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Cancer Stem Cell Populations in Lymphoma in Dogs and Impact of Cytotoxic Chemotherapy

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Abstract

Cancer relapse following chemotherapy has been attributed in part to the presence of cancer stem cells (CSC), which drive tumor growth and metastasis and are highly resistant to the effects of cytotoxic chemotherapy. As a result, treatment with cytotoxic chemotherapy selects for drug-resistant CSC populations that eventually drive tumor recurrence. Little is known currently regarding the role of CSC in dogs with lymphoma, nor the impact of chemotherapy on CSC populations. Therefore, we prospectively quantitated CSC populations in dogs with B cell and T cell lymphoma, using tumor aspirates and flow cytometric analysis with a panel of CSC markers. In addition, in vitro studies were done to determine the impact of chemotherapy resistance on the stem cell phenotype and stem cell properties of lymphoma cells. We found that the percentages of tumor cells expressing CSC markers were significantly increased in dogs with B cell lymphoma, compared to B cells from normal lymph nodes. Similar findings were observed in dogs with T cell lymphoma. In vitro studies revealed that lymphoma cells selected for resistance to CHOP chemotherapy had significantly upregulated expression of CSC markers, formed spheroids in culture more readily, and expressed significantly greater aldehyde dehydrogenase activity compared to chemotherapy-sensitive tumor cells. Similar results were observed in tumor samples dogs with relapsed B cell lymphoma. These findings suggest that cytotoxic chemotherapy can lead to a relative enrichment of tumor cells with CSC properties, which may be associated with lymphoma recurrence.

Keywords

dog; immune; tumor; B cell; chemotherapy

Introduction.

Lymphoma accounts for up to 90% of all hematopoietic neoplasms in dogs, with certain breeds showing a higher risk of developing the disease^{1,2}. The tumor is characterized by a clonal expansion of lymphoid cells, and the origin of the malignant cells (B cell versus T cell) is also strongly associated with response to therapy³. Most high-grade canine

lymphomas are of B-cell origin, and usually manifest a higher complete response rate to cytotoxic chemotherapy with longer remission and survival times compared to T cell lymphomas in dogs⁴⁻⁶. Following treatment with standard cytotoxic chemotherapy (typically protocols that include some combination of cyclophosphamide, vincristine, hydroxydoxorubicin, and prednisone⁷), approximately 85% of dogs with B cell lymphoma (BCL) experience complete tumor remission⁶. The duration of remission averages less than 12 months for dogs with BCL, and 6 months for dogs with T cell lymphoma (TCL)⁶. Virtually all treated dogs will relapse and ultimately succumb to the original lymphoma, regardless of treatment protocol^{8,9}. In lymphoma and other hematopoietic neoplasms in humans and in rodent models, tumor relapse and loss of sensitivity to chemotherapy has been attributed to selective expansion and/or enrichment of CSC populations¹⁰⁻¹².

Cancer stem cells are tumor cells that exhibit properties of self-renewal and asymmetric cell division, which allows them to generate both tumor populating cells as well as new CSC^{13,14}. The CSC population is thought to arise from either normal stem cells or from progenitor cells that have undergone multiple mutations^{15,16}. Alternatively, mutations in adult cells may drive cell de-differentiation to become more stem cell-like¹⁷⁻¹⁹. Though the concept of CSC is still not universally accepted, there is compelling evidence from certain models that CSC can drive the growth and recurrence of primary tumors, and lead to tumor metastasis^{13,20,21}. In addition, ablation of CSC has been shown experimentally to lead to the cessation of cancer growth, tumor regression, and decreased relapse of aggressive cancer, consistent with their key role in sustaining the overall tumor cell population²²⁻²⁵. The unusual nature of CSC cell division, and the presence of metabolic pathways that are associated with drug efflux, render these cells relatively resistant to chemotherapy, compared to cancer daughter cells. Therefore, treatment with cytotoxic chemotherapy has been shown to selectively enrich lymphomas and leukemias for CSC, which eventually leads to disease recurrence²⁶⁻²⁸.

Multiple different populations of CSC have been identified in lymphomas in humans and in rodent models, using cell surface expressed molecules and functional properties to identify CSC within the much larger population of differentiated tumor cells²⁹⁻³¹. Cell surface molecules used to define CSC in human lymphoma include CD20⁺/CD27⁺ B cells for Hodgkin's lymphoma and CD45⁺/CD19⁺ cells in mantle cell lymphoma cells^{32,33}. In human acute myeloid leukemia, CD34⁺/CD38⁻ cells were identified as leukemia stem cells³⁴, and one study found evidence suggesting that CD34⁺/CD90⁻ cells were the source of leukemia stem cells³⁵. However, another group reported that CD90 expression was linked to poor prognosis and high risk disease³⁶, and CD90 has been identified as a marker of CSC in other tumor types^{37,38}. Other markers used to identify leukemia stem cells include CD96, CD123, CD47, CD44, and CLL-1³⁹⁻⁴³.

A widely-accepted *vitro* assay to identify CSC *in vitro* is their ability to form tumor spheres under non-adherent and serum-free or low serum cell culture conditions^{44,45}. The cells that form tumor spheres display CSC properties including resistance to chemotherapy and increased aldehyde dehydrogenase (ALDH) activity^{32,46}. The ALDH family of enzymes is responsible for detoxifying cells, metabolizing chemotherapeutic drugs, and for retinoic acid signaling to maintain the CSC phenotype⁴⁷⁻⁴⁹. For Hodgkin's lymphoma, a circulating

population of CD20⁺/CD27⁺ B cells with high levels of ALDH activity was found to be the source of Hodgkin and Reed-Sternberg cells³².

The goal of the present study was to characterize CSC populations in canine B and T cell lymphoma using a combination of surface marker expression and functional assays. We previously defined CSC populations in dogs with melanoma and osteosarcoma, but a similar analysis has not been done for dogs with lymphoma⁵⁰. The current study leveraged many of the CSC properties defined in human lymphoma to evaluate canine lymphoma CSC. Our study was performed using flow cytometry and tumor aspirates from lymph nodes of 13 normal dogs, 44 dogs with untreated lymphoma, and 11 dogs with relapsed lymphoma following cytotoxic chemotherapy. The results of these studies indicated that CSC are present in relatively low numbers in dogs with BCL and TCL, and that CSC are enriched by cytotoxic chemotherapy. The presence of CSC subpopulations in canine lymphoma suggest that CSC could be considered an attractive target for selective targeted therapeutics.

Materials & Methods.

Study dogs.

Lymph node fine needle aspirates (FNA) and biopsies were obtained from dogs diagnosed with BCL or TCL by oncologists at the Veterinary Specialty & Emergency Hospital in Englewood, CO or the Flint Animal Cancer Center at the James Voss Veterinary Teaching Hospital at Colorado State University. Diagnosis of lymphoma was done by flow cytometry by the Clinical Immunology Laboratory at Colorado State University. Tumor relapsed dogs were defined as animals that initially experience complete remission following cytotoxic chemotherapy, and then later developed disease recurrence. All dogs were treated with either standard CHOP therapy protocols or with Tanovea (VetDC, Ft Collins, CO). Lymph node FNAs from healthy dogs were obtained from dogs owned by hospital staff, and the animals were deemed normal based on a normal physical exam and normal blood work conducted in the last year. All animal studies were approved by the Institutional Animal Care and Use Committee, and the Clinical Review Board, at Colorado State University.

The normal dog population included 8 females and 5 males. Ages for normal dogs ranged from 3 to 12 years, with the average age being 8 years. The BCL patient population included 20 untreated females, 3 relapsed females, 15 untreated males, and 6 relapsed males. The TCL patient population included 2 untreated females, 7 untreated males, 1 relapsed female, and 1 relapsed male. Ages for untreated lymphoma dogs ranged from 3 to 14 years with the average age being 9 years, and for relapsed lymphoma dogs from 5 to 13 years with the average age being 9 years. The mean age difference between normal, untreated lymphoma, and relapsed lymphoma was not statistically significant, Supplementary Figure 1.

Collection and processing of tumor samples.

Fine needle aspirates were obtained from the lymph nodes of 13 healthy dogs and from the affected lymph nodes of 35 dogs with untreated BCL, 9 dogs with untreated TCL, 9 dogs with relapsed BCL, and 2 dogs with relapsed TCL. Lymph node biopsies were obtained from 3 healthy dogs and from 3 dogs with untreated BCL. Samples were placed in tissue

culture medium (described below) at 4C and were processed by analysis after overnight storage. Tissue sampling procedures were kept consistent for all samples and were obtained by the same hospital personnel.

Lymphoma cell lines.

The canine lymphoma cell lines CLBL1 (BCL⁵¹), 1771 (BCL⁵²), and Oswald (TCL⁵³) were maintained in MEM medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO) and 5% CTM (10,000 ug/mL Pen/Strep, 200 mM L-glutamine, 10 mM essential amino acids without L-glutamine, 10 mM non-essential amino acids, and 7.5% bicarbonate solution (all from Gibco)⁵⁴. These cell lines were validated as canine and screened to be genetically unique by PCR⁵⁵.

Antibodies for flow cytometry and immunohistochemistry.

Antibodies used to quantitate expression of CSC markers included CD29 (clone Hmb1-1), CD34 (clone 1H6), CD90 (clone YK1X337.217), CD117 (clone ACK2), Sca1 (clone D7), CD133 (clone 13A4), and Oct3/4 (EM92) all obtained from eBioscience (San Diego, CA). Isotype matched, irrelevant antibodies were used at the same concentrations. To identify lymphocyte populations, B cell were identified as CD21⁺ (clone CA2.1D6) and T cells were identified as CD5⁺ (clone YKIX322.3) from Bio-Rad Laboratories (Hercules, CA), as described previously⁵⁶.

Flow cytometry.

Samples were prepared for flow cytometry as described previously⁵⁶. Briefly, fine needle aspirates were incubated with ammonium-chloride-potassium (ACK) lysis buffer (0.5% Phenol Red solution, 8% NH₄Cl, 1% KHCO₃, 0.037% Na₂EDTA in distilled water) to lyse red blood cells prior to incubation with 10% normal dog serum (Jackson ImmunoResearch) to minimize non-specific binding. The samples were then immunostained with antibodies diluted in fluorescence-activated cell sorting (FACS) buffer (1% BSA in PBS with 0.05% sodium azide). To exclude dead cells from analysis, 7-AAD viability dye (eBioscience) was added prior to analysis, and 7-AAD⁺ cells were not analyzed. Cells were analyzed for fluorescence expression using a Beckman Coulter Gallios flow cytometer (Brea, CA). Data were analyzed using FlowJo Software (Ashland, OR).

Generation of chemotherapy-resistant canine cell lines.

Chemotherapy-resistant cell lines were generated by culturing tumor cells in the presence of a mixture of 3 of the 4 cytotoxic chemotherapy drugs (doxorubicin, vincristine, and dexamethasone) which comprise the CHOP cocktail, with the exception of cyclophosphamide^{8,9}. The concentrations of each drug in the mixture was as follows: CLBL1 cells: 845 ng/ml dexamethasone, 3 ng/ml vincristine, and 80 ug/ml dexamethasone and Oswald cells: 30 ng/ml doxorubicin, 125 ng/ml vincristine, and 80 ug/ml dexamethasone). These concentrations were selected based on IC50 values for each drug against the individual lymphoma cell lines, as determined using *in vitro* cell survival assays. Selection of drug-resistant cell lines was done by treating cell lines with chemotherapy-selection drugs, with a minimum of 80% cell death achieved after the first chemo-selection

treatment. Drug selection continued for 14 days until the cells were considered fully resistant.

Immunohistochemistry.

Lymph node biopsies were harvested and fixed in periodate-lysine-paraformaldehyde (PLP) for 24 hours before transferring to a 30% glucose solution for another 24 hours, all at 4C. Afterwards, tissues were embedded in OCT (Optimal Cutting Temperature compound), frozen at -80C, and cryosectioned to a thickness of 5 microns. The tissues were mounted on glass slides and blocked with 5% donkey serum for 10 minutes prior to staining. Tissues were stained with unlabeled CD117 antibody or its matched isotype control for 1 hour at room temperature, followed by donkey-anti-rat FITC for 1 hour at room temperature. The tissues were then permeabilized with 0.025% saponin for 1 hour at room temperature and stained with unlabeled Oct3/4 antibody or its matched isotype control overnight at 4C. This was followed by donkey anti-rat Cy3 for 1 hour at room temperature. Finally, the tissues were stained with DAPI to identify nucleated cells and coverslipped with Prolong Diamond mounting media (LifeTech, Carlsbad, CA). Images were acquired on a confocal microscope and exposure times were set according to each tissue's matched isotype stain. Figures were then compiled using Adobe Photoshop.

Tumor sphere assay.

Matched numbers of untreated and chemotherapy-resistant Oswald cells were plated in low-adherence petri dishes (Falcon, Corning, NY) and maintained in cell culture medium alone or medium with chemotherapy. Images of the cells were then acquired with a light microscope after 3 days.

ALDH assay.

An Aldefluor kit (Stemcell Technologies, Vancouver, Canada) was used to measure ALDH activity in untreated and chemotherapy-resistant lymphoma cells. Verapamil (Sigma-Aldrich) was used to prevent export of the fluorescent Aldefluor reagent, and DEAB (Stemcell Technologies) was added immediately to the negative control wells to quench the ALDH activity. 7-AAD (eBioscience) was added prior to flow cytometric analysis for dead cell exclusion.

Doxorubicin efflux assay.

Efflux of doxorubicin by untreated and chemotherapy-resistant Oswald cells was measured by flow cytometry, as described previously⁵⁷. Briefly, cells were re-suspended in medium alone, medium with 2 mg/ml Doxorubicin, or medium with 2 mg/ml Doxorubicin and 50 ug/ml Verapamil for a positive control of Doxorubicin inside the cells. After 1 hour at 37C, cells were washed with medium alone or medium with Verapamil, allowed to rest for 1.5 hours at 37C, washed a second time, then analyzed by flow cytometry.

Statistical analysis.

Statistical comparisons between those data sets with two sample groups were done using non-parametric t-tests (Mann-Whitney test). Comparisons between 3 or more groups were

done using ANOVA, followed by Tukey multiple means post-test. Analyses were done using Prism7 software (GraphPad, La Jolla, CA) and statistical significance was determined for $p < 0.05$.

Results.

Expression of CSC molecules by healthy and malignant lymphocytes.

Malignant canine lymphocytes have been reported to exhibit a high forward scatter and medium side scatter properties⁵⁸. These criteria were therefore used to identify malignant lymphocytes in dogs with BCL and TCL⁵⁶. The malignant tumor cells were further classified as CD5⁺ T cells or CD21⁺ B cells (Figure 1A), using flow cytometry. Normal T and B cells were defined as CD5⁺ or CD21⁺ cells, respectively, when evaluating CSC populations in lymph nodes of healthy dogs. Tumor-infiltrating lymphocytes were defined as CD5⁺ T cells in tumors of dogs with B cell lymphoma. Expression of CSC markers by the gated populations of malignant B and T cells, as well as by normal B and T cells, was determined by analysis of flow cytometry data and was defined as percentage of CSC⁺ cells per overall cell population.

When expression of CSC markers by malignant B cells in dogs with BCL was compared to expression by healthy B cells in normal lymph nodes, we found that amongst the population of malignant B cells, there was a significantly higher percentage of cells that expressed CSC markers CD34, CD90, CD117, and Oct3/4, compared to the percentages of healthy canine B cells expressing these stem cell markers (Figure 1B and 1C). The greatest percentages of CSC expression by malignant B cells occurred in the CD90, CD117, and Oct3/4 positive populations.

In dogs with TCL, malignant T cells had upregulated intracellular expression of Oct3/4 compared to normal T cells, whereas there was not an overall increase in the percentages of malignant T cells expressing the other CSC molecules evaluated (Figure 1D and 1E). Interestingly, expression of the stem cell associated marker CD90 was downregulated on malignant T cells, compared to healthy T cells.

Thus, within both BCL and TCL, there existed cell subpopulations with phenotypic characteristics consistent with CSC. Notably, the CSC subpopulations found in BCL were very different from those present in TCL, with essentially no overlap. These findings suggest distinct pathways for the origin of CSC in these two types of lymphoma.

Immunohistochemical (IHC) evaluation of CSC populations in tumor tissues.

Lymph node biopsies from 3 normal dogs and from 3 dogs with BCL were evaluated using IHC for expression of CD117 (Figure 2A) and Oct3/4 (Figure 2B). In normal lymph nodes from 3 dogs, expression of neither marker could be detected using IHC. (It should be noted that IHC is generally less sensitive for detection of antibody binding than flow cytometry). However, in tumor tissues of one dog with BCL, CSC expressing CD117 and Oct3/4 could be detected, whereas in the other two tumor samples, expression was not detected using IHC.

Effects of chemotherapy on in vitro CSC selection.

Previous studies have found that the percentages of CSC in certain cancers (breast, colorectal, and lung) is significantly increased following treatment with cytotoxic chemotherapy^{59–61}. This phenomenon is thought to reflect selection for chemotherapy resistant CSC, and the expanded CSC populations are associated with an overall decrease in responsiveness to subsequent chemotherapy^{62,63}.

Studies were designed therefore to determine in vitro whether a similar response occurs in canine lymphoma following exposure to cytotoxic chemotherapy. To address this question, two canine lymphoma cell lines, one B cell (1771) and one T cell (Oswald) were exposed to high combined doses of cytotoxic chemotherapy drugs to select for chemotherapy resistant cells. The CSC phenotype of chemotherapy-resistant BCL and TCL cells was then compared to the phenotype of chemotherapy sensitive cells.

Following chemotherapy selection, there was a significant upregulation of expression of multiple important CSC associated molecules, including CD29, CD34, CD90, Oct3/4, Sca1, and CD133, by chemotherapy resistant BCL cells (1771), compared to unselected cells (Figure 3A–C). The most highly upregulated molecules were CD29 and CD34 in BCL cells. Similarly, upregulated expression of the CSC markers CD29, CD34, CD90, and Sca1 was noted in chemotherapy resistant TCL cells (OSW), with CD90 and CD34 exhibiting the highest degree of upregulation (Figure 3D).

Next, chemotherapy-resistant and -sensitive cells were evaluated for functional changes associated with CSC properties. These functional characteristics included tumor sphere formation in non-adherent culture conditions, doxorubicin efflux activity, and aldehyde dehydrogenase activity. We observed that chemotherapy-resistant Oswald cells formed spheres much more readily in soft agar than chemotherapy-sensitive cells, suggesting a higher self-renewal capacity (Figure 4A)⁴⁵. These chemotherapy resistant Oswald cells also had significantly increased ALDH activity (Figure 4B). The ALDH activity is important because ALDH enzyme is important for detoxifying and metabolizing chemotherapeutic drugs, and maintaining overall cell “stemness”^{47–49}. Chemotherapy selected Oswald cells also effluxed doxorubicin much more efficiently than chemotherapy-sensitive Oswald cells, consistent also with induction of efflux pump expression, another feature of CSC^{64,65}(Figure 4C).

Upregulated ALDH activity and efflux of doxorubicin were also observed in chemotherapy-resistant BCL cells (1771) compared to chemotherapy-sensitive cells. However, unlike the case with Oswald cells, the chemotherapy resistant 1771 cells did not acquire the ability to form spheres in culture (data not shown).

Overall, these results indicate that not only do cytotoxic chemotherapeutics select for upregulated expression of CSC markers, but they also select for TCL and BCL cells with the functional properties of CSC. Thus, these results predict that relapsed lymphoma cells resistant to chemotherapy in dogs following relapse are likely to exhibit accentuated CSC characteristics.

Cancer stem cell marker expression by naïve and relapsed lymphoma.

Indeed, when CSC marker expression was evaluated in dogs with relapsed BCL, and compared to CSC marker expression with dogs with untreated BCL, there were increased numbers of CSC in relapsed dogs (Figure 5A). For example, 33% of tumor cells in relapsed dogs expressed the CSC marker Oct3/4, compared to 15% expression by tumor cells in dogs with naïve BCL. These preliminary findings suggest that examination of larger numbers of animals with relapsed BCL would reveal expanded populations of CSC, relative to treatment naïve tumors. Such a response might elucidate an important mechanism by which canine BCL become resistant to chemotherapy, including different classes of cytotoxic agents.

Discussion.

There are a number of mechanisms invoked to account for lymphoma relapse following chemotherapy, including intrinsic drug resistance, or induced upregulation of drug efflux pumps^{67,68}, drug metabolizing enzymes⁶⁹, and drug-induced DNA damage repair mechanisms⁷⁰. Selective enrichment of CSC has also been invoked as an explanation for tumor relapse in lymphoma, based on evidence from experimental rodent models and human trials^{26,66,71–74}.

The studies reported here are the first to our knowledge to define the various subpopulations of CSC present in canine lymphoma and to compare the CSC subpopulations in BCL versus TCL in dogs. The key findings reported here were that in dogs with BCL, the primary CSC subpopulations identified were CD34⁺, CD90⁺, CD117⁺, and Oct3/4⁺ (Figure 1). In TCL, the most prominent CSC subpopulation was Oct3/4⁺ (Figure 1). Though there is some overlap between normal and malignant B and T cells with respect to CSC marker expression, in general the majority of CSC markers were upregulated by malignant cells.

Cytotoxic chemotherapy was found to strongly select for CSC enrichment by cultured lymphoma cell lines. For example, in chemotherapy resistant canine BLC tumor cell lines, there was strong selection for upregulated expression of multiple CSC markers, including CD29, CD34, CD90, CD117, Oct3/4, Sca1, and CD133. In the case of TCL lines, upregulated expression of the CSC markers CD29, CD34, CD90, and Sca1 was observed (Figure 3).

Moreover, in dogs with relapsed BCL that relapsed following chemotherapy, there was substantial upregulation of CSC populations, compared to treatment naïve tumors (Figure 5). Thus, canine lymphoma appears to respond to chemotherapy in a manner similar to that of human lymphoma, with selective enrichment for CSC.

Previous studies have identified canine BCL cell lines cells with increased expression levels of ABC transporters and genes associated with the CSC phenotype, including ABCG2 and Bmi-1⁷⁵. In addition, function studies of these cell lines have demonstrated the presence of a side-population of cells characteristic of CSC, and have also demonstrated efflux of DNA specific dyes⁷⁵. However, these previous studies have not compared the CSC phenotype of normal and malignant canine B cells and T cells. Nor have previous studies demonstrated

how selection for chemotherapy resistance leads to enrichment of tumor cells with a CSC phenotype and CSC functional properties.

The functional properties of CSC not only contribute to longevity, but they also render these cells more resistant to radiation treatment and chemotherapy drugs, allowing them to evade the effects of cytotoxic chemotherapy^{76,77}. Thus, it would be very desirable to identify drugs or other agents that could selectively target CSC for elimination. The ability to selectively enrich for lymphoma CSC using in vitro chemotherapy selection may provide one tool for identification of agents that more selectively target CSC. Furthermore, the ability to accurately phenotype CSC subpopulations in naive tumor samples using flow cytometry may provide a useful prognostic markers for predicting initial responses to chemotherapy as well as overall remission duration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cancer stem cell marker expression by normal and malignant canine lymph node B and T cells.

Fine needle aspirates were obtained from lymph nodes of 13 normal dogs, 35 dogs with untreated BCL, and 9 dogs with untreated TCL for flow cytometric analysis. The cells were stained for CD21 and CD5 to distinguish between B and T cells, and positive staining for cancer stem cell markers (CD29, CD34, CD90, CD117, Sca1, CD133, and Oct3/4) was determined with respect to cells stained with irrelevant isotype antibody. The data were analyzed using FlowJo Software and the gating strategy is shown in (A). Data shown

represents **(B)** normal lymph node B cells, **(C)** malignant B cells from untreated BCL, **(D)** normal lymph node T cells, and **(E)** malignant T cells from untreated TCL. Data are presented as mean $\pm$ SEM of the percentage of marker expression on lymphocytes.

Figure 2. Expression of CD117 and Oct3/4 by normal and malignant canine lymph nodes. Biopsies were obtained from the lymph nodes of 3 normal dogs and 3 dogs with BCL and prepared for immunofluorescent staining. The tissues were stained with antibodies against CD117 (**A**) and Oct3/4 (**B**), with positive staining determined with respect to isotype antibody-stained tissues for each dog. Positive staining is shown in red, and the cells were counterstained with DAPI for nuclear detection as noted in Methods. Images were acquired with a confocal microscope, and images shown are representative images of one normal dog and one dog with BCL.

Figure 3. Impact of chemotherapy resistance on CSC populations within lymphoma cell lines. Canine BCL cell lines CLBL1 and 1771 as well as the TCL cell line Oswald were selected for chemotherapy resistance using 4 times IC50 doses of doxorubicin, vincristine, and dexamethasone for 2 months. Untreated and chemotherapy-resistant cells were then immunostained for expression of cancer stem cell markers (CD29, CD34, CD90, CD117, Oct3/4, Sca1, and CD133). Cells were also immunostained with an isotype-matched irrelevant antibody. Representative histograms depict geometric mean fluorescence intensity (MFI) plots for 1771 cells (**A**). The percentage positive expression of noted CSC markers is

shown for CLBL1 (**B**), 1771 (**C**), and Oswald cells (**D**). Each experiment was conducted with 3 separately selected chemotherapy-resistant cell lines, with similar results each time.

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Figure 4. Cancer stem cell properties of a chemotherapy-resistant lymphoma cell line. The Oswald line was selected over 2 months for chemotherapy-resistant cells. **A)** Identical numbers of chemotherapy sensitive and resistant cells were plated and images were obtained after 3 days of initial culture to demonstrate colony formation: chemotherapy sensitive cells (left), chemotherapy-resistant cells (right). An aldefluor assay was used to measure ALDH activity by chemotherapy sensitive and resistant cells (**B**) and efflux of doxorubicin was measured in (**C**). Data are presented as mean $\pm$ SEM of the percentage of ALDH⁺ cells in (**B**) and fold change of fluorescence intensity in (**C**). Statistical analysis was performed

using Student's t-test and statistically significant differences were denoted as * = $p < 0.05$ and **** = $p < 0.0001$. These experiments were repeated 3 times, with similar results each time.

Figure 5. Cancer stem cell marker expression by normal lymphocytes, untreated lymphoma cells, and relapsed lymphoma cells.

Fine needle aspirates were collected from lymph nodes of normal dogs and from lymph nodes of dogs with BCL or TCL (untreated and relapsed animals) for flow cytometric analysis of CSC subpopulations. Cells were initially immunostained to identify CD21+ B cells and CD5+ T cells, as shown in Figure 1, Expression of cancer stem cell markers CD29, CD34, CD90, CD117, Oct3/4, Sca1, and CD133 was determined with respect to the level of antibody binding in cells immunostained with irrelevant isotype-matched antibodies. Flow cytometry data were analyzed using FlowJo Software. The data depicted were generated

from 12 healthy dogs, 35 dogs with untreated BCL, 9 dogs with untreated TCL, 9 dogs with relapsed BCL, and 2 dogs with relapsed TCL. (YOU SHOULD REPLOT GRAPH B WITHOUT THE RELAPSED TCL DOGS, SINCE YOU CANT PLOT SD WITH ONLY 2 SAMPLES) Expression of CSC markers by normal and malignant B cells is depicted in (A). In (B), CSC marker expression by normal and malignant T cells is depicted (B). Data are presented as mean $\pm$ SEM of the percentage of CSC marker expression on lymphocytes, and statistical analysis was performed using two-tailed ANOVA (excluding relapsed TCL patients), followed by Tukey's multiple means comparison. Statistically significant differences were denoted as * = $p < 0.05$, ** = $p < 0.005$, and **** = $p < 0.0001$.