were prepared as described previously. Samples
shipped to the laboratory were sent overnight on ice, and kept refrigerated until analysis. Samples were
received by the laboratory and analyzed within 72 hours of being obtained from the dog. For peripheral
blood samples, 400 μl of blood was lysed using 1 ml of lysis buffer (0.15 M NH₄Cl, 1 M KHO₃, 0.1 mM
Na₂EDTA, 1 N HCL at a pH of 7.2-7.4) for 5 minutes at room temperature. Lymph node aspirates were
obtained by the submitting clinics by aspirating material from the node into a solution of saline and 10%
sodium. This suspension was centrifuged and resuspended in 1 ml of lysis buffer for 5 minutes. Samples subsequently were centrifuged, lysed a second time and re-suspended in 200 μl of phosphate buffered saline (PBS)-2% fetal bovine serum (FBS). A 96-well plate
was used in which 25 μl of cell suspension was added to individual wells plus 25 μl of a cocktail of
antibodies. Samples were incubated for 15 minutes at room temperature and then washed twice.
Samples then were resuspended in PBS-2% FBS with 10 μg/ml of propidium iodide for dead cell
exclusion, and analyzed within 1 hour. Nine of the 20 samples were stained with a panel of antibodies
listed in Table 1 and acquired on a Coulter XL flow cytometer (panel 1). When the single laser Coulter XL
was replaced with a 3 laser Coulter Gallios, we were able to extend our panel to examine up to 6
antigens at 1 time. Therefore, the subsequent 11 samples were stained with the combination of
antibodies listed in Table 1, panel 2.

All data analysis was carried out with Kaluza software (Beckman Coulter). CD45 expression was assessed
in the same staining reaction as CD3 (panel 1) or CD5 (panel 2), but was not assessed in the same
reaction as the T cell subset antigens CD4 and CD8. Nonetheless, the subset antigens expressed by the CD45- population could be unambiguously assigned in all cases because of the substantial expansion of that subset, and the correlation in size when populations were backgated to the forward scatter vs. side scatter histogram.

Immunohistochemistry

For immunophenotyping of tissue samples, 5 μm thick sections from formalin-fixed, paraffin-embedded tissues were cut and immunostained utilizing antibodies directed against the CD3 antigen to stain T cells (clone LN10, Leica Bond) or Pax5 antigen to stain B cells (clone DAK-Pax5, Dako). Deparaffinization, antigen retrieval, immunohistochemistry (IHC) staining, and counterstaining was performed on the Bond maX Automated Staining System® using the Bond Polymer Detection System®. Antigen retrieval was accomplished on line using Bone Epitope Retrieval Solution 2 (EDTA based, pH 9.0 solution) using a 30 minute incubation.

Statistical analysis

The differences in expression of class II MHC and CD21 between neoplastic and non-neoplastic T cells in the same dog were compared by subtracting the log median fluorescence intensity of each population, and analyzing the difference using the Wilcoxon signed-rank test. The difference in CD25 expression was analyzed the same way, but the percent positive cells was used instead of the median fluorescence intensity. The difference in size was compared by using the ratio of the linear forward scatter value of the neoplastic T cells to the non-neoplastic T cells, and applying the Wilcoxon signed-rank test to those ratios.
Differences in breed, sex, and lymphocyte subset distribution between groups of dogs were analyzed using a chi-square test, except as otherwise noted in Tables 2 and 3. Median age between groups was compared using the Mann-Whitney test.

Results

Case characteristics

Twenty dogs had a disease with CD45- phenotype and had both flow cytometry and histology results. The median age was 9.9 years (range 5.3 – 11.8). Golden Retrievers comprised 45% of all the cases (Table 2). The majority of dogs were still alive at the time the study was conducted, but a median survival of 637 days was determined using the product limit method of Kaplan Meier. Two dogs were mildly anemic (11% of the 18 dogs for which this information was available); none were hypercalcemic (0/12 dogs for which this information was available). The median lymphocyte count for the 15 dogs for which that information was available was 5046 (a range of 1000 to 23000 cells/μl).

A cytologic description of blood or lymph node aspirates performed by a board-certified veterinary clinical pathologist was available for 12 cases. Although cytologic description of the cells varied considerably, none of the cases included a description of large granular lymphocytes (a cytologic feature only noted in T cell leukemia).

Treatments varied, and included no treatment, prednisone only, prednisone and chlorambucil, and CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). Because of the small number of dogs and wide variety of treatments, we did not attempt to correlate treatment with outcome.

Flow cytometric characteristics
Immunophenotyping was carried out on either blood (n=10), lymph node aspirates (n=8) or both (n=2).

In cases where both sites were analyzed, the phenotype was identical. In addition, 2 cases were analyzed twice, with an interval of approximately 6 months, and the phenotype remained consistent.

Nine of the cases had a CD8+ phenotype, 3 had a CD4+ phenotype, and 8 consisted of cells that were negative for both CD4 and CD8 (Table 2). Although we were not able to perform flow cytometry on the lymph nodes of all 20 dogs, several pieces of evidence support using peripheral blood to phenotype these lymphomas. All 20 cases were given an unequivocal histologic diagnosis of T cell lymphoma in the lymph node. The 12 cases where flow cytometry was performed on the node exhibited consistent phenotypic features, including loss of CD45 expression. In the remaining 8 cases, cells with the identical CD45- phenotype were found circulating in the blood. The loss of antigen expression is considered a hallmark of T cell neoplasia. Thus, these 8 cases had evidence of neoplastic cells in their blood (by flow cytometry) and lymph nodes (by histology). It is unlikely that the circulating cells represented a different neoplastic process from the one identified by histology in the lymph node.

Cases were chosen based on the presence of CD45- T cells. Aberrant antigen expression, including loss of CD45, is used routinely to identify neoplastic cells in human T cell malignancies. Based on this precedent, we assumed that the CD45- cells in our dogs were the neoplastic population. Therefore, we sought to compare expression of a variety of other antigens on these cells to the CD45+ T cells in the same dog. Of the 20 included in this study, 11 were analyzed with the multicolor panel (panel 2, Table 1) so that we were able to directly compare several features of CD45- and CD45+ T cells in the same dog. We found that when compared with CD45+ cells in the same dog, the neoplastic CD45- cells: 1) exhibited higher levels of CD21, class II MHC and CD25, and 2) were larger. Examples of each of these features are shown in Figures 1A and B, and a summary of the data is shown in Figure 1C. These same characteristics could be inferred indirectly from the samples analyzed with the 2 color panel (panel 1),
but data from those dogs is not included in the figure. In almost all cases (12/13), virtually 100% of the
CD45- cells expressed CD25, the interleukin 2 receptor.

Histologic and immunohistologic features of canine TZL

An unbiased, consensus assessment by 2 pathologists (EJE, DMS) indicated that all 13 CD45- cases
reviewed were TZL, whereas none of the 15, CD45+, TCL were classified as such. The CD45+ cases were
classified as either peripheral T cell lymphoma or lymphoblastic T cell lymphoma and are described
separately. Furthermore, board-certified anatomic pathologists at reference laboratories
unambiguously identified the remaining 7 cases as TZL, and none of these pathologists were aware of
the phenotyping, because the biopsy result accompanied the sample submitted for phenotyping.

All 13 TZL cases available for review demonstrated a well-defined pattern of architectural and
cytomorphologic findings typical of dogs with this entity. Specifically, samples demonstrated a diffuse
pattern of cellular proliferation with extensive thinning of the perinodal capsule without involvement of
the perinodal adipose tissue. Most characteristic of the T-zone origin of the proliferative population was
the extensive peripheral compression of remnant fading follicles (Figure 2A). Closer inspection of the
neoplastic population disclosed small cells (nuclei 1.0 to 1.5x the size of a red blood cell) with a
moderate amount of lightly-stained cytoplasm. Cell nuclei were oval to elliptical with sharp, shallow
indentations (Figure 2B) and contained finely granular, evenly dispersed chromatin. Nucleoli and mitotic
figures were not apparent. Immunohistochemically, the proliferative cell population expressed strong,
widespread CD3 immunoreactivity whereas most of the residual, non-neoplastic follicular cells were
Pax5 immunoreactive (Figure 2, C-D).

Population characteristics of CD45 negative T cell neoplasia
We hypothesized that the 20 cases we report are representative of all cases of CD45- T cell lymphoproliferative disease with regard to clinical outcome and histologic subtype. To verify this hypothesis, clinical follow up and histologic assessment should be carried out prospectively on a larger number of dogs, but some comparisons between the 20 cases described here and the larger population of CD45- cases can be made with currently available data. Thus selected characteristics of the 20 dogs in this study were compared to the 494 dogs in the Clinical Immunology database that were described as having CD45- T cells as the dominant population. Golden Retrievers and Shih tsu dogs were analyzed as 2 discrete breed categories because in our extended population, as well as that described by Flood-Knapik, these were the 2 most common breeds presenting with T zone lymphoma.

Table 2 shows that with regard to predominant breed, the percentage of female dogs, and the distribution of T cell subsets, the 20 dogs in the current study are no different than the larger group of animals with the same phenotype, although we acknowledge that with sample size disparity, our ability to detect a difference between the 2 populations is low. The dogs in this study were slightly younger (median age, 9.9 vs 10.6) and less likely to have lymphocytosis. Hypercalcemia was not noted in any of the 20 dogs in this study. The presence or absence of hypercalcemia was definitively indicated in 157 dogs in the larger group of cases, and hypercalcemia was present in 5% of these dogs (the difference between these 2 populations was not statistically significant when analyzed a using chi-square test).

Approximately 15% of dogs that present with circulating T cells exhibiting the CD45- phenotype are reported to have no lymphadenopathy (Table 2). We sought to determine if there are differences between dogs that present with and without lymphadenopathy. The rationale for this comparison is the tendency in veterinary medicine to classify dogs into “lymphoma” or “leukemia” categories, depending on the main site of involvement, and we hypothesize that such a distinction may not be relevant in this
disease. Therefore, we compared the population characteristics of dogs for which definitive information about lymph node involvement (clearly marked as “present” or “absent” by the submitting veterinarian) was provided with the submission (242 cases, Table 3). There was no statistical difference in the median age of presentation between dogs with and without lymph node involvement, nor were there statistically significant differences in the breed distribution, sex or T cell subset distribution (Table 3).

Discussion

Our study demonstrated that 100% of 20 cases of T cell neoplasia characterized by loss of CD45 expression were histologically defined as TZL, and none of the CD45+ cases were given this histologic diagnosis. The group of dogs in our study resembles dogs in a previous report of TZL \(^8\) in that Golden Retrievers were the dominant breed, 50% of the dogs had lymphocytosis, and the disease followed an indolent course. Furthermore, an earlier report by Valli et al.\(^7\) demonstrated similar prolonged survival in 10 dogs with TZL. These parallels demonstrate good consistency in the histologic definition of TZL by different pathologists.

This study advances our ability to recognize canine TZL by describing consistent immunophenotypic features that can be used to identify this disease by FC. The characteristic CD45- T cells are readily identified with 2 color flow cytometry, and knowledge of this phenotype can help resolve cases where the distinction between lymphoid hyperplasia and lymphoma is difficult to make histologically. The high levels of expression of CD21 and class II MHC, which are components of many flow cytometry panels, can further establish the TZL phenotype. Our findings closely mirror a recent study \(^21\) in which cases described cytologically as “small clear cell” have a consistent CD45-, CD21 high phenotype. The small clear cell cytologic appearance is thought to indicate T zone lymphoma, although histology was not available in this study. An earlier study however \(^22\) suggested that a subset of cases defined as “small
clear cell” by histology, lacked expression of CD45. Taken together, these studies, carried out by 2 different institutions using different flow cytometry panels, indicate consistent identification of this constellation of antigen expression. To avoid potentially misclassifying such tumors as B cell in origin by flow cytometry it is important to be aware that a subset of T cell lymphomas expresses high levels of CD21. The immunophenotype of this T cell disorder contrasts sharply with the immunophenotype of more aggressive CD4 TCL described in 15, making the distinction between these 2 types of T cell lymphoma straightforward.

The disease described in this population of dogs is called “T zone” because of its histologic similarity to the human disease of the same name. Histologically, human TZL is characterized by infiltration of affected tissues with a uniform population of small- to medium-sized cells with an abundant volume of clear cytoplasm which expands existing T-zones. Morphologically, the canine cases presented here mimicked their human counterpart and, moreover, they mimicked previous reports of canine TZL. 7 However despite these morphologic similarities, we recognize the hazards of applying a well-defined, human classification scheme developed over many years of experience to a much less well-characterized disease in dogs, and that the 2 diseases may not be comparable. In people, TZL is not a distinct classification, but a rare variant of a broader category of TCL called PTCL-NOS (peripheral T cell lymphoma – not otherwise specified) 1, which includes a number of other entities without a distinct category.

In human medicine, TZL comprises only 1.5% of all cases of PTCL-NOS. 23 Although we do not have histologically-confirmed incidence or prevalence data in dogs, 10% of all suspected lymphoproliferative disorders submitted to the Clinical Immunology service for immunophenotyping had the characteristic features of TZL (T cell, loss of CD45). Two other publications indicate that TZL is relatively common,
comprising between 15.5 and 62% of all canine indolent lymphomas, which themselves comprise up to
29% of all canine lymphomas. The very limited data available suggests that the overall survival for TZL
in people (14 months, 24, 20 – 30 months) is similar to what is seen in dogs (21 months, this study, and
33 months ). Owing to their shorter natural life span, although this outcome in people is considered
poor, in dogs it is considered good.

The neoplastic T cells exhibit aberrant antigen expression in that they do not express CD45. There does
not appear to be a normal, CD45- T cell counterpart described in mice or people, and in the course of
immunophenotyping canine lymphomas and leukemias, we have not seen evidence for CD45- T cells in
the blood or lymph nodes in normal dogs or in reactive lymph nodes. Thus, it seems likely that loss of
CD45 is an event related to neoplastic transformation of these cells, but we do not know if it plays a role
in this process, or if it is an epiphenomenon related to other changes. Preliminary data provided no
evidence of loss of the telomeric end of chromosome 7, where the CD45 gene is located (M. Breen and
S. Culver, personal communication).

CD45 is a tyrosine phosphatase with a complex role in the regulation of signaling through the T cell
receptor, and in the regulation of cytokine receptor activation. It is a heavily glycosylated protein that
is recognized by the carbohydrate binding protein gal-1, a member of the galectin family. One possible
mechanism linking the absence of CD45 with TCL is that binding of surface CD45 by galectin in both
immature (thymic) and activated T cells induces apoptosis. The loss of CD45 expression may allow T
cells to escape deletion in the thymus or to evade apoptotic signals in the periphery leading to eventual
neoplastic transformation. The role of CD45 in T cell signaling and apoptosis is extraordinarily complex
however, and any number of additional mechanisms may be proposed.
The CD45- T cells have many features of activated T cells. First, they express higher levels of class II MHC, a characteristic of activated human T cells. In the dog, T cells express class II MHC constitutively but an increase in the level of expression may be seen with antigen activation. Second, the T cells express high levels of CD25 when compared to non-neoplastic T cells in the same sample. CD25 is the alpha chain of the interleukin 2 receptor, and is expressed on activated effector CD4 and CD8 T cells as well as regulatory T cells. The heterogeneity in phenotype (CD4 and CD8 expression) suggests that the tumor cells are more likely to have arisen from activated effectors than regulatory T cells, but gene expression profiling and functional studies would be necessary to draw conclusions regarding the true lineage of these cells. Finally, the T cells express high levels of CD21. CD21 is a complement receptor, a receptor for Epstein-Barr virus, and a receptor for interferon alpha. In mice, CD21 is expressed at low levels on naïve T cells, and is upregulated significantly on memory T cells. Taken together this constellation of antigen expression suggests that these neoplastic T cells may arise from an activated precursor T cell. Additional studies focused on the origin of these cells will be very useful in identifying potential triggers for neoplastic transformation.

In this study, 53% of dogs with TZL presented with a lymphocytosis at the time of diagnosis, which is comparable to the data reported by Flood-Knapik, who reported lymphocytosis in 47.5% of their cases. Additionally, the median absolute lymphocyte count between the 2 reports (7753 lymphocytes/μL vs. 9212 lymphocytes/μL) are similar. Surprisingly, 100% of the dogs with histologically-confirmed TZL in which peripheral blood was available for FC analysis (n=12) had neoplastic cells in the peripheral blood (detected by the presence of CD45- T cells) despite the fact that nearly half had a normal absolute lymphocyte count. While there may be some academic debate regarding the nomenclature ascribed to the question of whether to call a disease stage V lymphoma with peripheral blood involvement or
leukemia, these findings emphasize the importance of not using peripheral blood count as a screening
tool for the absence of neoplastic cells.

Dogs that present without evidence of lymph node involvement have the same epidemiologic
characteristics as those that do. Thus, we hypothesize that both types of presentation (primary disease
in the lymph node and primary disease in the blood) are manifestations of a single disease entity.

Definitive demonstration of this would require: 1) Full clinical staging, including bone marrow
examination and follow-up on both types of cases and, 2) More detailed molecular description of the T
cells involved using gene expression profiling to establish that these cells have a common origin.

Nonetheless, the fact that both groups of patients have T cells with an identical, aberrant phenotype
and share epidemiologic characteristics suggests that further pursuing this idea would be worthwhile. A
similar situation is seen in human B cell chronic lymphocytic leukemia/small lymphocytic lymphoma,
which is now considered a single entity regardless of the major site of involvement. At least 1 group of
veterinary pathologists has proposed merging B cell CLL and small cell lymphoma in dogs.33

One remarkable finding of our population study was that Golden Retrievers comprise almost half of the
cases of TZL. This observation points to a strong genetic risk factor for this disease. The unique
immunophenotype of the neoplastic cells makes it feasible to recognize even small numbers of these
cells in the peripheral blood, and it is likely that the disease could be identified long before it is clinically
apparent. Prospective analysis of healthy Golden Retrievers as they age could help to determine if early
diagnosis is possible, and would provide a powerful model system in which to follow progressive
changes in neoplastic cells from their earliest detection.
When viewed with the accompanying report it is clear that T cell lymphoproliferative disease is heterogeneous, and thus determining only if a dog has T cell disease (using clonality studies or immunocytochemistry) without further characterization by histology or flow cytometry can create a misleading clinical picture. We demonstrate here that flow cytometry can be used to accurately identify a common form of indolent T cell lymphoma, providing clinicians with a minimally invasive way of obtaining a specific, clinically relevant diagnosis.

Footnote

a Vision BioSystems, Leica, Bannockburn, IL
b GraphPad Prism, San Diego, CA
Figure legends:

Figure 1. Immunophenotypic features of cells from patients with TZL.  
A. Plot of a lymph node aspirate demonstrating the CD45+ (purple) and CD45- T cells (green).  
The cells in red are B cells (as determined by expression of CD21 but not CD5, plot not shown) and are shown for comparison of CD21 expression.  
B. Representative plots of cell size, CD21 expression, class II MHC expression and CD25 expression on CD45- and CD45+ T cells in the same patient using color gates described in A.  
The histograms scaled so that populations with different cell numbers can be compared, so the Y axis does not have units.  
B cell expression of CD21 is shown (red), but not B cell expression of class II MHC or CD25.  
C. Summary data for all cases assessed with the multicolor panel.  
Each plot shows the level of expression of the indicated parameter in CD45+ and CD45- cells determined in the same dog, and the values for an individual dog are joined by lines.  
All parameters are mean fluorescence intensity except for CD25 expression, which is a percentage of positive cells.  
All comparisons between neoplastic and non-neoplastic cells were statistically significant (p < .05) as determined by the Wilcoxon signed-rank test applied to the difference in log median fluorescence (class II MHC and CD21), the difference in percent positive (CD25) or the ratio of the linear forward scatter value (size).

Figure 2. The histologic and immunohistologic features of canine TZL.  
Lymph node tissue is from a 9-year old, Shih-tzu breed with TZL which demonstrates light microscopic (A-B, hematoxylin and eosin (H&E) stained) and immunohistochemical (C-D) features representative of all thirteen cases.  
In A, note the thinned nodal capsule and the compressed remnant fading follicles (black arrows) owing to the eccentric population of proliferating cells.  
Higher magnification view of A reveals a proliferating population of small cells with abundant, clear cytoplasm and oval nuclei with frequent, sharp, shallow indentations (B, white arrows).  
Immunohistochemistry confirms the T-cell phenotype of the proliferating population through uniform, heavy CD3-immunoreactivity (C, brown) and
absent Pax5 immunoreactivity (D, red). However, note the heavy Pax5-immunoreactivity in the residual, compressed follicular B-cells (D, red). E and F show normal lymph nodes stained for Pax5 (E) and CD3 (F) for comparison. Bars: B = 25 μm, C-D = 300 μm.


Table 1. Antibody panels used for immunophenotyping

Panel 1 (two color)*

<table>
<thead>
<tr>
<th>Tube</th>
<th>Antibody specificity and fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>M** IgG1-FITC / CD45-PE€</td>
</tr>
<tr>
<td>3</td>
<td>CD18-FITC / M IgG1-PE</td>
</tr>
<tr>
<td>4</td>
<td>CD4-FITC / CD8-PE</td>
</tr>
<tr>
<td>5</td>
<td>CD5-FITC / CD21-PE</td>
</tr>
<tr>
<td>6</td>
<td>CD3-FITC / CD45-PE</td>
</tr>
<tr>
<td>7</td>
<td>CD4-FITC / CD14-PE</td>
</tr>
<tr>
<td>8</td>
<td>Class II MHC-FITC / CD34-PE</td>
</tr>
</tbody>
</table>

Panel 2 (multicolor)

<table>
<thead>
<tr>
<th>Tube</th>
<th>Antibody specificity and fluorochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M IgG1-FITC / M IgG1-PE / M IgG1-Alexa 647 / M IgG1-Alexa 700 / M IgG1-PE-750 / M IgG1-Pacific Blue</td>
</tr>
<tr>
<td>2</td>
<td>CD3-FITC / CD25-PE / CD5-APC / CD8-Alexa 700 / CD4-Pacific Blue</td>
</tr>
<tr>
<td>3</td>
<td>Class II MHC-FITC / CD22-PE / CD21-Alexa 647</td>
</tr>
<tr>
<td>4</td>
<td>Class II MHC-FITC / CD34-PE / CD5-APC – CD14-PE-Alexa 750</td>
</tr>
<tr>
<td>5</td>
<td>Class II MHC-FITC / CD18-PE / CD5-APC / CD14 PE-Alexa 750 / CD4-Pacific Blue</td>
</tr>
<tr>
<td>6</td>
<td>CD5-FITC / CD45-PE / CD21-Alexa 647</td>
</tr>
</tbody>
</table>

*The first 9 cases in the study were analyzed using this panel, and the remainder were analyzed with the more extensive panel 2.

**Mouse

€ Unless otherwise noted, all antibodies were purchased from AbD Serotec. 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), CD3 = CA17.2A12, CD14 = UCHM (human, used in panel 1) and CD14 = TUK4 (human, used in panel 2), class II MHC = YKIX334.2, CD34 = 1H6, CD25 = P2A10 (purchased from eBiosciences).
Table 2. Comparison of clinical characteristics of dogs in this study with all dogs having the same immunophenotype.

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Dogs in this study (20)</th>
<th>All other dogs (494)</th>
<th>p value for difference between populations*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytosis</td>
<td>53% (8/15) ¤</td>
<td>94% (407/432)</td>
<td>NA ¤</td>
</tr>
<tr>
<td>Lymphadenopathy**</td>
<td>100% (20/20)</td>
<td>76% (168/222)</td>
<td>NA</td>
</tr>
<tr>
<td>CD8 subset</td>
<td>45% (9/20)</td>
<td>33% (162/494)</td>
<td>NA</td>
</tr>
<tr>
<td>CD4 subset</td>
<td>15% (3/20)</td>
<td>16% (81/494)</td>
<td>0.6</td>
</tr>
<tr>
<td>CD4-CD8- subset</td>
<td>40% (8/20)</td>
<td>49% (245/494)</td>
<td></td>
</tr>
<tr>
<td>Golden Retrievers</td>
<td>45% (9/20)</td>
<td>40% (187/471)</td>
<td></td>
</tr>
<tr>
<td>Shih tsu</td>
<td>5% (1/20)</td>
<td>8% (38/471)</td>
<td>0.8</td>
</tr>
<tr>
<td>Other</td>
<td>50% (10/20)</td>
<td>52% (246/471)</td>
<td></td>
</tr>
<tr>
<td>Female#</td>
<td>55% (11/20)</td>
<td>47% (229/487)</td>
<td>0.65</td>
</tr>
<tr>
<td>Age (median)</td>
<td>9.9</td>
<td>10.6</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Statistical tests were: chi-square for comparison of the lymphocyte subset distribution, sex distribution and comparison of breeds, Fisher’s test for comparison of breeds, and t test for median age. NA = not analyzed, since these parameters were used to define the two groups.

** Lymphocytosis is defined as greater than 5000 cells/ul.

¤ The values represent the number of dogs for which the characteristic was present / the total number of dogs for which information about that characteristic was available. For example in this study, information about the presenting lymphocyte count was available for 15 dogs.

€€ NA = not analyzed since these parameters were used to define the population

# Fewer than 1% of dogs in the entire population were listed as non-neutered or unknown. Therefore sex was analyzed simply as male or female.
Table 3. Comparison of dogs with CD45- T cell disease that present with and without lymphadenopathy.

<table>
<thead>
<tr>
<th>Clinical Feature</th>
<th>Dogs with lymphadenopathy (190)</th>
<th>Dogs without lymphadenopathy (52)</th>
<th>p value for difference between populations *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphadenopathy **</td>
<td>100% (190/190) €</td>
<td>0% (0/52)</td>
<td>NA €</td>
</tr>
<tr>
<td>Lymphocytosis</td>
<td>72% (92/124)</td>
<td>100% (52/52)</td>
<td>NA</td>
</tr>
<tr>
<td>CD8 subset</td>
<td>37% (71/190)</td>
<td>23% (12/52)</td>
<td>0.06</td>
</tr>
<tr>
<td>CD4 subset</td>
<td>13% (24/190)</td>
<td>23% (12/52)</td>
<td></td>
</tr>
<tr>
<td>CD4-CD8- subset</td>
<td>49% (93/190)</td>
<td>52% (27/52)</td>
<td></td>
</tr>
<tr>
<td>Golden Retrievers</td>
<td>38% (71/185)</td>
<td>42% (22/52)</td>
<td></td>
</tr>
<tr>
<td>Shih tzu</td>
<td>9% (17/185)</td>
<td>4% (2/52)</td>
<td>0.44</td>
</tr>
<tr>
<td>Other</td>
<td>53% (97/185)</td>
<td>54% (28/52)</td>
<td></td>
</tr>
<tr>
<td>Female #</td>
<td>44% (83/189)</td>
<td>55% (28/51)</td>
<td>0.2</td>
</tr>
<tr>
<td>Age (median)</td>
<td>10.3</td>
<td>11.25</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Footnotes are the same as for Table 2.
Figure 1. Immunophenotypic features of cells from dogs with T-zone lymphoma. A. Plot of a lymph node aspirate demonstrating the CD45+ (purple) and CD45- T cells (green). The cells in red are B cells (as determined by expression of CD21 but not CD5, plot not shown) and are shown for comparison of CD21 expression. B. Representative plots of cell size, CD21 expression, class II MHC expression and CD25 expression on CD45- and CD45+ T cells in the same dog using color gates described in A. The histograms scaled so that populations with different cell numbers can be compared, so the Y axis does not have units. B cell expression of CD21 is shown (red), but not B cell expression of class II MHC or CD25. C. Summary data for all cases assessed with the multicolor panel. Each plot shows the level of expression of the indicated parameter in CD45+ and CD45- cells determined in the same dog, and the values for an individual dog are joined by lines. All parameters are mean fluorescence intensity except for CD25 expression, which is a percentage of positive cells. All comparisons between neoplastic and non-neoplastic cells were statistically significant (p < .05) as determined by the Wilcoxon signed-rank test applied to the difference in log median fluorescence (class II MHC and CD21), the difference in percent positive (CD25) or the ratio of the linear forward scatter value (size).
Figure 2. The histologic and immunohistologic features of canine T-zone lymphoma.
Lymph node tissue is from a 9-year old, Shih-tzu breed canine with canine T-zone lymphoma which demonstrates light microscopic (A-B, hematoxylin and eosin (H&E) stained) and immunohistochemical (C-D) features representative of all thirteen cases. In A, note the thinned nodal capsule and the compressed remnant fading follicles (black arrows) owing to the eccentric population of proliferating cells. Higher magnification view of A reveals a proliferating population of small cells with abundant, clear cytoplasm and oval nuclei with frequent, sharp, shallow indentations (B, white arrows). Immunohistochemistry confirms the T-cell phenotype of the proliferating population through uniform, heavy CD3-immunoreactivity (C, brown) and absent Pax5 immunoreactivity (D, red). However, note the heavy Pax5-immunoreactivity in the residual, compressed follicular B-cells (D, red). E and F show normal lymph nodes stained for Pax5 (E) and CD3 (F) for comparison. Bars: B = 25 μm, C-F = 300 μm.