DISSERTATION

CLINICAL AND MOLECULAR CHARACTERIZATION OF CANINE SMALL CELL B-CELL LYMPHOCYTOSIS DISORDERS

Submitted by

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ABSTRACT

CLINICAL AND MOLECULAR CHARACTERIZATION OF CANINE SMALL CELL B-CELL LYMPHOCYTOSIS DISORDERS

Canine neoplasms provide an opportunity to study genetic risk, pathogenesis, clinical progression of disease, and novel therapeutics, which may translate to human medicine. Canine B-cell chronic lymphocytic leukemia (BCLL) is a common neoplasm in dogs that is defined by an expansion of small-sized CD21+ B cells in the peripheral blood. This disease has a similar clinical presentation to human chronic lymphocytic leukemia (CLL), and we hypothesize that canine BCLL may be a useful natural animal model for human CLL. The aims of this thesis were to evaluate clinical progression and molecular features of canine BCLL, including immunoglobulin heavy variable region (IGHV) gene mutation status and gene expression profiling. We evaluated breed-specific differences in canine BCLL and compared findings to human CLL. Additionally, we identified a syndrome in English bulldogs characterized by small cell B-cell lymphocytosis, which is distinct from BCLL.

In Chapter 1, we investigated IGHV gene mutation status in canine BCLL. Human CLL is a heterogeneous disease, and molecular subtypes of CLL patients are defined by whether IGHV genes in malignant B cells have undergone somatic hypermutation. These subtypes, termed unmutated IGHV CLL and mutated IGHV CLL have different cellular origins, B-cell activation and clinical outcomes. We sequenced immunoglobulin heavy chain VDJ gene rearrangements in 55 canine patients with BCLL, including 36 non-Boxer and 19 Boxer dogs, 11 Boxers with large B-cell lymphoma, and 6 healthy control dogs (3 Boxers and 3 non-Boxers). The majority of non-Boxer BCLL cases (75%) had mutated IGHV genes, which is associated with favorable prognosis in human CLL. The majority of Boxer BCLL cases (79%) had

unmutated IGHV genes, which is associated with poor prognosis in human CLL. BCLL IGHV gene usage and mutation status were compared to Boxers with large B-cell lymphoma and the normal IGHV repertoire of control dogs. IGHV3-41 was preferentially used in Boxers with BCLL, large B-cell lymphoma and without lymphoproliferative disease. However, preferential use of unmutated IGHV genes was unique to Boxers with BCLL. These results suggest that Boxers with BCLL may be a valuable model to investigate unmutated IGHV CLL, and we hypothesized that Boxers would have more aggressive disease.

In Chapter 2, we examined clinical outcome in canine BCLL. Previous small-scale BCLL outcome studies suggested that BCLL has an indolent clinical course, but there was a wide range in survival times, similar to human CLL. We hypothesized that canine BCLL has a heterogeneous clinical progression and certain clinical or flow cytometric factors are prognostic. We performed a retrospective study, reviewing the medical records for 121 canine BCLL cases from 3 major breed groups: small breed dogs (n=55), due to increased risk of BCLL; Boxers (n=33), due to preferential use of unmutated IGHV genes; and other breeds (n=33), to compare outcome with small breed cases. We examined clinical data, laboratory data, treatment and survival data, and measured cellular proliferation by the percent of Ki67-expressing CD21+ B cells by flow cytometry in a subset of cases. The median overall survival time (MST) for all cases was 300 days, with a wide range in survival times (1-1644 days). Boxers had significantly shorter survival (MST, 178 days) compared to non-Boxers (MST, 423 days), and there was no significant survival difference between small breeds and other non-Boxer breeds. Boxers had significantly higher Ki67 expression than non-Boxers, indicating increased tumor cell proliferation. Cases with high Ki67 (>40% Ki67-expressing B cells) had significantly shorter survival (MST, 173 days) than cases with <40% Ki67 (MST undetermined), irrespective of breed. Additionally, a high presenting lymphocyte count and clinical signs at diagnosis were associated with poor prognosis. In summary, BCLL demonstrated a heterogeneous clinical course, like human CLL, and Boxer dogs and cases with high Ki67 expression had more aggressive disease.

In Chapter 3, we investigated the gene expression profile of canine BCLL, and compared these results to the human CLL gene signature. We measured expression of 327 genes relevant to human CLL by NanoString analysis in BCLL cases (n=26), large B-cell lymphoma cases (n=23) and sorted control lymph node B cells (n=8). We performed RNA Sequencing on 12 BCLL cases and 3 sorted control lymph node B cell samples. By both methods, we found that differentially expressed genes in canine BCLL cases were significantly enriched for human CLL gene signatures. Canine BCLL was not enriched for gene sets from other human B-cell neoplasms, including mantle cell lymphoma, splenic marginal zone lymphoma and follicular lymphoma. Canine BCLL had upregulation of cytokine signaling pathways, KRAS signaling, NF-κB signaling and B-cell receptor signaling pathways. Additionally, we identified two subgroups of BCLL cases by transcriptome analysis: BCLL subgroup 1 and 2. BCLL subgroup 2 had overexpression of cell cycle progression and proliferation genes and BCLL subgroup 1 had overexpression of genes regulated by NF-κB in response to TNF and ribosome and spliceosome genes. BCLL subgroup 2 had significantly higher MKI67 expression and an overrepresentation of Boxers, suggesting this molecular subgroup may have poorer prognosis. These results identified similarities in gene expression profiles between canine BCLL and human CLL and identified two molecular subgroups of BCLL for further study.

In Chapter 4, we investigated a syndrome of small cell B-cell lymphocytosis in English bulldogs. We previously found that this breed disproportionally develops an expansion of small B cells, which was previously interpreted as BCLL. However, English bulldogs had a unique clinical presentation compared to conventional BCLL cases, raising the possibility that these dogs have a different B-cell lymphoproliferative disorder. We examined 84 cases of B-cell lymphocytosis in English bulldogs, assessing clinical presentation, flow cytometry features, B-cell clonality, and immunoglobulin gammopathy patterns. Clonality analysis by the PCR for antigen receptor rearrangement assay determined that 70% of cases had polyclonal or restricted polyclonal immunoglobulin gene rearrangements, suggesting nonmalignant B-cell expansion. Expanded B cells expressed low levels of

class II MHC and CD25. Young male English bulldogs were overrepresented. Splenomegaly was common, and the majority of cases had hyperglobulinemia, characterized by IgA +/- IgM polyclonal or restricted polyclonal gammopathy patterns. We hypothesize that this syndrome, termed polyclonal B-cell lymphocytosis in English bulldogs (PBLEB), has a genetic basis.

In conclusion, this dissertation contributes to our understanding of small cell B-cell lymphocytosis syndromes in dogs. We examined clinical and molecular heterogeneity in canine BCLL, identifying clinical and molecular subgroups of cases, which helps improve management of canine BCLL and lays the foundation to further study pathogenesis in this common canine neoplasm. Additionally, we identified similarities between canine BCLL and human CLL, supporting the hypothesis that this canine population may be a useful translation model for CLL. Finally, we identified a unique B-cell syndrome in English bulldogs, broadening our understanding of the spectrum of small B-cell lymphocytosis diseases seen in dogs.

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INTRODUCTION

Human non-Hodgkin lymphoma

Non-Hodgkin lymphomas (NHLs) are comprised of a heterogeneous group of lymphoid tumors, including all lymphomas not classified as Hodgkin lymphoma. Non-Hodgkin lymphoma accounts for an estimated 4.3% of all new cancer cases in the United States, with 19.6 NHL cases per 100,000 men and women diagnosed per year.[1] Lymphomas are classified by the World Health Organization classification of lymphoid neoplasms, which was revised most recently in 2016.[2,3] This classification system includes more than 50 subtypes of NHL. A combination of histology, immunophenotyping, cytogenetics, and genetic mutation analysis are used to subtype lymphoma and provide prognosis. Proper classification is important as different NHL subtypes have highly variable clinical outcomes and treatment indications. Additionally, as targeted therapies have developed, identification of targetable genetic alterations is increasingly important.

Approximately 90% of NHL cases are of B-cell origin, with T-cell and natural killer cell lymphomas occurring at lower frequencies.[4] The frequency of lymphoma subtypes differs geographically. Diffuse large B-cell lymphoma (DLBCL) is the most common subtype in developed regions (28.9%), followed by follicular lymphoma (25.5%), marginal zone lymphoma of MALT type (8.8%) or nodal/splenic type (3.0%), mantle cell lymphoma (7.8%), and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) (7.0%).[4] Burkitt lymphoma (a B-cell subtype), precursor B- and T-lymphoblastic leukemia/lymphoma, and extranodal natural killer/T-cell lymphoma subtypes are significantly increased in developing regions, but still account for relatively small proportions of cases.[4]

Lymphomas are derived from a clonal expansion of lymphocytes. Neoplastic B cells and T cells typically have clonal immunoglobulin (IG) and T-cell receptor (TCR) gene rearrangements, respectively,

though immature precursor neoplasms may have clonal cross-lineage rearrangements or clonal rearrangements of both IG and TCR loci.[5] Clonality of antigen receptors is determined by evaluating the size of the gene rearrangements. Historically, the immunoglobulin heavy chain (IGH) and T-cell receptor gamma (TRG) loci were evaluated, but more recently clonality assessment has expanded to immunoglobulin light chain kappa (IGK) and lambda (IGL) loci and T-cell receptor beta (TRB).[6] Antigen receptor gene rearrangements are composed of variable (V), joining (J), and in some cases, diversity (D) genes. The IGH and TRB loci form a VDJ rearrangement and IGK, IGL and TRG loci form a VJ rearrangement.[7] The loci contain multiple V, D, and J genes and a recombinase enzyme complex containing RAG1 and RAG2 proteins rearrange individual genes in each lymphocyte to form a V(D)J rearrangement.[8] Nucleotides are then added or subtracted at gene junctions, further adding to the diversity of gene rearrangements between lymphocytes. A polyclonal or reactive population of lymphocytes will have diverse antigen receptor rearrangements, while a clonal or neoplastic population will have identical rearrangements.[9] Evaluating the homogeneity of antigen receptor gene rearrangements in a lymphocyte population by polymerase chain reaction is the foundation of clonality testing.[9] Clonality testing is very useful in supporting a diagnosis of lymphoma, particularly in challenging cases where histology and/or immunophenotyping are not definitive.

B-cell lymphoma: cell of origin

Different lymphoma subtypes are derived from a clonal expansion of lymphocytes at different stages of development. The normal cell counterpart, or cell of origin, has been well-characterized for many B-cell lymphoma subtypes. Development of B cells is characterized and guided by the ordered rearrangement of IG heavy chain and light chain loci to form a functional B-cell receptor (BCR).[7] B-cell precursors in the bone marrow rearrange IG genes and undergo negative selection to remove immature B cells capable of binding self-antigens. B cells migrate to peripheral lymphoid organs, where mature B cells are activated upon binding to foreign antigen. Within the germinal center, B cells expand, undergo

somatic hypermutation to increase antibody affinity for antigen, and undergo class switch recombination to isotype switch from IgM and IgD to IgG, IgA or IgE. Activated B cells give rise to plasma cells, capable of secreting antibody, and memory B cells, which can expand during secondary immune responses. Cellular morphology, immunophenotype, mutation status of IG gene rearrangements (reflecting somatic hypermutation) and gene expression profiling help to identify the cell of origin for lymphoma subtypes.

A number of B-cell lymphomas are derived from germinal center B cells, likely because of the high rate of B-cell proliferation and/or because processes occurring in the germinal center reaction are error-prone. Errors during somatic hypermutation and class switch recombination can allow for IG locus translocations, which bring proto-oncogenes undergo the control of IG enhancers.[10] Follicular lymphomas are derived from B cells with germinal center differentiation, and therefore, express markers normally found in the germinal center, including CD10, BCL6 and HGAL.[11] DLBCL contains two major subgroups, derived from germinal center B cells (GCB type) or activated peripheral B cells (ABC type), which have different prognoses.[12]

The cell of origin for CLL/SLL is still controversial, as there is no known normal B-cell counterpart with the immunophenotype of CLL/SLL cells. CLL has two molecular subtypes with different prognoses, which are defined by whether the immunoglobulin heavy chain variable (IGHV) genes have undergone somatic hypermutation.[13,14] This dichotomy in IGHV mutation status suggests that unmutated IGHV CLL and mutated IGHV CLL have different origins, derived from pre-germinal center B cells and post-germinal center B cells, respectively. Gene expression profiling by Seifert et al. suggested that unmutated CLL and mutated CLL were most similar to CD5+ B cells, which predominantly have unmutated IGHV genes.[15] However, this group identified a small subset of normal CD5+ B cells that express the memory B-cell marker CD27 and have mutated IGHV genes. They concluded that unmutated

CLL is derived from CD5+CD27- B cells and mutated CLL is derived from CD5+CD27+ post–germinal center memory B cells.[15]

B-cell lymphoma pathogenesis

While events such as chromosomal translocations often incite malignant transformation, many B-cell lymphoma subtypes are dependent upon BCR signaling and signals from the microenvironment for tumor cell survival and proliferation.[16] B cells may be stimulated via T-cell dependent or independent mechanisms.[7] T-cell dependent responses require interaction between CD40 ligand on T helper cells with CD40 expressed on B cells, resulting in T cells releasing cytokines that promote B cell proliferation, differentiation and class switching. T-cell independent activation can occur when an antigen binds pattern recognition receptors, such as Toll-like receptors, or when an antigen binds to and stimulates signaling through the BCR. The BCR is composed of surface immunoglobulin molecules and signal transduction cofactors (CD79A and CD79B). Following BCR crosslinking, signals are transmitted through intracellular tyrosine kinases, which activate downstream messengers such as the RAS/MEK/ERK pathway, AKT mTOR pathway, and protein kinase C pathway. Activation alters transcription factors such as NF-κB and MYC, which promote B-cell survival and proliferation. Tumors including CLL and mantle cell lymphoma are dependent on BCR signaling, as evidenced by the efficacy of BCR pathway inhibitors in treating these tumors.[17] Additionally, subgroups of unrelated patients have IG gene repertoire biases and highly similar BCR amino acid sequences.[18,19] These stereotyped BCRs are additional evidence that these neoplasms developed under antigen selection.

In tumors such as follicular lymphoma and CLL, malignant B cells require interactions with the tumor microenvironment to maintain tumor cell survival and proliferation. Follicular lymphoma cells require interaction with T cells and follicular dendritic cells in the germinal center.[20] Follicular lymphoma prognosis is predicted by the gene expression signatures of the non-malignant immune cells in the

tumor, highlighting how cells in the microenvironment can modulate tumor behavior.[21] CLL cells also require signals from the microenvironment in order to proliferate. CLL cells circulating in the peripheral blood have a quiescent phenotype.[22] Proliferation mainly occurs in proliferation centers within the lymph node and bone marrow, which contain CD4+ T cells that can activate CLL cells via CD40-CD40 ligand binding.[23,24] Expanded and highly active proliferation centers are associated with a more aggressive clinical course.[25] Stromal cells in the marrow support CLL cell survival through integrin interaction (VLA-4 and VCAM-1) and secretion of chemokines, such as CXCL12, which interacts with CXCR4 on CLL cells.[26–28] Nurselike cells found in secondary lymphoid organs activate BCR signaling and provide survival signals to CLL cells.[29–31]

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia is the most common leukemia of adults in western countries.[1,32] This neoplasm is defined by an accumulation of CD5+ B cells in the peripheral blood, bone marrow, and secondary lymphoid organs.[3] When lymphadenopathy is present and there are <5,000 B cells/μL in the peripheral blood, patients are diagnosed with small lymphocytic lymphoma (SLL).[33] CLL and SLL are considered different manifestations of the same disease. CLL typically affects older patients, with a median age at diagnosis of 72 years old.[32] Relatives of CLL patients have an increased risk of developing this tumor, suggesting a genetic predisposition.[34] Genome-wide association studies (GWAS) have identified over 20 loci associated with increased risk of CLL, including loci near genes associated with apoptosis.[35,36]

CLL is characterized by an expansion of small-sized lymphocytes with mature-appearing morphology in the blood.[32] CLL is usually diagnosed by immunophenotyping of the peripheral blood, which identifies an expansion of CD19+ CD5+ CD23+ B cells with dim CD20 expression.[37] Cases rarely have lymph node biopsies, except for the minority of cases affected with SLL. Unlike other NHL

subtypes, there is no one chromosomal translocation that defines CLL. The four most common genomic aberrations include: del13q14, resulting in loss of miR-15a and miR16-1, which regulate function of the anti-apoptotic gene BCL2;[38,39] trisomy 12, which affects NFAT signaling involved in cell cycle regulation and apoptosis;[40] del11q22-q23, which affects the tumor suppressor gene ATM involved in DNA damage response;[41,42] and del17p13, which affects the tumor suppressor gene TP53 and is associated with very poor prognosis.[43] CLL has high genetic heterogeneity and a large number of somatic mutations have been described.[44] Some of the most common recurrent mutations affect genes involved in NOTCH signaling (NOTCH1), DNA damage and cell cycle control (ATM, TP53), NF-κB regulation (BIRC3), RNA processing and export (SF3B1, FUBP1), MYC activity (PTPN11), MAPK-ERK signaling (NRAS, KRAS, BRAF), chromatin modification (IKZF3) and inflammatory pathways (MYD88).[32,44] Different genomic aberrations and recurrent somatic mutations are associated with widely different prognoses, accounting for some of the clinical heterogeneity in CLL. A key feature of CLL is the intraclonal heterogeneity of the tumor. Different CLL subpopulations are detected in the same patient, and those subclones will alter over time, accumulating additional mutations that contribute to progression of disease and resistance to therapy.[44,45]

CLL has a remarkably heterogeneous clinical course. Survival times can range from just a few months to decades. Approximately one-third of patients never require therapy and achieve a normal lifespan.[46] A combination of clinical, biological and genetic factors are used to predict prognosis and guide therapy. Early staging systems developed by Rai and Binet, which were solely based on physical exam findings and hematologic data, were highly prognostic.[47,48] These systems identified risk groups based on the degree of tumor cell infiltration in the peripheral blood/bone marrow, lymph nodes, liver and spleen, and the presence or absence of anemia and thrombocytopenia. The IGHV mutation status is prognostic and now routinely performed for CLL patients.[46] Cases with unmutated IGHV genes have a poor prognosis compared to mutated IGHV cases.[13,14] It is hypothesized that unmutated CLL tumors

have polyreactive BCRs, which allow for more antigen stimulation and BCR signaling, leading to progression of disease.[18,49] Additional genetic factors, such as del17p13 or TP53 mutations, NOTCH1 mutations, and SF3B1 and BIRC3 mutations, are associated with poor prognosis.[50] The CLL international prognostic index working group identified 5 independent prognostic factors that stratify patients into 4 risk groups with significantly different survival: TP53 status, IGHV mutational status, β_2-microglobulin concentration, clinical (Rai/Binet) stage, and age.[51]

Optimal treatment guidelines for CLL, including indications to treat, first-line treatment, and treatment for refractory or relapsing patients, continue to evolve.[52] Treatment with alkylating-based chemotherapies were shown to have no benefit in treating CLL and recent studies tracking mutations in CLL subclones over time demonstrate that early chemotherapy can be detrimental, promoting expansion of chemotherapy-resistant clones.[44,45,53] The development of inhibitor molecules, including ibrutinib, idelalisib and venetoclax, has revolutionized CLL treatment and started to replace chemotherapy.[32] Ibrutinib and idelalisib target BTK and PI3Kδ, respectively, to block BCR signaling and proliferation and induce CLL cell apoptosis.[54] Venetoclax blocks the function of the anti-apoptotic protein, BCL2.[55] As the number of novel therapies increases, the challenge comes in determining which drugs should be used in which settings. Many of these drugs are used in combination, and as patients become refractory, clinicians reach for different drugs throughout the course of disease. Determining the optimal combinations of drugs and order of administration in patient groups with different risk is an ongoing effort. Additionally, recent efforts to sequence patients over the course of therapy have revealed the acquisition of mutations and expansion of drug-resistant subclones over time. For example, patients on ibrutinib therapy can develop resistance mutations in the BTK gene, which may be detected before relapse.[56] Further work is needed to determine how to incorporate this sequencing data into practice; for example, whether treatment protocols should be switched in patients that have developed resistance mutations but are still in clinical remission. Dogs with naturally occurring CLL could provide a

useful model for preclinical assessment of novel treatments and study of different drug regimens as first or second line therapy.

Dogs as a natural model for human cancer

The field of comparative oncology studies the comparison of canine and human cancers, to investigate genetic risk, study mechanisms of tumor development, and assess novel therapies.[57–59] There are over 75 million pet dogs in the US, and cancer is the leading cause of death in dogs, accounting for 45% of deaths in dogs over 10 years old in one study.[60,61] This population provides an opportunity to study naturally occurring cancers, to improve human and animal health.

Dogs spontaneously develop many of the same cancer types seen in people, and tumor progression follows a similar course across species. This natural cancer model allows us to study environmental and genetic risk factors for different tumor types. Because dogs and humans usually live in a shared environment, they have similar environmental exposures. The practice of dog breeding to maintain stringent standards has generated purebred breeds with restricted genetic variation, providing an opportunity to study genetic risk.[57] Certain dog breeds have increased risk for different tumor types. For example, specific breeds have increased risk for particular hematopoietic neoplasms, including small breed dogs for B-cell chronic lymphocytic leukemia, Boxers for CD4+ peripheral T-cell lymphoma, Golden retrievers for T-zone lymphoma, and German shepherds for acute myeloid leukemia.[62–66] These high-risk breeds may be investigated through GWAS, to identify risk alleles for specific cancer types. Because of the genetic homogeneity within a purebred breed, these studies may be performed with smaller sample sizes in dogs compared to humans.[67] Genome-wide association studies have identified risk alleles for histiocytic sarcoma in Bernese mountain dogs, squamous cell carcinoma of the digit in standard poodles, osteosarcoma in Rottweilers, greyhounds and Irish wolfhounds, and mast cell tumors in Golden retrievers.[68–71]

Naturally occurring spontaneous cancers in dogs are also useful in studying clinical progression and response and resistance to therapy. Dogs frequently have a similar clinical presentation and disease course compared to people, with significant heterogeneity among patients with the same tumor type. Given their shorter lifespan, it is easier to follow disease progression and assess response to therapies, particularly for indolent cancers, which may progress over decades in humans. Dogs provide an opportunity to study the interactions between tumor cells and the immune system and investigate changes in the tumor microenvironment within the primary tumor and distant metastases. Because similar pathways are often dysregulated in human and canine cancers, dogs provide a valuable natural model to test novel targeted therapies which block or alter these pathways.[72] There is a large system in place for canine cancer clinical trials, making it feasible to test therapies and study acquired resistance to therapies. This data can help inform human studies and ultimately benefit both dogs and people.

Canine lymphoma/leukemia

Lymphoid neoplasms are among the most common tumor types diagnosed in dogs, accounting for 13% of all tumor diagnoses in a more recent study.[73] Fewer subtypes are recognized in dogs compared to people, but several subtypes have been established in both species.[74] In veterinary medicine, lymphoma subtyping is most commonly performed by flow cytometry immunophenotyping and/or histopathology. Many lymphoma subtypes can be identified by flow cytometry alone, while some require a histologic diagnosis. As in human medicine, clonality testing is a very useful adjunct diagnostic tool to support a diagnosis of lymphoid neoplasia. The PCR for antigen receptor rearrangements (PARR) assay is used routinely to assess the clonality of lymphoid expansions.[75]

The frequency of different lymphoma/leukemia subtypes varies between dogs and people (Figure 1). In dogs, B-cell lymphomas are most common, accounting for 64%-80% of hematopoietic neoplasms across large-scale histologic studies.[76,77] Diffuse large B-cell lymphoma is the most common

subtype in both species,[76–78] and recent studies have compared the mutations and dysregulated pathways between human and canine DLBCL.[79–82] Other B-cell neoplasms recognized in dogs include marginal zone lymphoma (nodal and splenic forms), mantle cell lymphoma, follicular cell lymphoma and B-cell chronic lymphocytic leukemia. T-cell lymphomas are more common in dogs than people. Peripheral T-cell lymphoma-not otherwise specified is a rare aggressive neoplasm in people that is common in Boxer dogs, providing a valuable model to study this disease. Other T-cell neoplasms, such as T-zone lymphoma and CD8+ T-cell chronic lymphocytic leukemia, are common in dogs but quite rare in people. Acute leukemias of lymphoid and myeloid origin are aggressive precursor neoplasms recognized in dogs and people.

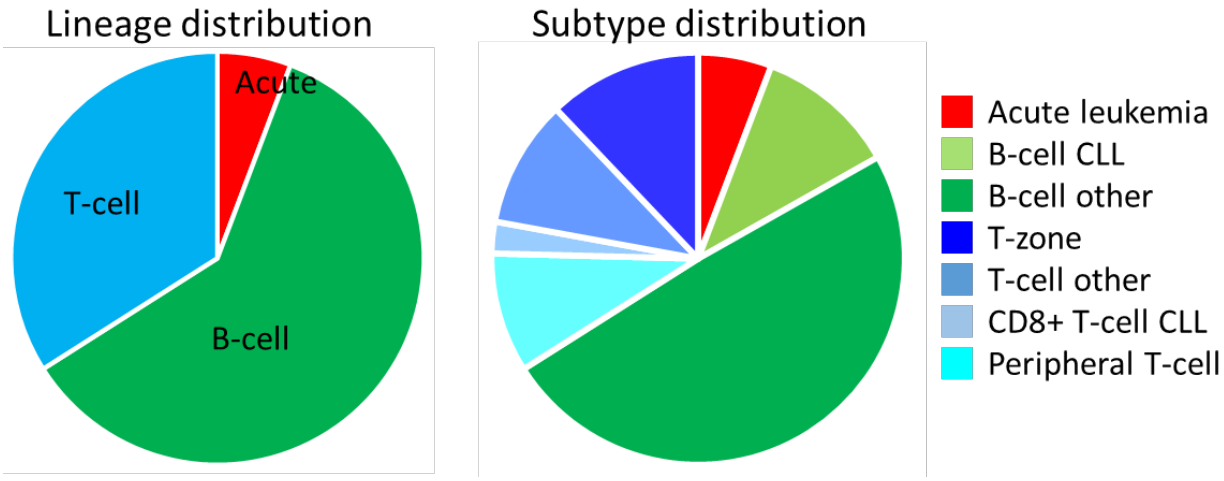

Figure 1. Distribution of canine lymphoproliferative diseases. Data represent lymphoma/leukemia diagnoses from approximately 25,000 unique canine cases submitted to the Colorado State University-Clinical Immunology laboratory. Diagnoses were determined by flow cytometry immunophenotyping. The broad distribution of cases by acute/precursor neoplasm, B-cell lineage or T-cell lineage is presented on the left. The distribution of subtypes is presented on the right. B-cell neoplasms are most common, and B-cell CLL accounts for approximately 10% of all cases. The B-cell other category includes the most common lymphoma subtype, diffuse large B-cell lymphoma. Common canine T-cell subtypes include T-zone lymphoma and peripheral T-cell lymphoma.

Canine CLL can be of B-cell (BCLL) or T-cell lineage. This contrasts with human classification systems, where the historical entity of T-cell CLL has been replaced by T-cell prolymphocytic leukemia

(small-cell variant), and therefore, the term CLL strictly refers to a neoplasm of B cells.[2,83] In dogs, BCLL is one of the most common lymphoid neoplasms and accounts for 10% of canine samples submitted to the Colorado State University-Clinical Immunology laboratory with a suspicion of lymphoproliferative disease (Figure 1).

There are currently no defined consensus criteria for BCLL diagnosis. In general, BCLL is diagnosed by an expansion of small-sized mature-appearing lymphocytes in the bone marrow and/or peripheral blood. Immunophenotyping is performed by flow cytometry or immunocytochemistry/immunohistochemistry to determine B-cell origin. Canine BCLL cells do not express the CD5 antigen like human CLL cells. Some canine BCLL studies have excluded cases with lymphadenopathy and/or splenomegaly, due to concerns that these cases may have a different B-cell lymphoma subtype with extension into the blood (stage V), rather than BCLL, which was historically considered a disease of the blood and bone marrow.[84] However, human CLL/SLL involves secondary lymphoid organs and exclusion of canine cases with lymph node or spleen involvement likely excludes true cases of BCLL. Differentials for B-cell lymphoma subtypes with a leukemic component include CLL/SLL, mantle cell lymphoma, follicular lymphoma and splenic marginal zone lymphoma.[2] The latter three subtypes appear quite rare in dogs according to several large-scale histologic studies.[76–78] Diagnostic criteria for BCLL in our laboratory include: >5,000 lymphocytes/µL in the peripheral blood, a homogeneous expansion (>60%) of CD21+ lymphocytes, and small size by flow cytometry (forward light scatter ratio of B cells to neutrophils <0.60). This definition may include cases of other B-cell lymphoma subtypes, and work is needed to define BCLL criteria and identify markers to discriminate this tumor from other subtypes.

Despite its prevalence, there are only a small number of studies in the veterinary literature describing BCLL. One study from our laboratory evaluated the clinical presentation and breed distribution of BCLL in 491 dogs.[62] BCLL cases had a median age of 11 years at diagnosis, corroborating

other studies that show BCLL generally affects older patients, similar to human CLL.[62,66,84] Approximately half of cases had peripheral lymphadenopathy and splenomegaly, 26% had anemia, and thrombocytopenia was rare. Twelve breeds had significantly increased odds of BCLL compared to mixed breed dogs, and 10/12 were small breeds.[62] When compared to Labrador retrievers, 16 breeds had increased risk and 12/16 were small breeds. The 4 larger breeds were English bulldogs, Boxers, Pit bulls and Dobermans. English bulldogs had a unique clinical presentation, including a younger age at diagnosis (median, 6 years old) and significantly lower class II MHC and CD25 expression on B cells, raising the possibility that bulldogs have a different manifestation of BCLL or a different B-cell disorder.[62]

Two small-scale studies evaluating outcome in 17 and 21 BCLL patients found that BCLL generally has an indolent clinical course.[84,85] However, there was a wide range in survival times in both studies, suggesting that canine BCLL may have a heterogeneous clinical course similar to human CLL. Comazzi et al. found that young age was associated with poor prognosis.[84] A larger scale outcome study is needed to evaluate the spectrum of clinical outcomes in BCLL and assess additional prognostic factors. Additionally, investigation of IGHV mutation status, gene expression profile, and underlying mutations are needed to further characterize canine BCLL and evaluate similarities to human CLL. A previous study assessing copy number change in canine BCLL identified a loss of the region containing miR-15a and miR16-1, similar to the del13q14 aberrancy seen in human CLL.[86] Larger studies are needed to investigate the range of mutations in canine BCLL and evaluate functional effects.

Conclusions

Canine BCLL shares a similar clinical presentation with human CLL, but further work is needed to evaluate the dog as a model for CLL. A canine BCLL model may be useful in studying genetic risk, pathogenesis of disease, novel therapeutics, and acquired resistance to therapy. Genetic risk factors could be investigated in high-risk small breeds. Additionally, human CLL tumor cell stimulation and

proliferation occurs in lymph nodes, which are not routinely sampled in human patients. Dog owners are often highly motivated to participate in clinical trials, and collection of lymph node samples, particularly at different time points, may be more feasible in canine patients. These samples could provide an opportunity to study mechanisms of tumor development and progression in the lymph node microenvironment, and assess changes in subclones and the mutational landscape over time. Finally, as the number of novel therapies for CLL expands, dogs could provide a useful preclinical model to assess response to therapy and evaluate combinations of different agents.

The goals of this thesis were to assess molecular features and outcome in canine BCLL, and compare these findings to human CLL. We investigated the role of IGHV mutation status in canine BCLL, performed a large-scale BCLL outcome study, and performed gene expression profiling. Additionally, we investigated English bulldogs with small cell B-cell lymphocytosis and identified a new syndrome, termed polyclonal B-cell lymphocytosis of English bulldogs (PBLEB). This work advances our understanding of BCLL in dogs, to improve the diagnosis and treatment of canine patients, and helps establish the dog as a model for human CLL.

CHAPTER 1: Preferential use of unmutated immunoglobulin heavy variable region genes in Boxer dogs with B-cell chronic lymphocytic leukemia[1]

Summary

Human chronic lymphocytic leukemia (CLL) is a clinically heterogeneous disease, and immunoglobulin heavy variable region (IGHV) gene mutational status is an important prognostic marker. IGHV mutational status has not been previously examined in canine CLL. We sequenced the IGHV-D-J rearrangements from 55 canine patients with CLL, including 36 non-Boxer and 19 Boxer dogs. The majority of non-Boxers (75%) had mutated IGHV genes, whereas the majority of Boxers (79%) had unmutated IGHV genes. IGHV3-41 and IGHV3-67 gene usage was significantly higher in Boxers with CLL compared to non-Boxers. Additionally, 11 Boxers with large B-cell lymphoma and the normal IGHV repertoire of 6 control dogs (3 Boxers and 3 non-Boxers) were sequenced. IGHV3-41 was preferentially used in Boxers with other forms of lymphoma and without lymphoproliferative disease. However, preferential use of unmutated IGHV genes was unique to Boxers with CLL, suggesting Boxers may be a valuable model to investigate unmutated CLL.

Introduction

Human chronic lymphocytic leukemia (CLL) is the most common leukemia of adults in the Western world.[2,87] The disease has a variable clinical course, with wide ranges in time to progression and survival.[88] Analysis of the immunoglobulin genes has been crucial in understanding CLL pathogenesis and identifying subsets of patients with different clinical courses. Early studies identified restricted immunoglobulin heavy variable region (IGHV) gene usage in CLL compared to normal B cells.[89] Later,

studies demonstrated that the mutational status of the IGHV genes is highly prognostic and divides patients into subsets with different clinical outcomes.[13,14] Patients with mutated IGHV genes have a more favourable clinical course, while patients with unmutated IGHV genes have a poorer prognosis. Subsequently, subsets of unrelated CLL individuals were found to have highly similar to identical B-cell receptor immunoglobulins (stereotyped BCR),[90] which allowed for further stratification of patients and prognostication for certain subsets.

IGHV mutational status continues to be a major prognostic factor in human CLL[51] and more recently was shown to predict response to therapeutic agents.[91] The European Research Initiative on CLL has established standard methods for accurate analysis of mutational status.[92,93] Mutational status is determined by amplifying and sequencing the IGHV region, aligning the sequence to immunoglobulin gene databases, and calculating the percent identity between the case sequence and closest germline IGHV gene. Germline identity >98% is consistent with unmutated CLL, while cases with <98% identity constitute mutated CLL cases.

Canine B-cell chronic lymphocytic leukemia (BCLL) shares many features with human CLL. The disease is characterized by a clonal expansion of small B cells in the peripheral blood. In people, the expanded B-cell population usually co-expresses CD5 and CD23.[33] Canine BCLL cells do not express the CD5 antigen and a CD23 antibody is not available in dogs. However, the clinical presentation and clinical course in dogs appear similar to that seen in human patients. The disease affects older dogs, with a median age at diagnosis ranging from 10-11.9 years.[62,66,84] Lymphadenopathy and splenomegaly are common, affecting approximately 50% of patients.[62] Cytologic review reveals the majority of lymphocytes are small with condensed chromatin and no apparent nucleoli, with fewer yet variable numbers of pro-lymphocytes. Anemia is relatively common, affecting 25-53% of patients across two studies, and thrombocytopenia and neutropenia are rare.[62,84] While it appears that many patients have indolent disease,[84,85] one study found a wide range in survival times (25 to >1000 days).[85]

IGHV gene usage and mutational status have not previously been studied in canine BCLL patients. Bao et al. characterized the canine immunoglobulin heavy chain variable region, identifying 80 IGHV genes, 6 IGHD genes, and 3 IGHJ genes.[94] These gene names have been modified to adhere to the conventions of the international ImMunoGeneTics (IMGT) information system (http://imgt.cines.fr,[95–97]), and the new names are used in this study (personal correspondence from M-P Lefranc; unreferenced). IGHV genes were classified into three subgroups, with 76/80 genes belonging to subgroup IGHV3 (previously VH1). Recently, Martin et al. expanded the canine immunoglobulin locus annotation, describing 83 IGHV genes and 6 IGHJ genes.[98] Three studies have shown that canine IGHV-D-J rearrangements predominantly use IGHV3 subgroup genes,[94,99,100] with IGHV3-38 (previously VH1-44) and IGHV3-19 (previously VH1-62) preferentially used in one study.[94] Heavy chain complementarity-determining region 3 (CDR3) length ranged from 7 to 17 amino acids (AA) in one study,[94] and 5 to 27 AA in another.[99] IGHV gene usage and mutational status were investigated in canine diffuse large B-cell lymphoma, where IGHV3-38 was most frequently used.[101,102]

We investigated IGHV gene usage and mutational status in a cohort of canine BCLL patients, and compared the repertoire to patients with large B-cell lymphoma and to normal B-cells. We hypothesized that canine BCLL patients would have a skewed IGHV gene repertoire and variable mutational status.

Methods

Diagnostic criteria for BCLL cases

BCLL cases were selected from peripheral blood samples submitted to the Colorado State University-Clinical Immunology (CSU-CI) laboratory for flow cytometric immunophenotyping. Flow cytometry was performed as previously described and antibody combinations are listed in Table 1.[65] BCLL cases were defined as having >5,000 lymphocytes/μL, with a homogeneous expansion (>60%) of small lymphocytes expressing the B-cell marker CD21. Antibodies for CD19 and CD20 are not available in the dog but anti-CD21 reliably detects B cells when combined with T-cell antibodies. Intracellular flow

cytometry with CD79A and PAX5 antibodies is also available in the dog to detect B cells, but these antibodies are not used in routine immunophenotyping in our laboratory. B-cell size was classified as small when the ratio of the geometric mean of B-cell to neutrophil forward scatter (FS) was <0.60, which correlates to a B-cell FS value <400 on our flow cytometer. In previous studies, dogs meeting these diagnostic criteria predominantly had an indolent clinical course[85] and clinical characteristics[62] similar to mutated human CLL.

Table 1. Antibody panels used for immunophenotyping. Unless otherwise noted, all antibodies were purchased from Bio-Rad, Hercules, CA. Clones are as follows: CD45, YKIX716.13; CD18, YFC118.3 (human CD18); CD4, YKIX302.9; CD8, YCATE55.9; CD5, YKIX322.3; CD21, CA2.1D6; CD22, RFB4 (human CD22, purchased from AbCam, Cambridge, MA); 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 eBioscience, San Diego, CA).

Tube	Antibody specificity and fluorochrome
Panel 1 (two color)[a]	
1	None
2	M[c] IgG1-FITC/CD45-PE
3	CD18-FITC/M IgG1-PE
4	CD4-FITC/CD8-PE
5	CD5-FITC/CD21-PE
6	CD3-FITC/CD45-PE
7	CD4-FITC/CD14-PE
8	Class II MHC-FITC/CD34-PE
Panel 2 (multicolor)[b]	

1	M IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-Alexa-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]Panel 1 samples were analyzed using a single laser Coulter XL (Beckman Coulter Inc., Brea, CA). [b]Panel 2 samples were analyzed using a 3-laser Coulter Gallios (Beckman Coulter Inc., Brea, CA). [c]M, mouse.

Clonality was confirmed in all cases using a PCR-based assay termed the PCR for antigen receptor rearrangements (PARR) assay,[75,103] which detects clonal immunoglobulin gene rearrangements based on size and is similar to the method used in people.[9]

BCLL cases were selected at random from the CSU-CI database initially, with additional Boxers sequenced after discovering preferential IGHV gene usage in this breed.

Case selection for large B-cell lymphoma and control dog cohorts

Cases with large B-cell lymphoma were identified among lymph node aspirate samples submitted to the CSU-CI laboratory for immunophenotyping. To meet the criteria for large B-cell lymphoma, >60% of the large cells in the sample expressed CD21,[104] and the median FS of the CD21+ lymphocytes was >450. Histopathology was not performed, so further subtyping could not be determined.

Control dogs without evidence of lymphoproliferative disease were identified at necropsy or biopsy and lymph node sections were collected.

Characterization of the canine IGHV locus

In this study, we annotated the 80 germline genomic IGHV genes previously identified [94] using the guidelines established by the IMGT information system (http://imgt.cines.fr,[95–97]). A subset of these annotations are depicted in Figure 2. Functional rearrangements used for analysis had these conserved IMGT AA and motifs, and an open reading frame absent of stop codons or frameshift mutations.

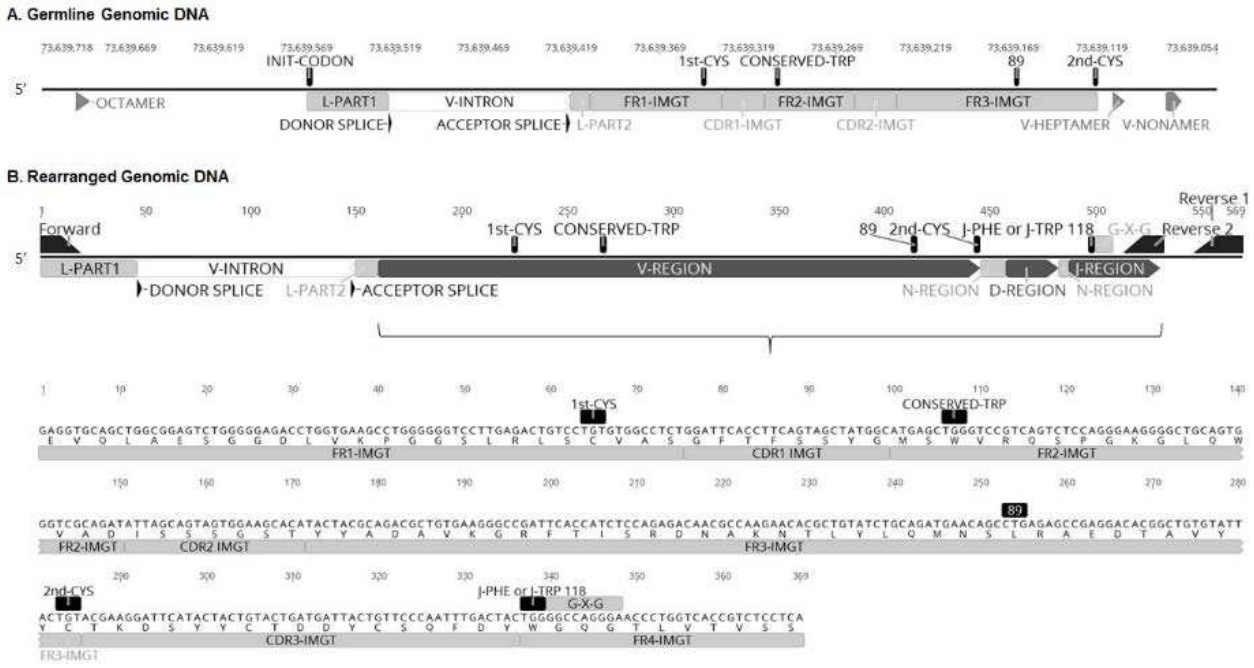

Figure 2. Dog IG V-REGION depictions in germline genomic DNA (A) and rearranged genomic DNA (B). (A) The V-DOMAIN of canine IGHV3-38 is shown as an example. IMGT standardized labels are shown, including framework regions (FR), complementarity determining regions (CDR), and the four conserved positions: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic) and 104 (2nd-CYS). Other labels include: the OCTAMER in the 5'UTR of the V-GENE; INIT-CODON, initiation codon (ATG sequence); L-PART1, first exon of the leader sequence; DONOR and ACCEPTOR SPLICE sites flanking the INTRON; L-PART2, second part of the leader sequence; V-HEPTAMER and V-NONAMER, recombination sites. (B) An IGHV-D-J rearrangement from a BCLL case is shown as an example. The location of the PCR sequencing primers are shown, including the forward primer (used for both amplification protocols) and the reverse primers

for protocol 1 (reverse 1) and protocol 2 (reverse 2) (Table 2). Below, the genomic sequence and amino acid translation are shown for a portion of the rearrangement. The conserved position, 118 (J-PHE or J-TRP), and G-X-G motif of the J-REGION are shown.

Additionally, we identified new IGHV and IGHJ genes compared to previous annotations.[94,98] Contiguous germline DNA sequence from dog chromosome 8 (NCBI Reference Sequence: NW_003726071.1) encoding all previously identified IGHV genes through the immunoglobulin heavy constant mu (IGHM) gene was analyzed using the Geneious 'Dotplot' function with consensus IGHV and IGHJ gene probes generated via multiple sequence alignments (http://www.geneious.com, version 5.5.8[105]). All identified genes were compared to those reported previously.[94,98] Newly identified genes were confirmed using BLAST search of dog genomic sequences (available at ncbi.nlm.nih.gov/projects/mapview,[106]) and/or dog BLAT search (available at genome.ucsc.edu,[107]), using the CanFam3.1 assembly (GenBank Assembly ID GCA_000002285.2).

Sequencing of canine IGHV genes

For BCLL cases, genomic DNA was extracted from 200 µL peripheral whole blood using the QIAamp DNA mini kit (Qiagen, Germantown, MD). DNA was amplified by PCR with a consensus IGHV3 subgroup-specific forward primer binding the leader exon (L-PART1) and either a pool of reverse primers binding the IGHJ intron regions (protocol 1) or the IGHJ coding regions (protocol 2) (Table 2; Figure 2). Earlier cases were sequenced using IGHJ intron primers, but these primers did not amplify rearrangements as effectively as IGHJ coding region primers, so the majority of cases were sequenced with protocol 2. While either leader primers or FR1 region primers may be used to amplify the IGHV region, leader primers were selected because they allow analysis of the whole IGHV gene, while FR1 primers exclude the 5' portion.[92] Amplified products were purified using the DNA Clean & Concentrator-5 kit (Zymo Research Corp, Irvine, CA), ligated into a pDrive T-vector (Qiagen, Germantown, MD) and

transformed into competent TG1 bacteria (Zymo Research Corp, Irvine, CA). Inserts were directly sequenced by Sanger sequencing from multiple independent clones (8-16 total). Tumor-associated IGHV-D-J rearrangements were identified as predominant repeated sequences with identical CDR3 sequences and a nucleotide length equal to the fragment size detected in the PARR assay. The cloning and sequencing protocol was identical for large B-cell lymphoma cases, except that DNA was isolated from fresh lymph node aspirates.

Table 2. PCR amplification primer sequences and cycling conditions for IGHV sequencing.

Primer sequence	Primer location	Cycling conditions
Protocol 1		
Forward: ATGGAGTCTGTGCTCGGCT	L-PART1	Denaturation step of 15 minutes at 95°C, followed by 10 cycles of 94°C for 30 seconds, 64°C for 30 seconds with the temperature decreasing 0.5°C every cycle, and 72°C for 1.5 minutes each, followed by 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds and 72°C for 1.5 minutes each, and final extension step of 7 minutes at 72°C.
Reverse primer pool[a]*:* TGGATCTCAGGCTAACGGA CGGAAAGACAGACACAGGTAG CCCAGGAGTCTCTGGAAATTG CCCAGAGAGAAAGAGAAGAGAAG ATCTCTGCCTCCACTTCCT CCCAGAGAAAGGAGCAGAAA	IGHJ1-INTRON IGHJ2-INTRON IGHJ3-INTRON IGHJ4-INTRON IGHJ5-INTRON IGHJ6-INTRON	
Protocol 2		

Forward: ATGGAGTCTGTGCTCGGCT	L-PART1	Denaturation step of 5 minutes at 95°C, followed by 40 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 1 minute each, and final extension step of 10 minutes at 72°C.
Reverse primer pool: ACCTGAGGAGACGGTGACC TGAGGACACGAAGAGTGAGG	J-REGION IGHJ2, IGHJ4 J-REGION IGHJ6	

[a]Protocol 1 reverse primer pool: amplification was first attempted with a pool of IGHJ2, IGHJ4, and IGHJ6 primers. If the clone was not identified, amplification was attempted in a second wave with a pool of IGHJ1, IGHJ3, and IGHJ5 primers. These primer sequences are located in the introns downstream of the IGHJ genes (see Figure 2B). L-PART1: first exon, encoding the first part of the leader sequence in an IGHV gene; J-REGION: coding region of the IGHJ genes (see Figure 2B).

For control dogs without lymphoproliferative disease, more IGHV-D-J rearrangements were sampled per dog, since there was not a clonal B-cell population present. For each case, the PCR and cloning conditions were the same, except that DNA was extracted from a section of fresh whole lymph node and 100 independent clones were selected for sequencing, rather than 8-16 clones.

Alignment and determination of mutational status and CDR3 length

Patient sequence from the first nucleotide of FR1 through the conserved 2nd-CYS in FR3 was queried against the CanFam3.1 assembly using NCBI BLAST (https://blast.ncbi.nlm.nih.gov). The most similar reference nucleotide sequence identified in this BLAST search was compared to our annotated library of germline reference IGHV genes (Supplemental table S1) to determine IGHV gene usage in the patient. When more than one reference germline IGHV gene was identified as a possible match, the intron sequence was used to confirm the patient IGHV gene identity.

Mutational status was determined using guidelines adapted from human medicine.[92] The percentage of identity was calculated based on the number of nucleotide differences between the

patient sequence and reference sequence in the V-REGION. Percent identity was calculated from the first nucleotide of FR1 to both the 2nd-CYS[108] and to codon 105,[92] but this boundary difference only changed the mutational status for one of the 389 sequences analyzed in this study. The sequence affected was a sequence from a control dog and did not have a statistical impact, so all sequences presented here were analyzed one way, to the 2nd-CYS. The following formula was used: IGHV identity (%) = 100 – (mutations/aligned IGHV region length × 100), with an insertion or deletion of multiple nucleotides counted as one mutation. Cases were classified as unmutated when the percent identity was >98% and mutated when percent identity was <98%, according to the convention used for human CLL.[92,109] Mutated BCLL cases were further categorized into those with percent identity between 96%-97.9% and those with <96% sequence homology.[110,111] The CDR3 length was identified by determining the number of codons from the first codon following 2nd-CYS to the last codon preceding TRP-118.[97] Additionally, a subset of IGHV-D-J rearrangements were analyzed using the NCBI IgBLAST web-based program (http://www.ncbi.nlm.nih.gov/igblast/,[112]) and IMGT/V-QUEST (http://www.imgt.org/IMGT_vquest/vquest, version 3.4.8,[108,113]), to confirm that the same results were obtained by other analysis methods. Custom reference databases were used in IgBLAST by uploading the canine germline IGHV, IGHD and IGHJ gene libraries.

Statistical analysis

A chi-square test and Fisher exact tests were performed to determine the statistical significance of differences in IGHV gene repertoire and mutational status.

Results

Study population

A total of 55 patients with BCLL were included in the study, including 29 females and 26 males. The median age at the time of sample collection was 10.5 years (range, 4.9-15.6 years; one unknown).

Initially, BCLL cases were selected at random, with no bias for breed. However, as it became apparent that Boxer dogs had a unique IGHV gene usage, additional Boxer dogs were sequenced. Therefore, of the 55 BCLL patients sequenced, 19 dogs (35%) were Boxers. Twenty-one breeds were represented in the non-Boxer group (Table 3). The peripheral B-cell count ranged from 7,300-816,600 cells/µL.

Table 3. Breed, mutational status, and IGHV gene rearrangements in 55 canine patients with chronic lymphocytic leukemia.

Case No.	Breed[a]	Identity (%)	Mutational status[b]	IGHV	IGHJ	CDR3 length[c]
1	SHI	91.7	Mutated	IGHV3-75	IGHJ6	17
2	CDT	88.9	Mutated	IGHV3-47	IGHJ4	16
3	MIX	92.4	Mutated	IGHV3-47	IGHJ6	13
4	COC	93.1	Mutated	IGHV3-47	IGHJ4	22
5	RAT	87.8	Mutated	IGHV3-41	IGHJ4	11
6	BDC	99.0	Unmutated	IGHV3-41	IGHJ4	16
7	BIC	99.0	Unmutated	IGHV3-41	IGHJ4	18
8	AIR	100.0	Unmutated	IGHV3-41	IGHJ4	14
9	MIX	89.2	Mutated	IGHV3-38	IGHJ2	16
10	CKP	89.9	Mutated	IGHV3-38	IGHJ4	14
11	SHI	91.3	Mutated	IGHV3-38	IGHJ6	16
12	MLT	93.1	Mutated	IGHV3-38	IGHJ6	17

13	JRT	93.8	Mutated	IGHV3-38	IGHJ4	14
14	BIC	95.8	Mutated	IGHV3-38	IGHJ4	12
15	BIC	95.8	Mutated	IGHV3-38	IGHJ4	17
16	CRN	96.5	Borderline	IGHV3-38	IGHJ4	13
17	MIX	97.6	Borderline	IGHV3-38	IGHJ2	12
18	CSH	97.6	Borderline	IGHV3-38	IGHJ4	23
19	LAB	100.0	Unmutated	IGHV3-38	IGHJ4	15
20	WET	100.0	Unmutated	IGHV3-38	IGHJ4	16
21	CCR	94.8	Mutated	IGHV3-35	IGHJ4	14
22	MIX	91.0	Mutated	IGHV3-19	IGHJ4	13
23	SHI	91.0	Mutated	IGHV3-19	IGHJ2	14
24	CRN	94.1	Mutated	IGHV3-19	IGHJ4	13
25	SHI	94.8	Mutated	IGHV3-19	IGHJ4	13
26	MIX	94.8	Mutated	IGHV3-19	IGHJ4	14
27	PIT	95.5	Mutated	IGHV3-19	IGHJ4	10
28	MIX	100.0	Unmutated	IGHV3-19	IGHJ4	14
29	MIX	97.9	Borderline	IGHV3-12	IGHJ2	15
30	POM	98.6	Unmutated	IGHV3-9	IGHJ4	17

31	MIX	99.0	Unmutated	IGHV3-9	IGHJ6	12
32	LBD	92.0	Mutated	IGHV3-5	IGHJ4	16
33	BOR	95.1	Mutated	IGHV3-5	IGHJ4	13
34	SHI	95.5	Mutated	IGHV3-5	IGHJ2	14
35	STS	98.6	Unmutated	IGHV3-5	IGHJ2	27
36	LAB	95.8	Mutated	IGHV3-2	IGHJ4	15
37	BOX	99.0	Unmutated	IGHV3-67	IGHJ4	11
38	BOX	99.7	Unmutated	IGHV3-67	IGHJ4	10
39	BOX	100.0	Unmutated	IGHV3-67	IGHJ4	10
40	BOX	96.9	Borderline	IGHV3-41	IGHJ4	14
41	BOX	97.9	Borderline	IGHV3-41	IGHJ2	17
42	BOX	98.3	Unmutated	IGHV3-41	IGHJ4	17
43	BOX	99.3	Unmutated	IGHV3-41	IGHJ6	18
44	BOX	99.7	Unmutated	IGHV3-41	IGHJ4	11
45	BOX	99.7	Unmutated	IGHV3-41	IGHJ4	11
46	BOX	99.7	Unmutated	IGHV3-41	IGHJ6	22
47	BOX	100.0	Unmutated	IGHV3-41	IGHJ4	12
48	BOX	100.0	Unmutated	IGHV3-41	IGHJ4	13

49	BOX	100.0	Unmutated	IGHV3-41	IGHJ4	16
50	BOX	100.0	Unmutated	IGHV3-41	IGHJ6	17
51	BOX	97.9	Borderline	IGHV3-38	IGHJ4	13
52	BOX	100.0	Unmutated	IGHV3-38	IGHJ4	10
53	BOX	100.0	Unmutated	IGHV3-38	IGHJ4	14
54	BOX	96.5	Borderline	IGHV3-19	IGHJ4	12
55	BOX	99.3	Unmutated	IGHV3-5	IGHJ4	10

[a]Breed abbreviations: SHI, Shih-Tzu; CDT, Coton de Tulear; MIX, Mixed breed; COC, Cocker Spaniel; RAT, Rat Terrier; BDC, Bearded Collie; BIC, Bichon Frise; AIR, Airedale Terrier; CKP, Cockapoo; MLT, Maltese; JRT, Jack Russell Terrier; CRN, Cairn Terrier; CSH, Chihuahua, Shorthair; LAB, Labrador Retriever; WET, Soft Coated Wheaten Terrier; CCR, Chinese Crested; PIT, Pit Bull Terrier; POM, Pomeranian; LBD, Labradoodle; BOR, Border Collie; STS, Schnauzer; BOX, Boxer. [b]Mutational status: mutated (<96% similarity); borderline (96-98% similarity); unmutated (>98% similarity). [c]Heavy chain complementary-determining region 3 amino acid length.

Eleven Boxer dogs with large B-cell lymphoma were sequenced, including 4 females and 7 males. The median age at the time of sample collection was 8.5 years (range, 7.1-13.1 years).

Six dogs without lymphoproliferative disease were sequenced, including 3 Boxer dogs and 3 non-Boxer dogs. The Boxers ranged in age from 6.0-8.4 years and included one female and two males. The non-Boxers included one Labrador retriever, one mixed breed dog, and one Chihuahua. The non-Boxers ranged in age from 9.0-13.0 years and included two females and one male. Five of 6 animals were deceased at the time of lymph node collection. One animal was alive at sample collection, diagnosed with mast cell tumor disease, and had 5 months follow up since sample collection. Two dogs died of heart failure and the remaining 4 dogs had non-lymphoid neoplasms. All 6 dogs had polyclonal

immunoglobulin and T-cell receptor rearrangements by PARR, providing additional support that the dogs did not have lymphoproliferative disease.

Newly identified IGHV and IGHJ genes

A combination of BLAST/BLAT searching and Dotplot analyses were employed to identify 7 new germline IGHV genes and 3 new germline IGHJ genes compared to those originally described by Bao et al.,[94] bringing the totals to 87 IGHV and 6 IGHJ genes. All 7 newly identified IGHV genes belong to the predominant IGHV3 subgroup, and were named: IGHV3-76, IGHV3-71, IGHV3-47-1, IGHV3-21-1, IGHV3-4, IGHV3-NL1, and IGHV3-NL2 (Supplemental table S1). Three of these 7 new IGHV genes were also annotated recently by Martin et al.[98] Five of the new IGHV genes mapped to chromosome 8 and 2 (designated NL) mapped to an unplaced genomic scaffold. Only IGHV3-76 and IGHV3-NL1 were considered functional. These 2 genes had open reading frames and conserved IMGT motifs, including the conserved amino acids: 23 (1st-CYS), 41 (CONSERVED-TRP), 89 (hydrophobic) and 104 (2nd-CYS). Two IGHV genes previously described[94] were reordered to reflect the genomic location on chromosome 8: IGHV3-32 (previously VH1-6) and IGHV3-78 (previously VH1-49P). The truncated sequence of VH1-42 that was previously published[94] was expanded and the more complete gene was renamed IGHV3-40. The 3 new IGHJ genes were clustered with the previously reported IGHJ genes on a 1.8 kb span 3' to the IGHV region, and the IGHJ numbering system was revised from that which is already published[94] to IGHJ1-IGHJ6, 5' to 3' (Figure 3). This new annotation of the IGHJ genes is consistent with that described by Martin et al.[98]

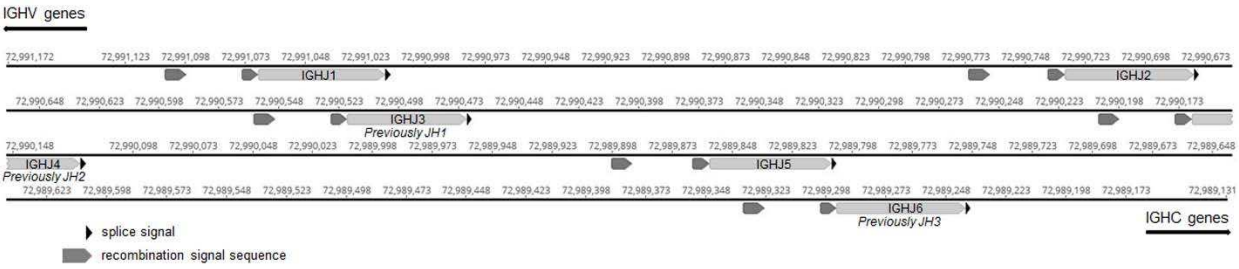

Figure 3. Dog IGHJ locus. The organization of the 6 canine IGHJ genes is shown, with the genomic locations on chromosome 8 identified (CanFam3.1, NCBI Accession NC_006590.3). Gene segments are shown as light grey bars, recombination signal sequences as dark grey bars, and splice signal sequences as black triangles. The 3 new IGHJ genes are IGHJ1, IGHJ2 and IGHJ5. The 3 IGHJ genes previously described[94] have been renamed from JH1, JH2 and JH3 to IGHJ3, IGHJ4 and IGHJ6, respectively, based on their genomic position. IGHC, immunoglobulin heavy constant genes.

BCLL IGHV repertoire and mutational status

IGHV-D-J rearrangements from 55 BCLL patients were examined (Table 3). Eleven IGHV genes were represented in the BCLL patient cohort. In non-Boxers, IGHV3-38 (33.3%) and IGHV3-19 (19.4%) were the most commonly used IGHV genes, followed by IGHV3-41 (11.1%) and IGHV3-5 (11.1%) (Figure 4A). In Boxers with BCLL, IGHV3-41 (57.9%) was most commonly used, followed by IGHV3-38 (15.8%) and IGHV3-67 (15.8%) (Figure 4B). IGHV gene usage was significantly different between non-Boxers and Boxers for IGHV3-67 ($p=0.037$) and IGHV3-41 ($p<0.001$).

Among non-Boxers with BCLL, 9/36 (25%) cases were classified as unmutated and 27/36 (75%) cases were mutated. Among Boxers with BCLL, 15/19 (79%) cases were unmutated, while 4/19 (21%) cases were mutated. The majority of mutated non-Boxer BCLL cases had a percent identity <96% (24/36 (66.7%) cases), and few cases had a 96%-97.9% identity (3/36 (8.3%) cases). All 4 mutated Boxer BCLL cases had a percent identity between 96%-97.9%, with none of the cases having a percent identity <96%. The frequency of unmutated cases was significantly higher in Boxers with BCLL compared to other breeds ($p<0.001$).

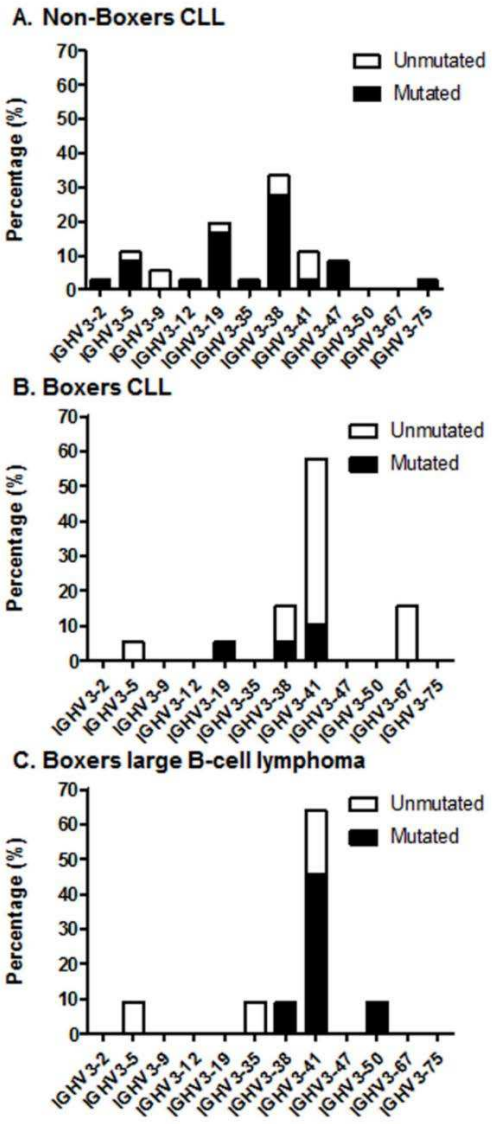

Figure 4. Distribution of IGHV gene usage and mutational status in (A) non-Boxer dogs with BCLL (n=36), (B) Boxer dogs with BCLL (n=19), and (C) Boxer dogs with large B-cell lymphoma (n=11). IGHV gene usage is reported as the percentage of patients using an IGHV gene within that cohort. There were significant differences in the IGHV gene usage between non-Boxers and Boxers with BCLL for IGHV3-67 ($p=0.037$) and IGHV3-41 ($p<0.001$). There were significantly more unmutated cases in the Boxer BCLL cohort, compared to non-Boxers with BCLL ($p<0.001$) and Boxers with large B-cell lymphoma ($p=0.026$).

The IGHJ gene repertoire was not significantly different between non-Boxer and Boxer BCLL patients. Across the cohort of 55 patients, 3 IGHJ genes rearranged. IGHJ4 (72.7%) was the most frequently used IGHJ gene, followed by IGHJ6 (14.5%) and IGHJ2 (12.7%). The heavy chain CDR3 mean length was 14.6 AA (range, 10-27 AA) across all BCLL cases. There was not a significant difference in CDR3 length between breed groups or between mutated and unmutated cases.

These results indicate that Boxers with BCLL have preferential rearrangement of IGHV3-41 and that the majority of cases are unmutated, regardless of IGHV gene usage.

Large B-cell lymphoma IGHV repertoire and mutational status

To determine whether Boxers with other forms of lymphoproliferative disease have preferential rearrangement of IGHV3-41 or unmutated IGHV genes, IGHV-D-J rearrangements from 11 Boxers with large B-cell lymphoma were examined. IGHV3-41 (63.6%) was most commonly used, at a frequency similar to that seen in the Boxer BCLL cohort (Figure 4C). 4/11 (36.4%) cases were classified as unmutated, and 7/11 (63.6%) cases were mutated. Among mutated cases, one case had a percent identity between 96%-97.9%, and remaining mutated cases had <96% homology to germline. The IGHJ gene repertoire was similar to BCLL, with IGHJ4 (72.7%) most frequently used, followed by IGHJ6 (18.2%) and IGHJ2 (9.1%). The CDR3 mean length was 13.8 AA (range, 10-20 AA). These results indicate that Boxers with large B-cell lymphoma preferentially use IGHV3-41, as seen in Boxers with BCLL, but in the majority of large B-cell lymphoma cases the IGHV genes are mutated rather than unmutated.

Normal canine IGHV repertoire and mutational status

IGHV-D-J rearrangements from 6 control dogs without lymphoproliferative disease were examined, including 3 non-Boxers and 3 Boxers. The number of unique productive clones obtained for each animal ranged from 27 to 65 (Figure 5). Across the 6 dogs, which included 323 unique IGHV-D-J rearrangements, the most commonly used IGHV genes were IGHV3-19 (28.2%), IGHV3-47 (24.5%), and IGHV3-41 (24.1%), followed by IGHV3-38 (6.5%), IGHV3-5 (6.2%) and IGHV3-2 (5.0%). There were significant differences in IGHV gene usage between individual animals and between breeds. Boxers (Figure 5A-C) used IGHV3-41 ($p<0.001$) and IGHV3-47 ($p<0.001$) significantly more than non-Boxers, and non-Boxers (Figure 5D-F) used IGHV3-19 ($p<0.001$) and IGHV3-38 ($p<0.001$) significantly more than Boxers. There was no significant difference in mutational status between Boxers and non-Boxers. These

results indicate that Boxers with normal B cells preferentially use IGHV3-41 and IGHV3-47 compared to other breeds, and the majority of rearrangements are mutated.

Discussion

In this study, we investigated IGHV gene usage and mutational status in canine patients with BCLL. Among non-Boxer patients with BCLL, we found that the majority of cases (75%) were mutated, using the homology cutoff value of 98% as established in human CLL. The ratio of mutated to unmutated cases was slightly higher than seen in the human population.[14,114] Boxers were analyzed separately as they were preferentially sequenced over other breeds due to their skewed use of unmutated IGHV genes. Among Boxers with BCLL, 79% of cases were unmutated and all of the mutated cases had a percent identity between 96%-97.9%, demonstrating that none of the Boxer BCLL cases sequenced were highly mutated. These data suggest that the Boxer breed may be a useful model for unmutated CLL. However, the reference dog genome sequence was obtained by sequencing a Boxer;[67] therefore, we were concerned that the high homology between the Boxer BCLL cases and reference genome may be due to breed bias. To address this question, we sequenced IGHV genes from additional Boxer dogs with large B-cell lymphoma and without lymphoproliferative disease and found that these dogs used predominantly mutated IGHV genes. The majority of IGHV genes from Boxers with large B-cell lymphoma (64%) were mutated. There was no significant difference in the number of unmutated IGHV gene rearrangements in normal Boxers compared to other breeds, suggesting use of unmutated IGHV genes in Boxers is specific to BCLL.

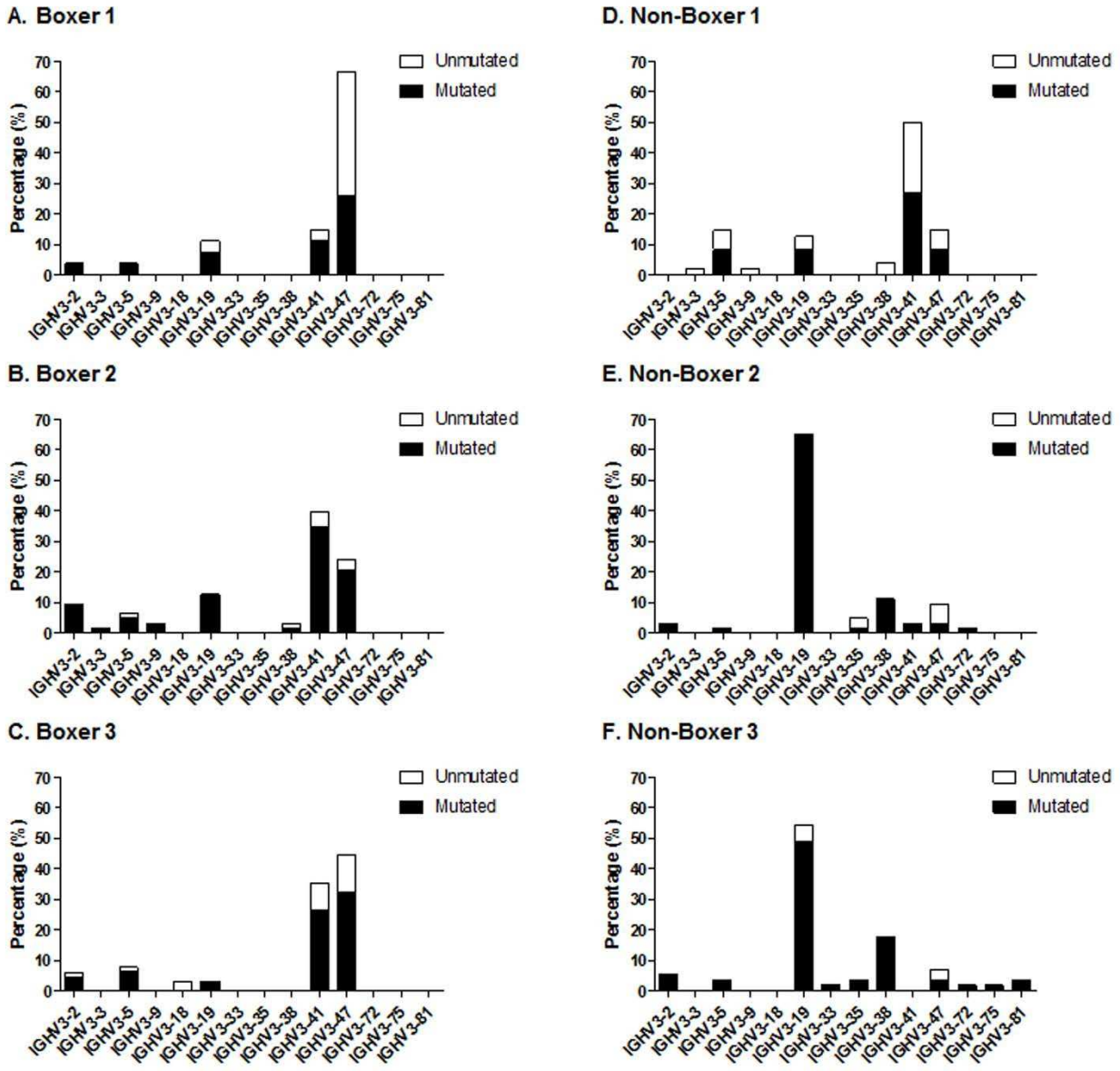

Figure 5. Distribution of IGHV gene usage and mutational status in 3 Boxer dogs (A-C) and 3 non-Boxer dogs (D-F) without lymphoproliferative disease. IGHV gene segment usage is reported as the percentage of unique rearrangements using an IGHV gene within a single dog's repertoire. There were significant differences in the IGHV gene usage between Boxers and non-Boxers for IGHV3-47, IGHV3-41, IGHV3-38, and IGHV3-19 ($p<0.001$ for all genes). There was no significant difference in the mutational status between Boxers and non-Boxers. The number of unique rearrangements examined for each case included: Boxer 1, n=27; Boxer 2, n=63; Boxer 3, n=65; non-Boxer 1 (Labrador retriever), n=48; non-Boxer 2 (Mixed breed), n=63; non-Boxer 3 (Chihuahua), n=57.

We examined gene usage in BCLL patients as well as the normal IGHV gene repertoire in 6 control dogs. IGHV3-38 and IGHV3-19 were most commonly used in non-Boxers with BCLL and in the normal gene repertoire of 2 of the 3 non-Boxer control dogs. One of the 3 control non-Boxers had a gene repertoire more similar to the Boxers. Bao et al.[94] demonstrated a bias for IGHV3-38 and IGHV3-19 amongst 3 healthy dogs, and IGHV3-38 was preferentially rearranged in canine cases with diffuse large B-cell lymphoma.[101,102] Boxers with BCLL, large B-cell lymphoma, and without lymphoproliferative disease preferentially used IGHV3-41, indicating IGHV3-41 gene usage is high in Boxers, regardless of disease status. IGHV3-47 was commonly used in all 3 control Boxers without lymphoproliferative disease, but was not used in Boxers with BCLL or large B-cell lymphoma. IGHV3-67 was one of the more commonly used IGHV genes in Boxers with BCLL, but was not used in any of the non-Boxers with BCLL, Boxers with large B-cell lymphoma, or normal B-cell repertoires examined. This suggests IGHV3-67 may be preferentially used in Boxers with BCLL, but the number of cases is small and additional cases are needed to verify this finding. A limitation of this study is that the leader primer used for sequencing is specific to the IGHV3 subgroup, which likely had a small effect on the normal repertoire of control dogs. The IGHV3 subgroup is the dominant subgroup in multiple studies,[94,99,100] accounting for 90% or more of rearranged genes in one study,[99] and we were able to identify the clone in 92% of BCLL cases we attempted to sequence with an IGHV3-specific primer.

This study contributes to our understanding of the canine immunoglobulin heavy chain variable region. Seven new IGHV genes and 3 new IGHJ genes were identified compared to the original annotation from Bao et al.[94] Some of the additions may be attributed to different reference genome builds. Martin et al. recently described 3 of these new IGHV genes and the new IGHJ genes.[98] Slight differences in our annotations may be due to different consensuses used to search for new IGHV genes. Additionally, all of the IGHV genes were assessed for functionality and annotated using the IMGT guidelines. All productive rearrangements used for analysis had the 4 previously described conserved AA

and the conserved Gly-X-Gly motif following codon 118. Eight of 389 sequences examined in this study had a residue other than the highly conserved PHE or TRP at codon 118, but were considered adequate for interpretation because the Gly-X-Gly was conserved.[93]

A challenge of mutational analysis in dogs is the lack of knowledge about polymorphisms in IGHV genes across dog breeds and individuals. Ideally, canine BCLL sequences would be compared to reference genome sequences from the same breed, but those are not available at this time. Additionally, the homology cutoff of 98% which distinguishes clinically distinct subsets of human CLL patients[13,14,115] may need to be adjusted to distinguish subsets of canine patients with different prognoses. The next phase of this study is to perform a large-scale outcome study in dogs with BCLL to correlate clinical outcome with mutational status. An additional challenge is the lack of antibodies available to differentiate BCLL from other canine B-cell neoplasms. The case criteria used in this study define an entity in dogs that most closely resembles CLL in people in its clinical presentation.[62] However, this entity may represent a different small B-cell neoplasm with lymphocytosis, such as leukemic mantle cell lymphoma,[3] or group of small B-cell neoplasms. We consider leukemic mantle cell lymphoma a less likely differential because this histologic subtype appears quite rare in dogs.[77,78,116] Future steps include obtaining histopathology and gene expression profiling to correlate canine findings with that seen in human CLL.

These results contribute to our understanding of the canine immunoglobulin genes and are the first to examine mutational status in a canine population with BCLL. This study identified Boxers with BCLL as having predominantly unmutated IGHV gene rearrangements and no highly mutated rearrangements. This suggests that this breed may be a valuable model to study CLL associated with unmutated IGHV genes.

CHAPTER 2: Clinical outcome and prognostic factors in dogs with B-cell chronic lymphocytic leukemia: A retrospective study

Summary

Canine B-cell chronic lymphocytic leukemia (BCLL) is generally considered an indolent disease, but previous studies demonstrate a wide range in survival times. We hypothesized that BCLL has a heterogeneous clinical course, similar to human chronic lymphocytic leukemia (CLL). Our objectives were to assess clinical progression and outcome in a large number of canine BCLL patients and evaluate the prognostic significance of clinical and flow cytometric factors. This retrospective study reviewed the medical records for 121 canine BCLL cases, defined as >5,000 lymphocytes/µL in the blood with a homogeneous expansion of small-sized CD21+ B cells (>60% of lymphocytes) by flow cytometry. Three breed groups were represented: small breed dogs (n=55) due to increased risk of BCLL; Boxers (n=33) due to preferential use of unmutated immunoglobulin genes; other breeds (n=33) to compare outcome to small breed cases. We retrieved signalment, laboratory data, physical exam and imaging findings, treatments and survival information. Cellular proliferation, determined by the percent of Ki67-expressing CD21+ B cells by flow cytometry, was measured in 39/121 cases. The median overall survival time (MST) for all cases was 300 days (range, 1-1644 days). Boxers had significantly shorter survival (MST, 178 days) compared to non-Boxers (MST, 423 days; p=0.0003), and there was no significant survival difference between small breeds and other non-Boxer breeds. Cases with high Ki67 (>40% Ki67-expressing B cells) had significantly shorter survival (MST, 173 days) than cases with <40% Ki67 (MST undetermined) (p=0.023), irrespective of breed. Cases with a high lymphocyte count (>60,000 lymphocytes/µL) or clinical signs at presentation had significantly shorter survival. In summary, BCLL demonstrated a variable clinical course and Boxer dogs and cases with high Ki67 had more aggressive disease.

Introduction

B-cell chronic lymphocytic leukemia is a malignancy of small-sized B cells, which are clonally expanded in the blood and/or bone marrow. BCLL is a common form of lymphoid leukemia in dogs. Among samples submitted for flow cytometric immunophenotyping, BCLL affected 8% of all canine submissions from any site with a suspicion of lymphoproliferative disease in one study, and 36% of blood submissions from 153 canine CLL cases in another study.[62,117] Diagnostic criteria for canine BCLL are variable across studies, generally requiring >5,000-6,000 lymphocytes/µL in the peripheral blood and identification of a significant expansion of B cells by immunophenotyping.[62,66,84,118,119] Some studies incorporate small mature cytomorphology or small cell size by flow cytometry in the inclusion criteria, and some exclude cases with moderate or severe lymphadenopathy or splenomegaly. BCLL generally affects older dogs, with a median age at diagnosis ranging from 10-11.9 years across studies.[62,66,84] A previous study assessing the breed distribution and clinical presentation of canine BCLL in 491 dogs demonstrated that small breed dogs have an increased risk of BCLL and approximately half of patients have lymphadenopathy or splenomegaly and approximately one quarter have anemia.[62]

In people, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) is a common neoplasm of clonally-expanded B cells in the blood, bone marrow, lymph nodes and spleen.[52] A diagnosis of CLL requires >5,000 lymphocytes/µL in the blood and SLL requires lymphadenopathy and/or splenomegaly with <5,000 circulating B cells/µL, but these two entities are considered different manifestations of the same disease. This human neoplasm has a highly variable clinical course and a combination of genetic and clinical variables are used to assess prognosis.[51,52,88] Canine BCLL is generally considered an indolent disease, but two studies assessing outcome demonstrate a wide range in survival times.[84,85] One study evaluating 17 BCLL patients found a MST of 480 days and young age was associated with a poor prognosis.[84] A second study evaluating outcome in 21 cases with small cell CD21+ B-cell lymphocytosis found that the majority of dogs had a prolonged clinical course (MST not reached), but

survival times ranged from 25 days to >1000 days.[85] We hypothesize that canine BCLL may have a variable clinical course like human CLL.

The goal of this study was to assess overall survival and prognostic factors in a larger number of canine BCLL cases. For this study, we included cases with an expansion of small-sized B cells in the blood by flow cytometry. We did not exclude cases with lymphadenopathy or splenomegaly or cytopenias, as these criteria may exclude true BCLL cases with more progressive disease. While it's possible some of the cases in this study have a different small B-cell neoplasm, we wanted to include all cases with a diagnosis of small cell B-cell lymphocytosis in the blood, as it is unknown whether cases with tissue infiltration are a different manifestation of BCLL versus an entirely different neoplasm. Additionally, we currently do not have markers in veterinary medicine to differentiate subtypes of small B-cell neoplasms, so all of these cases receive the same flow cytometry diagnosis. We evaluated clinical features and Ki67 expression for association with survival. Ki67 expression is a marker of proliferation and was shown to have prognostic value in canine high-grade B-cell lymphoma when measured by flow cytometry.[120] We previously found that Boxers with BCLL preferentially rearrange unmutated immunoglobulin heavy variable region (IGHV) genes, which is a poor prognostic indicator in human CLL, and this study also aims to examine breed-related differences in clinical presentation and outcome.[13,14,121]

Methods

Study population

The Colorado State University-Clinical Immunology (CSU-CI) laboratory database was queried for canine BCLL cases with blood samples submitted for immunophenotyping by flow cytometry between October 2010 and October 2018. Inclusion criteria included: (1) >5,000 lymphocytes/µL on CBC; (2) >60% of lymphocytes in the blood sample expressed CD21 by flow cytometry; (3) no cells in the population expressed CD34; (4) CD21+ B cells were small-sized, defined by a ratio of the geometric

median of forward scatter of B cells:neutrophils ≤0.60. Cases were randomly selected and veterinarians were contacted for medical records. Additional Boxer cases were contacted to investigate breed-specific differences in outcome. English bulldogs were excluded from this study because we identified a condition of polyclonal B-cell expansion in this breed, described in chapter 4.

For controls to evaluate CD21, CD25 and class II MHC expression on normal B cells, routine flow cytometry was performed on blood from 30 healthy dogs without lymphocytosis, which ranged in age from 1.9-8.9 years old, included 57% females, and represented a range of breeds. These dogs had lymphocyte subsets within normal limits by flow cytometry and polyclonal immunoglobulin rearrangements by PCR for antigen receptor rearrangements (PARR). Samples were processed and analyzed on the Coulter Gallios instrument with the same protocol used for clinical samples.

Between June 2017 and August 2019, the CSU-CI laboratory performed Ki67 staining by flow cytometry on a subset of randomly selected BCLL cases and nodal large B-cell lymphoma cases, as described below. Large B-cell lymphoma cases were defined by flow cytometry as lymph node samples with a homogeneous expansion of CD21+ B cells with a median forward scatter >500. In a separate study, the majority of cases with these flow cytometric features were diffuse large B-cell lymphoma, with smaller numbers diagnosed as nodal marginal zone lymphoma or Burkitt-like lymphoma.[122] Additionally, Ki67 staining was performed on 7 lymph node aspirates and 5 blood samples from 12 control dogs with no evidence of lymphoproliferative disease. For all BCLL cases with Ki67 data obtained between September 2017 and October 2018, veterinarians were contacted for medical records and those cases with available data were included in the outcome study.

Clinical variables

For all samples, the CSU-CI laboratory submission form and medical records were reviewed for signalment, physical exam findings, laboratory data, clinical signs, imaging findings and treatment and

survival data. Anemia was defined by a hematocrit below the lower limit of the reference interval for the laboratory performing the CBC. Thrombocytopenia was defined by a platelet count below the laboratory's reference interval with no evidence of platelet clumping on blood smear review. Hyperglobulinemia was defined using the upper limit of the reference interval for globulins on available biochemistry panels. Lymph node, spleen, and liver abnormalities, including enlargement or masses, were identified by palpation, ultrasound examination or radiology.

Flow cytometry

All cases had a blood sample submitted for flow cytometry at the CSU-CI laboratory. A small number of cases also had lymph node aspirates submitted for flow cytometry. Sample collection, storage, and staining were performed as previously described, using antibody panels listed in Table 4.[65] Samples submitted prior to May 11, 2012 were stained with antibody panel 1 and analyzed on a single laser Coulter XL (Beckman Coulter Inc., Brea, CA), samples submitted from May 11, 2012 to March 7, 2017 were stained with antibody panel 2 and analyzed on a three laser Coulter Gallios (Beckman Coulter Inc., Brea, CA), and samples submitted after March 7, 2017 were stained with antibody panel 3 and analyzed on a three laser Coulter Gallios. Data were analyzed using Kaluza Analysis Software (Beckman Coulter Inc., Brea, CA). Expression of CD21, class II MHC, and CD25 on B cells was determined for all cases submitted after May 11, 2012. Expression of CD21 and class II MHC was determined by median fluorescence intensity. Because anti-CD25 is not in the same staining reaction as anti-CD21, the percentage of B cells expressing CD25 was determined by gating on lymphocytes in tube 2, excluding cells expressing CD3, CD4, CD5 and CD8, and calculating %CD25 on remaining cells. The B-cell:neutrophil size ratio was determined by dividing the median forward scatter of CD21+ B cells by CD4+ CD5- CD18+ neutrophils. There was no significant difference in neutrophil size between cases run on the Coulter XL and the Coulter Gallios instruments, so size data from all cases were used for statistical analysis.

Table 4. Antibody panels for canine flow cytometry immunophenotyping.

Antibody tube	Antibody specificity and fluorochrome
Panel 1	
1	None
2	M[a] IgG1-FITC/CD45-PE
3	CD18-FITC/M IgG1-PE
4	CD4-FITC/CD8-PE
5	CD5-FITC/CD21-PE
6	CD3-FITC/CD45-PE
7	CD4-FITC/CD14-PE
8	Class II MHC-FITC/CD34-PE
Panel 2	
1	M IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-Alexa-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
Panel 3	
1	M IgG1-FITC/M IgG1-PE/M IgG1-Alexa 647/M IgG1-Alexa 700/M IgG1-PE-Alexa-750/M IgG1-Pacific Blue
2	CD3-FITC/CD25-PE/CD5-APC/CD8-Alexa 700/CD4-Pacific Blue
3	CD21-Alexa 647/Class II MHC-FITC/CD34-PE
4	Class II MHC-FITC/CD18-PE/CD5-APC/CD14-Alexa 700/CD4-Pacific Blue
5	CD5-FITC/CD45-PE/CD21-Alexa 647

[a]M, mouse. Antibody clones and sources are provided in Chapter 1, Table 1.

Clonality testing and immunoglobulin mutation status

B-cell clonality was determined by evaluating clonality of immunoglobulin receptor rearrangements. The CSU-CI laboratory PARR assay was performed as previously described.[123] A small number of cases had PARR requested at the time of flow cytometry. For the remaining cases, PARR was performed retrospectively for all cases that had material still available at the time of data collection.

Cloning and sequencing were performed on a subset of cases with material available to determine IGHV mutation status, as previously described.[121] The percent identity between the patient IGHV gene sequence and the reference germline IGHV gene sequence was calculated. Cases with a percent identity >98% were classified as unmutated and cases with <98% were classified as mutated.

Ki67 expression by flow cytometry

Intracellular Ki67 was measured by flow cytometry on the same blood samples and lymph node aspirates used for diagnostic immunophenotyping. Red blood cells were lysed as described for routine immunophenotyping and the leukocyte pellet was washed in 1 mL 1x phosphate buffered saline (PBS), centrifuged and resuspended in 200 µL diluted Zombie Violet (1:1,000 in 1x PBS; Zombie Violet™ Fixable Viability Kit, BioLegend, San Diego, CA). Samples were incubated in the dark for 30 minutes at 25°C, washed with 1000 µL flow buffer (PBS-2%, FBS-0.1%, NaAZ), and centrifuged. The cell pellet was resuspended in 25 µL flow buffer and combined with 25 µL of surface antibody cocktail, incubated in the dark for 15 minutes at 25°C, and washed in 1 mL flow buffer. The surface antibody cocktail contains: CD5 Percp-eFluor710 (YKIX322.3 clone, eBioscience), CD18 PE (YFC118.3 clone, Bio-Rad Serotec, Hercules, CA), and CD21 Alexa Fluor 647 (CA2.1D6 clone, Bio-Rad Serotec). Samples were fixed and permeabilized by resuspending the cell pellet in 1 mL of diluted permeabilization reagent and fixation reagent, prepared according the manufacturer recommendations (FoxP3 Staining kit, eBioscience, San Diego, CA), and incubated in the dark overnight at 4°C. To stain cells for nuclear antigens, samples were centrifuged, washed twice with 1 mL permeabilization buffer, and resuspended in 200 µL permeabilization buffer. 90 µL of the resuspended cell pellet was combined with 5 µL of blocking reagent and incubated in the dark for 30 minutes at 4°C. Without washing, 5 µL of intracellular Ki67 FITC antibody (SolA15 clone, eBioscience) or isotype-FITC were added, samples were incubated in the dark for 30 minutes at 4°C, washed twice with 1 mL permeabilization buffer, and resuspended in 220 µL flow buffer. Samples were analyzed on a three laser Coulter Gallios (Beckman Coulter Inc.) and analyzed using Kaluza Analysis software (Beckman Coulter Inc.). The isotype control was strictly used to determine the percent positive for Ki67 expression. Ki67 expression was measured for research purposes and results were not reported, and therefore, did not affect treatment decisions.

Statistical analysis

Signalment, physical exam findings, hematologic data, and flow cytometry data were summarized for all BCLL cases. To evaluate breed specific differences, we grouped cases into 3 broad breed groups: (1) small dog breeds with increased risk of BCLL, as previously defined;[62] (2) Boxer dogs; and (3) other breeds. To compare findings across breed groups, continuous variables were assessed using Kruskal Wallis or Mann Whitney tests and categorical variables were assessed using chi-square tests. Overall survival was calculated as days from the time of the flow cytometry diagnosis, since this date was available for all cases. Cases that were lost to follow-up or alive at the time of data collection were censored at the date of last contact. Continuous risk factors were divided into groups above and below the median for survival analysis, except for lymphocyte count. For lymphocyte count, cases were grouped above and below 60,000 lymphs/μL, as this cutoff was significant, but a cutoff at the median was not. Treatments were categorized as: (1) no treatment; (2) corticosteroid only; (3) corticosteroids and chlorambucil; or (4) maximum tolerated dose (MTD) injectable/oral chemotherapy. Individuals with missing/unknown data were excluded from analysis. Blood smear lymphocyte morphology obtained from the pathology report was categorized as: (1) intermediate to large-sized lymphocytes; or (2) small, small to intermediate, or intermediate-sized lymphocytes. Associations between patient data and survival were assessed using Kaplan-Meier log rank tests. For Ki67 expression analysis and survival analysis, the 'other breeds' group was further divided into small breeds and medium-large breeds. Small breeds were defined by an upper body weight range limit <15 kg, based on the American Kennel Club breed weight chart, and medium-large breeds had a lower body weight range limit >15 kg (AKC Material, URL: akc.org/expert-advice/nutrition/breed-weight-chart/). Statistical analysis was performed in GraphPad Prism version 7 or R version 3.5.2 and two-sided p-values <0.05 were considered statistically significant.

Results

Clinical presentation

Medical records were obtained for 121 BCLL cases, representing 3 major breed groups: small breeds with increased risk of BCLL (n=55), Boxer dogs (n=33), and other breeds (n=33). Signalment, laboratory data and physical exam findings at the time of diagnosis are summarized in Table 5. The median age of all cases in the study was 10.4 years (interquartile range [IQR], 8.6-12.4 years; range, 2.3-16.4 years). Fifty-five percent of all cases were male, including 57 castrated males, 7 intact males, and 2 males with unknown castration data. Of the female cases, 53 were spayed and 2 were intact.

Table 5. Signalment data, laboratory data, physical exam findings and treatment data for 121 BCLL patients in the outcome study.

	Boxers (n=33)		Small breed dogs (n=55)		Other breeds (n=33)		Non-Boxers (n=88)	
	n[a]	% affected or median (IQR)	n[a]	% affected or median (IQR)	n[a]	% affected or median (IQR)	n[a]	% affected or median (IQR)
Age	33	9.3 (8.3-10.7)	55	10.9 (9.1-13.2)	33	10.6 (8.4-12.5)	88	10.6 (9.0-13.0)
Male	33	45%	55	47%	33	76%	88	58%
Lymphocyte count, $x10^3/\mu L$	33	72.7 (32.9-138.1)	55	32.0 (14.3-75.0)	33	19.4 (11.5-63.5)	88	27.0 (12.8-64.6)

Anemia	33	45%	55	45%	33	30%	88	40%
Thrombocytopenia	33	36%	55	18%	33	21%	88	19%
Hyperglobulinemia	32	19%	52	23%	30	13%	82	20%
B cell size ratio	33	0.52 (0.48-0.56)	55	0.48 (0.44-0.51)	33	0.50 (0.45-0.57)	88	0.49 (0.45-0.54)
CD25 expression	30	72 (26-96)	46	55 (21-81)	23	68 (52-91)	69	62 (25-82)
Class II MHC expression	30	127 (100-254)	46	159 (102-220)	23	212 (82-327)	69	160 (90-260)
CD21 expression	30	46 (31-64)	46	50 (35-70)	23	42 (32-56)	69	49 (33-68)
Peripheral lymphadenopathy	32	66%	52	54%	33	39%	85	48%
Visceral lymphadenopathy	20	55%	31	39%	16	50%	47	43%

Splenic abnormalities	30	67%	41	59%	25	32%	66	48%
Hepatic abnormalities	30	30%	41	41%	26	23%	67	34%
Clinical signs at presentation	33	42%	54	37%	32	34%	86	36%
Treatment type: No treatment Steroids only Steroid/chloramb MTD chemo.	32	 9% 22% 22% 47%	55	 20% 13% 35% 33%	33	 21% 18% 30% 30%	88	 20% 15% 33% 32%

[a] n, number of cases with available data

Laboratory data: The median lymphocyte count for all cases on CBC was 39,675 lymphocytes/µL (IQR, 14,944-85,710/µL; range, 5,120-903,000/µL). Forty-one percent of cases were anemic and the anemia was generally mild to moderate (hematocrit median, 31.5%; IQR, 26-36%; range, 18-39%). Thirty-four of 50 anemic cases had a reticulocyte count performed and 50% had a regenerative anemia based on the reference interval of the laboratory, with reticulocyte counts ranging from 64,000-698,000 reticulocytes/µL (median, 119,000/µL; IQR, 102,950-246,500/µL). Twenty-four percent of cases were thrombocytopenic, with a decreased platelet count and no evidence of clumping. Thrombocytopenia severity was variable, but mild to moderate in most cases (median, 113,000 platelets/µL; IQR, 91,500-156,000/µL; range, 30,000-198,000/µL).

Blood smear review by a board-certified clinical pathologist was available for 107 cases. Ninety-two percent of cases were described as small, small to intermediate, or intermediate in size, and of those, 93% were described as having mature chromatin patterns. Nine cases were described as having intermediate to large sized lymphocytes cytologically, but these cases were included in the study, as the cells were small-sized by flow cytometry, with a B-cell:neutrophil size ratio ranging from 0.44-0.60. Small numbers of cases had descriptions of atypical chromatin patterns, including smooth, fine, or stippled. Moderate numbers of cases were described as having rare cells with a faint nucleolus, but 2 cases were described as having consistent nucleoli. Bone marrow cytology reports were available for 11 cases, and 3 samples were diagnosed with lymphocytic leukemia, 2 samples with lymphocytosis suspicious for CLL (40-45% small lymphocytes), 3 samples with mild lymphocytosis (7-16% small lymphocytes), 1 sample with few atypical lymphocytes consistent with possible lymphoid neoplasia, and 2 samples with lymphocytes considered within normal limits.

At diagnosis, 19% (n=22) of cases were hyperglobulinemic. Serum protein electrophoresis was performed in 8 cases. Six of 8 cases had a monoclonal gammopathy (the 1 case with immunofixation labeled with anti-IgM), 1/8 cases had a biclonal gammopathy (immunofixation not performed), and 1/8 cases had an IgM monoclonal gammopathy and a biclonal IgA gammopathy. Additional cases developed hyperglobulinemia after diagnosis, so that 25% of all cases had hyperglobulinemia during the course of disease. Thirteen cases had proteinuria on urinalysis, 3/13 had urine protein-creatinine ratio measured, which was elevated in all 3 cases, and none had urine protein electrophoresis performed.

Physical exam findings: Peripheral lymphadenopathy was detected in 53% of cases at diagnosis, and an additional 11 cases developed lymphadenopathy during the study, resulting in 62% of cases having peripheral lymphadenopathy at some time point. Lymph node cytology was evaluated in 37 cases, and 43% were diagnosed as lymphoma, 22% as possible/probable lymphoma, 24% as lymphoid hyperplasia/reactive lymphoid hyperplasia, and 11% as within normal limits. By histology (n=3), 1 case

was diagnosed with low grade small cell lymphoma, 1 with high grade small cell lymphoma (high mitotic rate), and 1 with probable small cell lymphoma. Fifty-four percent of cases had splenic abnormalities at diagnosis, an additional 5 cases developed splenic abnormalities after BCLL diagnosis (splenic abnormalities in 58% of total cases), and 2 cases had a previous splenectomy at the time of flow cytometry diagnosis. Splenic cytology was evaluated in 17 cases, and 29% were diagnosed as lymphoma, 41% as possible lymphoma, and 29% as lymphoid hyperplasia. By histology (n=3), 2 cases were diagnosed with low to intermediate grade lymphoma of small to medium sized lymphocytes and 1 case was diagnosed with lymphoid hyperplasia.

Forty-six percent of cases had thoracic or abdominal lymphadenopathy, and 33% of cases had hepatic abnormalities. Ten cases (8%) were presumed to have rectal involvement based on palpation, and half of these cases (4% of all cases) had rectal prolapse. Lymphoma was diagnosed in rectal tissue in 3 cases by cytology (n=1) or histology (n=2). Five cases had peritoneal effusion and 2 cases had bicavitary effusion. Five cases had multiple dermal or subcutaneous nodules interpreted as lymphoproliferative disease by the clinician based on gross appearance, and 2 cases with cytology were diagnosed as lymphoma. Six cases had ocular changes presumed secondary to lymphoproliferative disease or hyperglobulinemia/hyperviscosity syndrome by the clinician. Five cases had neurologic signs that were suspected to be secondary to lymphoproliferative disease at some point during the disease course; however, only 1 case had advanced imaging and that patient had multifocal disease throughout the spinal cord presumed secondary to progression of lymphoproliferative disease. Seven cases had pulmonary infiltrates on thoracic radiographs that were interpreted as possible neoplastic infiltrate by the radiologist.

Clinical signs: Sixty-two percent of patients were asymptomatic at diagnosis and leukocytosis +/- lymphadenopathy was an incidental finding. Thirty-eight percent of patients had clinical signs at presentation. Lethargy (n=21) and decreased appetite (n=20) were the most common clinical signs, with

weight loss (n=12), vomiting (n=9), diarrhea (n=9), and labored breathing (n=6) affecting smaller numbers of cases.

Flow cytometry

By flow cytometry, CD21+ B cells accounted for 64%-99% of lymphocytes in the blood in BCLL cases. The B-cell:neutrophil size ratio ranged from 0.37-0.60 (median, 0.50) in BCLL cases (n=121), compared to 0.39-0.54 (median, 0.44) in healthy controls (n=30). The percentage of B cells expressing CD25 was variable across BCLL cases, ranging from 0.6% to 99% (median, 64%; n=99), compared to healthy controls (median, 11%; range, 2.0%-24%) which had significantly fewer CD25-expressing B cells ($p<0.0001$) (Figure 6A). Class II MHC expression on B cells was significantly lower in BCLL cases (median, 158; range, 12-1168; n=99) compared to controls (median, 383; range, 256-511) ($p<0.0001$) (Figure 6B). CD21 expression was not significantly different between BCLL cases and control B cells, though BCLL cases had a wider range in CD21 expression (median, 48; range, 7-114; n=99) compared to controls (median, 46; range, 30-57) ($p=0.212$) (Figure 6C).

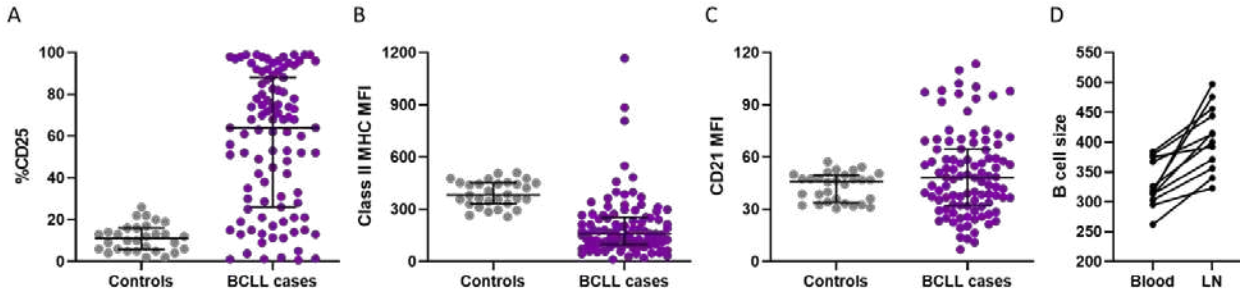

Figure 6. B-cell CD25, class II MHC and CD21 expression in BCLL cases compared to control B cells and comparison of B-cell size in paired blood and lymph node samples from BCLL cases. (A-C) Expression of CD25 (A), class II MHC (B) and CD21 (C) by flow cytometry is plotted for individual cases. Lines depict the median and interquartile range for each group. BCLL cases had significantly higher CD25 expression and significantly lower class II MHC expression than B cells from healthy controls. (D) Median forward light scatter of B cells is plotted for paired blood and lymph node samples from BCLL patients. B cells in the lymph node were significantly larger than those in the blood.

Flow cytometry and cytology were performed simultaneously on lymph node aspirates in 12 cases. All lymph node samples had a marked expansion of small-sized B cells, consistent with a small B-cell neoplasm. CD21+ B cells accounted for 88%-97% of all leukocytes in the sample and the median forward light scatter of B cells was <500 (median, 407; range, 323-497) in all cases. B cells in the lymph node were larger than the same patient's B cells in the peripheral blood by flow cytometry in all cases (Figure 6D). Nine of the 12 cases had flow cytometry and cytology performed on a lymph node aspirate at the same time. Five cases were diagnosed as lymphoma of small to intermediate-sized lymphocytes (n=1), intermediate lymphocytes (n=1), or intermediate to large-sized lymphocytes (n=3). One case was diagnosed with probable lymphoma, 2 cases as lymphoid hyperplasia, and 1 case as normal lymphocyte distribution.

Breed specific differences

Signalment, laboratory data, physical exam findings, and flow cytometry data were compared between 3 breed groups: small breeds with increased risk of BCLL, Boxer dogs, and other breeds. Across all variables, there were no significant differences between small breeds and other breeds, except for sex (males over-represented in the other breeds group, p=0.015); therefore, small breed and other breed cases were combined into a non-Boxer group (n=88). Comparing Boxer and non-Boxer cases, Boxers were significantly younger (median, 9.3 years) than non-Boxers (median, 10.6 years) (p=0.010). Boxers had a significantly higher presenting lymphocyte count (median, 72,700/µL) compared to non-Boxers (27,000/µL) (p<0.001) (Figure 7A). There was no difference in the frequency of anemia, thrombocytopenia, hyperglobulinemia, lymphadenopathy or splenic changes.

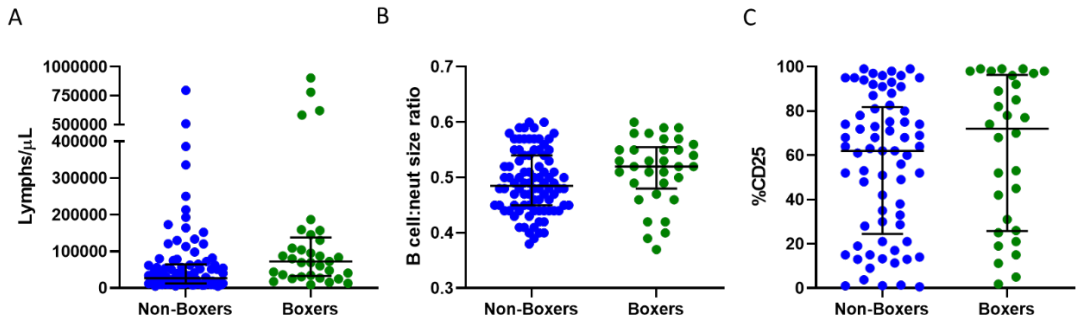

Figure 7. Breed-specific differences in lymphocyte count, B-cell size and B-cell CD25 expression between Boxers and non-Boxers with BCLL. Presenting lymphocyte count (A), B-cell:neutrophil forward side scatter ratio (B) and B-cell CD25 expression (C) are plotted for non-Boxer and Boxer BCLL cases. Lines depict the median and interquartile range for each group. Boxers had significantly higher presenting lymphocyte counts and larger sized B cells than non-Boxers. There was a significantly larger proportion of Boxer cases with very high CD25 expression (≥98% CD25-expressing B cells) compared to non-Boxers.

Comparing flow cytometry features across the 2 breed groups, Boxers had significantly larger B cells by flow cytometry compared to non-Boxers (p=0.037) (Figure 7B). Expression of CD21 and class II MHC on B cells was not significantly different between breed groups. The median percentage of CD25-expressing B cells was not significantly different between breed groups, however there was a larger proportion of Boxer cases (20%) with very high CD25 expression (≥98% CD25-expressing B cells) compared to non-Boxers (4%) (p=0.042) (Figure 7C).

Clonality and immunoglobulin mutation status

Sixty-four cases had sample available for PARR analysis. The PARR assay was performed on a blood sample in 61/64 cases, a splenic aspirate in 2 cases, and a lymph node aspirate in 1 case. 63/64 cases (98%) had clonal immunoglobulin rearrangements, supporting the diagnosis of B-cell neoplasia. The IGHV mutation status was determined in 14 cases, including 5 Boxers and 9 non-Boxers. Nine of 14 cases had unmutated IGHV genes, including all of the Boxers examined. Five cases had mutated IGHV genes.

Ki67 expression

The percentage of CD21+ B cells expressing Ki67 (Ki67%) was determined by flow cytometry in controls (n=12), BCLL cases (n=202) and large B-cell lymphoma cases (n=101) collected between 2017-2019. There was no significant difference in Ki67 expression between normal B cells in the control blood (n=5) and lymph node (n=7) samples, so these samples were combined for analysis. BCLL cases included small breeds with increased risk of BCLL (n=84), other small breeds (n=32), non-Boxer medium-large breeds (n=38) and Boxers (n=48). The median Ki67% in control B cells was 8.5%, and 11/12 control cases had low Ki67 expression, defined as <20% Ki67-expressing B cells (Figure 8).[120] Among non-Boxer BCLL breed groups, median Ki67% ranged from 14-16%, and there were no significant differences between small breeds with increased BCLL risk, other small breeds, or non-Boxer medium-large breeds. Boxers with BCLL had significantly higher Ki67% (median, 42%) compared to non-Boxer BCLL cases ($p<0.0001$). Large B-cell lymphoma cases had significantly higher Ki67% (median, 64%) than all BCLL groups ($p<0.0001$), and 87% of large B-cell lymphoma cases had >40% Ki67-expressing B cells. We used a Ki67% cutoff of >40% to identify high Ki67 expression cases.[120] The percentage of high Ki67 cases was significantly higher in Boxers with BCLL (58%) compared to non-Boxers with BCLL (27%) ($p<0.0001$), and the majority of non-Boxer BCLL cases (59%) had low ki67% (<20%).

Clinical outcome and survival analysis

The median overall survival time from flow cytometry diagnosis for all 121 outcome cases examined was 300 days (range, 1-1644 days). Boxers had significantly shorter survival (MST, 178 days) compared to non-Boxers (MST, 423 days) ($p<0.0001$) (Figure 9A). There was no significant difference in survival between small breed dogs and non-Boxer medium-large breed dogs ($p=0.46$), so all non-Boxer cases were combined for further analysis. Among Boxers, 47% of cases received MTD chemotherapy, 44% received corticosteroids or corticosteroids + chlorambucil, and 9% were untreated (Table 5).

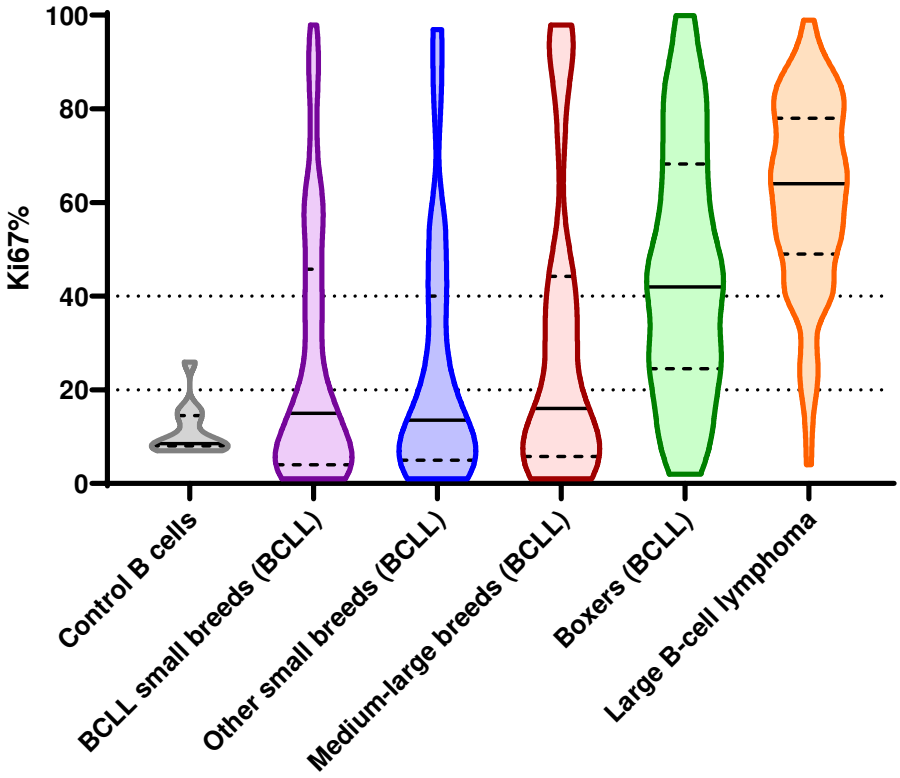

Figure 8. B-cell Ki67% expression measured by flow cytometry in control B cells, BCLL cases, and large B-cell lymphoma cases. The percentage of CD21+ B cells expressing Ki67 is on the vertical axis. The majority of non-Boxer BCLL cases had <20% Ki67 and there was no significant difference in Ki67 expression between small breeds with increased BCLL risk, other small breeds and non-Boxer medium-large breeds. Boxers with BCLL had significantly higher Ki67%. The vast majority of large B-cell lymphoma cases had high Ki67 (>40%). Violin plots show the median (solid line) and quartiles (dashed lines) for each group.

Among non-Boxers, 32% of cases received MTD chemotherapy, 48% received corticosteroids or corticosteroids + chlorambucil, and 20% were untreated. There was a trend toward more Boxers receiving MTD chemotherapy and more non-Boxers receiving no treatment, but neither finding was statistically significant (p=0.150 and p=0.164, respectively). The IGHV mutation status was not associated with outcome in the cases examined (p=0.562).

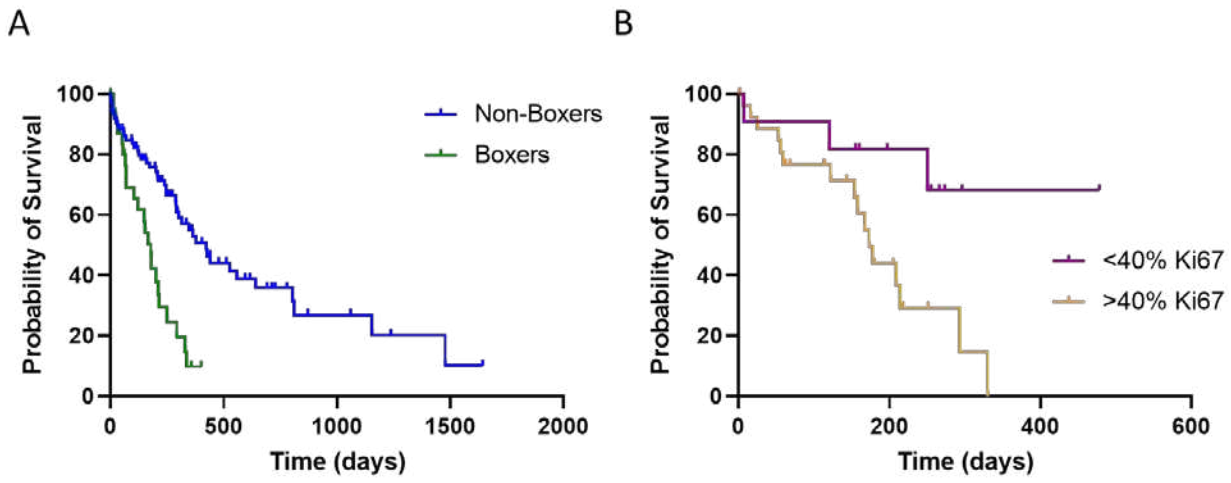

Figure 9. Kaplan-Meier curves showing overall survival in cases with BCLL. (A) Boxers had significantly shorter survival than non-Boxers. (B) Cases with high Ki67 (>40%) had significantly shorter survival than cases with low-intermediate Ki67 (<40%).

Ki67 data were available for 39 cases where survival data was also available, including 15 Boxers and 24 non-Boxers. High Ki67% (>40%) cases had significantly shorter survival (MST, 173 days; n=28) compared to cases with <40% Ki67 (MST, undefined; n=11) (p=0.026) (Figure 9B). The 3 cases with <40% Ki67 expression that died included a non-Boxer with heartworm disease and bicavitary effusion that died at home with no necropsy and the only 2 Boxers in the <40% Ki67 group, which were both managed aggressively with multi-agent MTD protocols. When Boxers were omitted from Ki67 survival analysis, the results did not change, indicating that non-Boxers with high Ki67% also have poorer prognosis. Low-intermediate Ki67 cases (<40% Ki67) were managed with corticosteroids +/- chlorambucil (36%) or no treatment (36%), except for the 2 Boxers in the group (18%, MTD chemotherapy) and 1 non-Boxer that received lomustine for a lung tumor but no treatment for BCLL. Among high Ki67 cases (>40% Ki67), 46% received MTD chemotherapy, 43% received corticosteroids +/- chlorambucil, and 11% were untreated. While a greater proportion of high Ki67 cases received MTD chemotherapy, this trend was not significant (p=0.30).

Signalment, laboratory, physical exam and flow cytometry variables were assessed for association with survival in all cases, non-Boxer cases only, and Boxer cases only, and results are summarized in Table 6. Among all cases, high presenting lymphocyte count, presence of clinical signs at diagnosis, and high CD25 expression were associated with significantly shorter survival. Presence of hyperglobulinemia was associated with significantly longer survival. Among non-Boxers only, lymphocyte count, clinical signs and hyperglobulinemia were still similarly associated with survival, and low CD21 expression was associated with shorter survival. Among non-Boxers only, CD25 expression was no longer significantly associated with survival. Among Boxers only, presence of clinical signs and absence of splenomegaly or splenic masses were associated with significantly shorter survival. Across all cases, though the number was small, cases with lymphocytes described as intermediate to large in size on blood smear review had significantly shorter survival than cases described as small, small to intermediate, or intermediate in size. When intermediate sized cases were combined in the intermediate-large group, this finding was no longer significant. Six of the 9 intermediate-large sized cases were Boxers, and when these were omitted, lymphocyte size by morphology was no longer significant, though the number of cases was small.

Table 6. Log rank test results evaluating factors for potential association with survival.

		All cases (n=121)			Non-Boxers (n=88)			Boxers (n=33)		
Variable	Cutoff	n	MST	p	n	MST	p	n	MST	p
Age	≥10 years	70	364	0.46	57	423	0.38	13	181	0.25
	<10 years	51	238		31	804		20	167	
Sex	Male	66	330	0.11	51	441	0.55	15	181	0.19
	Female	55	250		37	377		18	154	

Lymphocyte count	≥60,000 <60,000	45 76	173 423	<0.001 *	25 63	173 526	<0.001 *	20 13	178 167	0.70
Anemia	Present Absent	50 71	288 300	0.94	35 53	314 423	0.48	15 18	250 149	0.14
Thrombocytopenia	Present Absent	29 92	210 337	0.21	17 71	238 423	0.59	12 21	210 154	0.98
B cell size (flow cytometry)	≥0.50 <0.50	63 58	289 364	0.42	40 48	423 377	0.91	23 10	178 95	0.66
Lymphocyte size (blood smear)	Int-large Small-int	9 98	154 346	0.0025 *	3 75	59 427	0.10	6 23	160 181	0.53
Hyperglobulinemia	Present Absent	22 92	804 289	0.018*	16 66	NA 364	0.029*	6 26	250 178	0.12
Peripheral LAD	Present Absent	62 55	245 423	0.087	41 44	300 642	0.095	21 11	178 167	0.92
Visceral LAD	Present Absent	31 36	250 337	0.15	20 27	292 526	0.091	11 9	250 202	0.78
Splenic abnormalities	Present Absent	52 44	300 288	0.57	32 34	423 364	0.58	20 10	214 149	0.020*
Clinical signs	Present Absent	45 74	149 346	0.0031 *	31 55	200 427	0.026*	14 19	68 210	0.005*
CD25	≥64%	50	202	0.024*	33	314	0.19	17	104	0.054

	<64%	49	377		36	427		13	272	
CD21	≥48	50	364	0.12	36	526	0.045*	14	167	0.44
	<48	49	207		33	300		16	178	
Class II MHC	≥15.8	50	250	0.57	37	364	0.58	13	167	0.51
	<15.8	49	314		32	427		17	178	
Treatment	MTD	43	250	0.11	28	314	0.30	15	202	0.024*
	Stero/chlor	36	526		29	526		7	167	
	Steroids	20	178		13	364		7	69	
	None	21	293		9	423		3	210	

n, number of cases; MST, median survival time (days); LAD, lymphadenopathy; MTD, maximum tolerated dose chemotherapy; stero/chlor, corticosteroids and chlorambucil; *p value <0.05 was considered significant.

Discussion

This study found that BCLL, as defined by an expansion of small-sized CD21+ B cells in the blood by flow cytometry, has a variable clinical presentation and outcome. Cases demonstrated wide variability in overall survival times, and Ki67 expression was useful in identifying cases with a poor prognosis. Additionally, Boxers with BCLL had significantly shorter overall survival.

The overall survival time in this study for all 121 BCLL cases was 300 days (range, 1-1644 days), however, Boxers were intentionally over-represented in this population. When survival was assessed in non-Boxers, the MST of 423 days was similar to that previously reported in canine BCLL.[84] A subset of patients in this study had more indolent disease and were frequently asymptomatic, managed with corticosteroids, corticosteroids + chlorambucil or no treatment, and had prolonged survival. However, a

subset had more aggressive disease and shorter overall survival. We aimed to find factors that would help identify these patients.

B-cell Ki67 expression, as measured by flow cytometry, was variable in BCLL cases and associated with prognosis. The majority of non-Boxers had low-intermediate Ki67 expression (Ki67% <40%), but a subset of non-Boxers and the majority of Boxers had high Ki67 expression (Ki67% >40%). Cases with high Ki67 expression, indicating increased cellular proliferation, had significantly shorter overall survival. A previous study which evaluated the prognostic significance of Ki67 expression by flow cytometry in canine high-grade B-cell lymphoma also used Ki67% >40% to define high Ki67 cases.[120] That group defined low Ki67% as <20%, and our study found that peripheral blood B cells in healthy control dogs and the majority of non-Boxer BCLL cases had Ki67% <20%. We did not have enough low-intermediate Ki67 BCLL cases with outcome to determine whether low Ki67 cases (Ki67% <20%) and intermediate Ki67 cases (Ki67% 20-40%) have different prognoses, but rather, these cases were combined for analysis. Future studies could evaluate whether distinguishing low and intermediate Ki67 expression is significant in BCLL prognosis. In human CLL, high Ki67 expression is associated with poorer outcome, whether measured in neoplastic peripheral lymphocytes, lymph node proliferation centers, or as circulating Ki67 levels in plasma.[25,124,125]

We hypothesized that Boxers with BCLL have more aggressive disease, due to preferential use of unmutated IGHV genes in neoplastic B cells,[121] which is associated with poor prognosis in human CLL.[13,14] In this study, Boxers had significantly higher presenting lymphocyte counts, higher Ki67 expression and shorter overall survival compared to non-Boxers. However, when we evaluated survival in a group of Boxers and non-Boxers with IGHV sequencing, mutation status was not predictive of outcome. It is possible that too few cases were examined to detect a difference in survival, or, IGHV mutation status may not drive tumor biology in canine BCLL at all or as much as other factors. Additionally, we looked to

see whether other medium-large breed dogs had poorer prognosis similar to Boxers, but these other breeds had similar Ki67 expression and survival as small breed dogs.

We hypothesize that canine BCLL can have different manifestations, as seen with human CLL, and Ki67 expression is one marker useful in distinguishing them. In addition to shorter survival, a higher proportion of Boxers and high Ki67 cases were treated with MTD chemotherapy, suggesting clinicians independently interpreted these cases as having more advanced or aggressive disease. In another subset of BCLL cases, including those with low Ki67 expression, which had more indolent disease, no treatment or treatment with corticosteroids +/- chlorambucil appeared to be an appropriate therapy. It is possible that these different subsets actually have different small cell B-cell neoplasms, rather than different clinical manifestations of BCLL. Gene expression profiling and identification of driver mutations would be useful in determining whether these cases represent a spectrum of one disease or distinct diseases. Currently, we do not have antibodies available to differentiate these BCLL subsets by routine immunophenotyping. Among the B-cell antigens we detect in routine immunophenotyping, CD25 tended to be expressed at a higher level in Boxers compared to non-Boxers, but there was significant overlap between groups. Gene expression profiling may differentiate BCLL subsets, but until then, Ki67 expression appears useful in identifying more aggressive cases.

In addition to Ki67 expression and breed, we found that lymphocyte count, presence of clinical signs and hyperglobulinemia were associated with prognosis. A high presenting lymphocyte count (>60,000/µL) was associated with shorter survival in non-Boxer BCLL cases. Lymphocyte count was not prognostic in two prior studies of canine BCLL.[84,85] It is possible that these studies did not detect a difference due to smaller sample sizes (n=17 and n=21, respectively), or because they assessed association with survival using a different cutoff (30,000/µL) or lymphocyte count as a continuous variable. In our study, lymphocyte count was not prognostic when using the median lymphocyte count of the study population as a cutoff (40,000/µL), but was prognostic when the cutoff was raised to

60,000/µL. BCLL patients presenting with clinical signs had significantly shorter survival, which is an important prognostic factor in other types of canine lymphoma.[126,127] In human CLL, high lymphocyte count and clinical symptoms are associated with poorer prognosis by univariable analysis and help guide treatment decisions, but they are not factors used in the historic Rai or Binet staging systems nor more recent prognostic staging systems.[47,48,51,52,128] Hyperglobulinemia affected 19% of canine BCLL patients at diagnosis and was associated with longer survival. Serum protein electrophoresis was performed on only a subset of hyperglobulinemic samples, but all those examined had paraprotein detected. Paraproteins in this study and a separate study from our laboratory (chapter 4) were identified with immunofixation as IgM or less frequently IgA. In human CLL, serum free light chains are the most commonly detected paraproteins and are associated with poor prognosis.[129] IgM or IgG paraproteins are detected in a smaller proportion of cases and are also associated with poorer prognosis.[130,131] It was surprising that canine BCLL patients with hyperglobulinemia had prolonged survival, however, only a small subset of cases had SPE performed so it is unknown whether the hyperglobulinemia was secondary to a reactive process or paraprotein. If cases with paraprotein have prolonged survival, perhaps those cases have different signals driving B-cell stimulation and expansion or neoplastic B cells are more differentiated, affecting the clinical course of disease. High CD25 expression was associated with shorter survival across all cases, but not when assessed in non-Boxers only. Increased CD25 expression was associated with more progressive disease and poor prognosis in some human CLL studies but not others, as well as poor prognosis in canine diffuse large B-cell lymphoma, but more work is needed to fully elucidate its prognostic significance in canine BCLL.[122,132–134] Age was not prognostic in our study as it was in a prior study.[84] The vast majority of cases in our study were middle-aged to older (only 7/121 (6%) were <6 years old), so perhaps there were too few young cases to appreciate a difference in survival.

In this study, we included all cases with a flow cytometry diagnosis of BCLL, irrespective of tissue involvement. Human CLL commonly affects secondary lymphoid tissues and SLL, which is defined by

lymphadenopathy and/or splenomegaly, is considered the same neoplasm as CLL without a leukemic component.[2] Therefore, we did not want to exclude canine BCLL cases with secondary lymphoid organ enlargement and found that lymphadenopathy and splenomegaly or splenic masses were common in this study population. Other less common sites included rectal tissue, with rectal prolapse affecting 4% of cases, and apparent dermal, subcutaneous and pulmonary tissues. All 12 cases with flow cytometry performed on a lymph node aspirate had a marked expansion of small-sized B cells, consistent with neoplasia. These cases had a wide range of cytology diagnoses, demonstrating the difficulty in diagnosing small cell neoplasms by cytology alone. In the peripheral blood, lymphocyte cell size was also variable across blood smear pathology reports. It was not unusual for cases to be described as having intermediate-sized lymphocytes; these cases had a small B-cell size by flow cytometry and had the same prognosis as cases described as small-sized. These findings suggest that BCLL tumors may have intermediate-sized lymphocytes described in lymph node cytology or blood smear evaluation and this morphology does not necessarily indicate a different tumor or prognosis. A small number of cases were described as having intermediate to large-sized lymphocytes in the blood and these cases did have a poorer prognosis, with an over-representation of Boxers. These cases may represent more aggressive BCLL disease or a different B-cell neoplasm, such as mantle cell lymphoma, marginal zone lymphoma, or follicular lymphoma, all of which may have a leukemic component in humans.[2] Nuclear descriptions were variable or lacking in these cases, making it difficult to fully characterize the cells in this retrospective study. Future studies that objectively characterize lymphocyte morphology on fresh blood films would be needed to thoroughly evaluate the utility of morphologic features in prognosis. Human CLL may have admixed larger cells and up to 55% prolymphocytes, and cases with increased prolymphocytes have poorer prognosis.[135] A previous study of canine CLL found that prolymphocytes were not prognostic, but only 1/17 BCLL cases had >10% prolymphocytes.[84]

Limitations of this study include the retrospective study design, incomplete staging for some cases, variable treatment protocols and lack of necropsies. Treatments were variable and could have been influenced by owners' willingness to pursue therapy, making it difficult to assess the efficacy of different treatment types. In many cases, death or euthanasia was attributed to BCLL progression, but was not confirmed by necropsy. In some cases, patients were euthanized due to declining clinical condition, but given the old age and co-morbidities in these patients, cause of death is not certain. Additionally, by including cases with extensive tissue involvement and/or atypical cellular morphology, we may have included cases with a different small cell B-cell neoplasm. However, we currently cannot differentiate subtypes of small cell B-cell neoplasms by immunophenotyping, and therefore wanted to include any cases that would have a diagnosis of BCLL by routine flow cytometry.

In conclusion, this study demonstrated that canine BCLL, as defined by flow cytometry, has a variable clinical course. High Ki67 expression and Boxer breed were associated with more aggressive disease. We hypothesize that BCLL is a heterogeneous tumor, as is seen with human CLL. Future work to evaluate differences in gene expression and underlying mutations between subsets with different clinical behavior will be particularly important.

CHAPTER 3: Gene expression profiling in canine B-cell chronic lymphocytic leukemia

Summary

B-cell chronic lymphocytic leukemia is a common tumor in dogs and humans, defined by an expansion of small mature-appearing B cells in the blood and/or bone marrow. These diseases share similar presentations and heterogeneous clinical courses, and we hypothesize that spontaneous canine BCLL may serve as a natural model to study human CLL. However, little is known about the pathways driving canine BCLL development and progression. Our objective was to examine the gene expression profile of canine BCLL by NanoString and RNA Sequencing (RNA Seq) technology, to identify key molecular pathways in canine BCLL and compare the transcriptomes of canine BCLL and human CLL. Additionally, we examined heterogeneity and breed-specific differences in gene expression among canine BCLL samples, targeting small breeds and Boxers. For the NanoString study, RNA was extracted from peripheral blood in BCLL cases (n=26), lymph node samples in large B-cell lymphoma cases (n=23), and sorted CD21+ B cells from healthy dog lymph nodes (n=8). Expression was determined for a set of 327 genes hand-curated from the human CLL literature. For the RNA Seq study, RNA was extracted from 12 dogs with BCLL and sorted B cells from 3 healthy dog lymph node samples. Gene set enrichment analysis was used to identify enriched pathways and biological functions in canine BCLL and test whether human CLL gene sets were differentially expressed between canine BCLL cases and control B cells. Gene expression analysis segregated BCLL cases from large B-cell lymphoma cases and control B cells. By both methods, canine BCLL gene expression profiles were significantly enriched for the human CLL gene sets. By RNA Seq, 1,671 genes were differentially expressed between canine BCLL samples and control B cells. Canine BCLL exhibited upregulation of cytokine signaling pathways, KRAS signaling, NF-κB signaling and B-cell receptor signaling pathways. Clustering of BCLL samples by NanoString and RNA Seq analyses identified two subgroups, termed BCLL subgroup 1 and 2. We identified 3,541 genes

differentially expressed between subgroups by RNA Seq; BCLL subgroup 2 had overexpression of cell cycle progression and proliferation genes, and BCLL subgroup 1 had overexpression of the TNFA signaling via NF-κB pathway and ribosome and spliceosome genes. BCLL subgroup 2 had significantly higher Ki67 expression and an over-representation of Boxer dogs, previously shown to be associated with poor prognosis. These results identified similarities in gene expression profiles between canine BCLL and human CLL and identified two molecular subgroups of BCLL for further study.

Introduction

B-cell chronic lymphocytic leukemia is a common tumor in dogs and humans. In dogs, hematologic malignancies are among the most common tumors diagnosed, and BCLL accounts for approximately 8% of canine samples submitted for immunophenotyping with a suspicion of lymphoproliferative disease.[62,136] In humans, CLL is the most common adult leukemia in western countries and approximately 5.0 new cases per 100,000 men and women are diagnosed per year.[1,88] The dog is increasingly recognized as a model to study naturally occurring cancers, including to identify underlying genetic predispositions, to investigate mechanisms of tumor development, and to test new therapies.[57,58] Similarities between canine and human tumors have been identified for several tumor types, including osteosarcoma, hemangiosarcoma, and urinary bladder tumors.[57,137–139] To determine the utility of the canine model for particular tumor types, studies have assessed similarities in clinical presentation, histology or immunophenotype of neoplastic cells, risk factors, somatic or genomic changes, and dysregulated pathways driving tumor development.

Previous studies evaluating the clinical presentation and outcome of canine BCLL have identified similarities to human CLL. In both species, these neoplasms are defined by an expansion of small-sized B cells in the blood and bone marrow, with lymph nodes and spleen often affected.[52,62] B cells generally have small mature-appearing cytomorphology, with smaller proportions of intermediate-sized

lymphocytes or prolymphocytes.[52,140] Human CLL cells express B-cell surface antigens CD19, CD20 and CD23, as well as the T-cell antigen CD5.[33] Canine BCLL cells express the B-cell surface antigen CD21, but not the CD5 antigen.[62] Antibodies to CD19, CD20, and CD23 are not readily available for routine immunophenotyping in the dog. These tumors typically affect older patients, with a median age at diagnosis of 67-72 years in people and 10-12 years in dogs.[52,62,66,84] Humans with a family history of CLL have an increased incidence of disease, and small breed dogs have increased risk of BCLL suggesting a genetic predisposition.[62,141]

Human and canine CLL have diverse clinical presentations and outcomes, ranging from indolent disease to more aggressive disease requiring therapy (chapter 2).[52] Early staging systems in human CLL were based on the extent of blood and/or bone marrow, lymph node, spleen and liver involvement and presence of anemia and/or thrombocytopenia.[47,48] More recently, numerous additional prognostic factors have been evaluated and an international consortium developed the CLL International Prognostic Index (CLL-IPI), which incorporates clinical, biological and genetic variables.[51] This prognostic score incorporates TP53 status, immunoglobulin heavy variable region (IGHV) mutation status, serum β_2-microglobulin concentration, clinical stage, and age, and identifies four risk groups with significantly different overall survival. Canine BCLL also has a variable clinical outcome with a wide range in survival times.[84,85] Some of the human prognostic factors have not been assessed or were not prognostic in canine BCLL, but age and IGHV mutation status potentially play a role. Age was prognostic in one study,[84] but not another (chapter 2). We previously found that Boxers preferentially rearrange unmutated IGHV genes and have poor prognosis, however, IGHV mutation status was not prognostic in a small number of non-Boxers, so the prognostic utility of IGHV mutation status in canine BCLL needs further investigation.[121] We demonstrated that Ki67 expression, a marker of proliferation, was prognostic in canine BCLL and Boxers had significantly higher Ki67 expression than small breed dogs (chapter 2).

Several studies have evaluated the gene expression profile of human CLL and examined differential gene expression between prognostically different patient subsets. Two earlier studies using microarrays found that CLL samples displayed a common gene expression profile distinct from other B-cell neoplasms and only a small number of genes distinguished IGHV mutated and unmutated cases, suggesting that mutated and unmutated CLL are in fact the same disease.[22,142] Human CLL appeared to have an antiapoptotic phenotype with upregulation of genes in signal transduction pathways, including B-cell receptor (BCR) signaling.[22] Rosenwald et al. found overexpression of B-cell activation genes in CLL patients with unmutated IGHV, suggesting the aggressive disease course in these patients may be attributed to enhanced BCR signaling.[142] Two studies performed with RNA Seq highlighted alternative splicing events in CLL, which can alter protein structure and phosphorylation targets and ultimately affect numerous signaling pathways.[143,144] Gene expression studies have also sought to determine the cell of origin for CLL. An early study found that tumor cells were most similar to memory B cells,[22] while a more recent study comparing CLL to additional normal B-cell subsets determined that CLL is derived from CD5+ B cells, which represent a distinct differentiation stage in humans.[15] IGHV unmutated CLL was derived from mature CD5+CD27- B cells, while mutated CLL was derived from a previously unrecognized post-germinal center memory CD5+ subset expressing the memory B-cell marker CD27 and carrying somatically mutated IGHV genes.[15]

The goal of this study was to evaluate the gene expression profile of canine BCLL, which has not previously been examined. Gene expression was evaluated by NanoString analysis and RNA Seq analysis. NanoString studies evaluate expression of a pre-defined set of genes, which limits the scope of the analysis, but allows for evaluation of a larger number of samples and may be performed on lower quantity and lower quality RNA samples.[145] RNA Seq allows for global assessment of the transcriptome, but costs can limit the number of samples analyzed. Given the overlap in clinical presentation and disease progression between human and canine CLL, one aim of this study was to compare human CLL

and canine BCLL gene expression profiles. A second aim was to evaluate the heterogeneity among canine BCLL samples, and to determine whether BCLL cases with different Ki67 expression have distinct gene expression profiles.

Methods

Cases and controls

Cases included BCLL cases and large B-cell lymphoma cases that had immunophenotyping by flow cytometry performed at the Colorado State University-Clinical Immunology laboratory between July 2014 and August 2017. BCLL was defined by an expansion of small-sized CD21+ B cells in the peripheral blood, as previously described.[62] BCLL samples from Boxers or small breeds with an increased risk of BCLL were randomly selected. Ten BCLL samples were used for NanoString and RNA Seq experiments, 16 were used for NanoString only, and 2 were used for RNA Seq only. BCLL cases with available DNA had IGHV mutation status determined, as previously described.[121] Large B-cell lymphoma was defined by an expansion of CD21+ B cells with a median forward scatter >500 and high class II MHC expression (median fluorescence intensity, >100) in a lymph node aspirate. Cases with these flow cytometry features were previously shown to be predominantly of the diffuse large B-cell lymphoma (DLBCL) histologic subtype.[122] Twenty-three large B-cell lymphoma samples were used for the NanoString experiment: 13 samples were randomly selected from Golden retrievers, as Golden retriever results were being used for a second study; 3 samples were randomly selected from Boxers; and, an additional 7 cases irrespective of breed were randomly selected. Study population patient information is summarized in Table 7.

Table 7. Summary patient information for BCLL cases and large B-cell lymphoma cases analyzed in NanoString and RNA Sequencing experiments.

	NanoString BCLL cases (n=26)	NanoString large B-cell lymphoma cases	RNA Seq BCLL cases (n=12)

		(n=23)	
Age (years), median (IQR; range)	10.3 (8.3-11.3; 5.2-15.0)	8.2 (7.4-10.7; 2.8-14.5)	11.1 (8.8-13.2; 7.4-15.0)
Sex (%)	73% female	43% female	75% female
Breed distribution (n)[a]	BIC (1), BOST (1), BOX (11), CRN (1), COC (1), CSH (1), DACH (1), JRT (2), MLT (2), POM (1), SHI (3), TIBT (1)	BASS (1), BOR (1), BOX (3), CCR (1), GLDR (13), LAB (1), MAST (1), NEWFIE (1), VSL (1)	BOX (4), CRN (1), COC (1), DACH (1), JRT (1), MLT (2), POM (1), SHI (1)
CBC lymphocytes/µL, median (IQR; range)	58,100 (30,289-108,150; 6,450-473,690)	NA	43,430 (22,170-71,025; 6,450-276,298)
Lymphadenopathy, (% affected)[b]	11/20 (55%)	23/23 (100%)	4/10 (40%)
Anemia, (% affected)[b]	7/26 (27%)	2/15 (13%)	3/12 (25%)
Thrombocytopenia, (% affected)[b]	6/26 (23%)	2/14 (14%)	1/12 (8%)

IQR, interquartile range; n, number; NA, not applicable. [a] Breed abbreviation: BASS, Bassett hound; BIC, Bichon frise; BOR, Border collie; BOST, Boston terrier; BOX, Boxer; CCR, Chinese crested; COC, Cocker spaniel; CRN, Cairn terrier; CSH, Chihuahua shorthair; DACH, Dachshund; GLDR, Golden retriever; JRT, Jack Russell terrier; LAB, Labrador retriever; MAST, Mastiff; MLT, Maltese; NEWFIE, Newfoundland; POM, Pomeranian; SHI, Shih Tzu; TIBT, Tibetan terrier; VSL, Vizsla. [b] For lymphadenopathy, anemia, and thrombocytopenia, the number of affected cases/the number of cases with available data is provided; the percent affected among cases with available data is reported.

Control B cells were obtained from peripheral lymph node biopsies from healthy dogs that were used for IACUC-approved continuing education surgery courses. These samples had no evidence of

lymphoproliferative disease by histology, flow cytometry or clonality testing by PCR for antigen receptor rearrangements.

Cell sorting and RNA isolation

Peripheral blood samples were sorted to purify B cells in the majority (20/28) of BCLL cases; the remaining 8 BCLL cases that were not sorted had >80% B cells comprising the leukocyte population. In sorted samples, B cells were purified from leukocyte pellets by magnetic depletion of T cells and CD18-expressing myeloid cells using the Miltenyi Biotec protocol (Miltenyi Biotec, San Diego, CA). The leukocyte cell pellet was re-suspended in 145 µL flow buffer (PBS-2%FBS-0.1%NaAZ) with the following antibodies (purchased from Bio-Rad, Hercules, CA): anti-CD21 (A647, CA2.1D6 clone), anti-CD4 (FITC, YKIX302.9 clone), anti-CD8 (PE, YCATE55.9 clone), and anti-CD18 (PE, YFC118.3 clone). Samples were incubated for 10 minutes in the dark at 4^{o}C and washed with MACS buffer (phosphate-buffered saline [PBS]-0.5% BSA-2 mM ethylenediaminetetraacetic acid [EDTA] −1 mM). The cell pellet was resuspended in 50 µL MACS buffer, 20 µL PE beads (Miltenyi Biotec), and 20 µL FITC beads (Miltenyi Biotec), incubated for 10 minutes in the dark at 4^{o}C, washed and resuspended in 500 µL MACS buffer, and loaded onto the prepared LD column in a MidiMACS separator. The column was rinsed twice with 1 mL MACS buffer and the sorted B cells that were not bound by beads were collected. The median purity, determined by flow cytometry, was 93% (interquartile range [IQR], 88-96%). All NanoString BCLL samples had purity >80% except for 3 cases (60%, 77%, 79%) and all RNA Seq BCLL samples had purity >80% except for 1 case (77%). The large B-cell lymphoma samples used for NanoString analysis were not sorted and contained ≥80% CD21+ B cells (median, 90.5% B cells; range, 80.0-97.6% B cells).

CD21+ B cells were purified from control healthy dog lymph nodes by fluorescence-activated cell sorting. Samples were stained with anti-CD21-PE (B cells, clone CA2.1D6), anti-CD5-APC (T cells, clone YKIX322.3), anti-CD4-Pacific Blue (CD4 T cells, clone YKIZ302.9) and anti-CD8-FITC (CD8 T cells, clone

YCATE 55.9) and sorted on a MoFlo cell sorter (Beckman Coulter, Brea, CA). Purity was determined by flow cytometry and sorted samples with >90% CD21+ B cells were used for RNA isolation.

RNA was isolated using the Purelink RNA mini kit (Life Technologies, Carlsbad, CA). Cell samples were suspended in prepared RNA lysis buffer and stored at -80^{o}C until RNA isolation. RNA was isolated according to manufacturer instructions, RNA concentration was determined using a Nanodrop and/or Qubit fluorometer instrument (Thermo Fisher Scientific, Waltham, MA), and RNA integrity was assessed using an Agilent RNA ScreenTape assay (Agilent Technologies, Santa Clara, CA). NanoString sample concentrations ranged from 3.2-690 ng/µL and RNA integrity numbers (RINs) ranged from 3.3-8.8. RNA Seq sample concentrations ranged from 28-672 ng/µL and RINs ranged from 8.3-10.

NanoString: Gene expression and statistical analysis

NanoString technology was used to measure the expression of a custom set of 327 genes. Genes were selected that define the human CLL gene expression signature, have prognostic significance in human CLL, and/or are differentially expressed between normal B-cell subsets.[15,22,142,143,146–151] Six housekeeping genes were selected that demonstrated low variability between samples in a previous canine NanoString experiment: EEF1G, GUSB, HPRT1, POLR2A, RPL19, SDHA.[152] Approximately 100 ng of sample RNA was hybridized to custom-built mRNA probes and analyzed with the nCounter Digital Analyzer system (NanoString Technologies, Seattle, WA) through the University of Arizona Genetics Core.

NanoString quality control, normalization, and data analysis were performed using nCounter software (version 4.0, NanoString Technologies). Quality control metrics assessing imaging, binding density, positive control linearity and limit of detection were acceptable, and all data were used for analysis. A background threshold of 20 was used to correct for probes with very low counts. Standard two-step normalization was performed using a combination of positive control normalization (synthetic

control targets) and CodeSet content normalization (housekeeping genes). Three housekeeping genes were differentially expressed between BCLL cases and controls and were omitted (EEF1G, HPRT1, SDHA). Remaining housekeeping genes (GUSB, POLR2A, RPL19) had little variability and were used for normalization. Normalized data were used to calculate ratios between control B-cell, BCLL and large B-cell lymphoma groups to assess differential expression. Ratios were subsequently converted to $\log_2$ fold-changes. The Benjamini-Yekutieli False Discovery Rate (FDR) method was used to compare groups and an FDR adjusted p value <0.05 was considered significant.

NanoString experiment heat maps were generated by unsupervised hierarchical clustering (Pearson correlation, average linkage) in Morpheus (https://software.broadinstitute.org/morpheus) using normalized $\log_2$ gene counts. Very low expressing genes (≤20 counts in >80% of cases) were omitted and the resulting 312 genes were included in the heat maps. To compare expression of individual genes between groups, normality was assessed using a Shapiro Wilk test and a t test or Mann Whitney test was performed using normalized $\log_2$ gene counts (GraphPad Prism, version 8.1, La Jolla, CA). A p value <0.05 was considered significant. Gene Set Enrichment Analysis (GSEA) software (version 4.0.3, https://www.gsea-msigdb.org/gsea/index.jsp) was used to determine whether a defined gene set was differentially expressed between BCLL cases and controls.[153,154] Three gene sets were hand-curated using the human CLL literature: (1) genes overexpressed in CLL vs. control B cells; (2) genes underexpressed in CLL vs. control B cells; (3) genes overexpressed in CLL vs. DLBCL.[15,22,142,143,146–148] Enriched gene sets were identified using 1000 permutations of the phenotype labels. Among the GSEA statistics, the normalized enrichment score (NES) reflects the degree to which the tested gene set was overrepresented in the phenotype of interest. The statistical significance of the enrichment score is estimated by the nominal p value. The false discovery rate (FDR) estimates the probability of a false positive result, and an FDR <0.25 was considered significant.

RNA Sequencing: Gene expression and statistical analysis

Sample libraries were prepared and sequenced at Novogene Corporation Inc. (Sacramento, CA). For library construction, mRNA was enriched using oligo(dT) beads and fragmented, and cDNA was synthesized using random hexamers and reverse transcriptase, followed by second-strand synthesis using an Illumina buffer containing dNTPs, RNase H and Escherichia coli polymerase I. The cDNA library was subjected to purification, end repair, addition of a single A base, ligation of sequencing adapters, size selection and PCR enrichment. Libraries were sequenced on an Illumina HiSeq. Data quality control information for each sample is provided in Supplemental table S2. Sequencing reads were aligned to the CanFam3.1 reference genome using TopHat2 and mapping result details are provided in Supplemental table S3.[155] GTF files providing genes features were obtained from the Ensembl website (release 82; http://www.ensembl.org/info/data/ftp/index.html).[156]

Sequencing data were uploaded to the Galaxy web platform (https://usegalaxy.org/) for further analysis.[157] Read counts per gene were generated using HTSeq-count.[158] Read count data normalization and differential gene expression analysis were performed with DESeq2.[159] A Benjamini-Hochberg adjusted p value <0.05 was considered significant to identify differentially expressed genes. Pairwise comparisons were made between controls and all BCLL samples or BCLL subgroups. Similarity among transcriptional profiles was visualized by unsupervised hierarchical clustering (Morpheus software) and principal component analysis (DESeq2). Unsupervised hierarchical clustering and heat maps were generated in Morpheus using either fragments per kilobase million (FPKM) values for filtered genes or rlog normalized counts of differentially expressed genes. For FPKM data, we omitted genes with low expression or low variability by excluding genes where FPKM equaled 0 in >20% of samples or the standard deviation across samples was <10. The FPKM values for the remaining 4,622 genes was log transformed. Differentially expressed gene lists generated by DESeq2 were analyzed by GSEA software to identify altered pathways and biological functions. GSEA was performed using curated gene sets from

the Molecular Signature Database and gene sets from the Staudt laboratory (https://lymphochip.nih.gov/signaturedb/index.html).[153] Venn diagrams were created to visualize overlap in differentially expressed gene lists (http://genevenn.sourceforge.net/). We used GSEA to assess whether the canine BCLL gene expression profile was significantly enriched for human B-cell leukemia/lymphoma gene sets. Sets of genes that are overexpressed or underexpressed in tumor samples compared to controls were compiled for CLL, mantle cell lymphoma, splenic marginal zone lymphoma, and follicular lymphoma.[143,160–162] To compare expression of individual genes between groups, normality was assessed using a Shapiro Wilk test, and a t test or Mann Whitney test was performed using rlog normalized counts (GraphPad Prism). The normalized log transformed counts of individual genes were compared between NanoString and RNA Seq methods using a Pearson correlation (GraphPad Prism).

Results

NanoString and RNA Seq identify genes differentially expressed in BCLL and control B cells

Hierarchical clustering of gene expression data clearly separated the controls and BCLL samples in the NanoString and RNA Seq experiments (Figure 10). Large B-cell lymphoma samples were also clearly separate from controls and BCLL samples in the NanoString study (Figure 10A). The gene expression profile of normal B cells from healthy control dog lymph nodes was relatively homogeneous, while BCLL samples demonstrated more heterogeneity. Clustering identified 2 main subgroups of BCLL cases (Figure 10). By RNA Seq, 2 BCLL samples formed their own clade, but their gene expression profiles were most similar to BCLL subgroup 2 visually and by principal component analysis, so these cases were grouped for further analysis. The 10 BCLL samples that were included in the NanoString and RNA Seq studies were clustered into the same BCLL subgroup by either method. There was no distinct separation of Boxers and small breeds with BCLL by either method, however 9/11 (82%) and 3/4 (75%) Boxers clustered in the BCLL 2 subgroup by NanoString and RNA Seq, respectively. Small breed dogs were

intermixed between BCLL subgroups. Of BCLL cases where IGHV mutation was determined, 4/4 (100%) BCLL subgroup 1 cases had mutated IGHV genes, and 5/12 (42%) BCLL subgroup 2 cases had mutated IGHV genes.

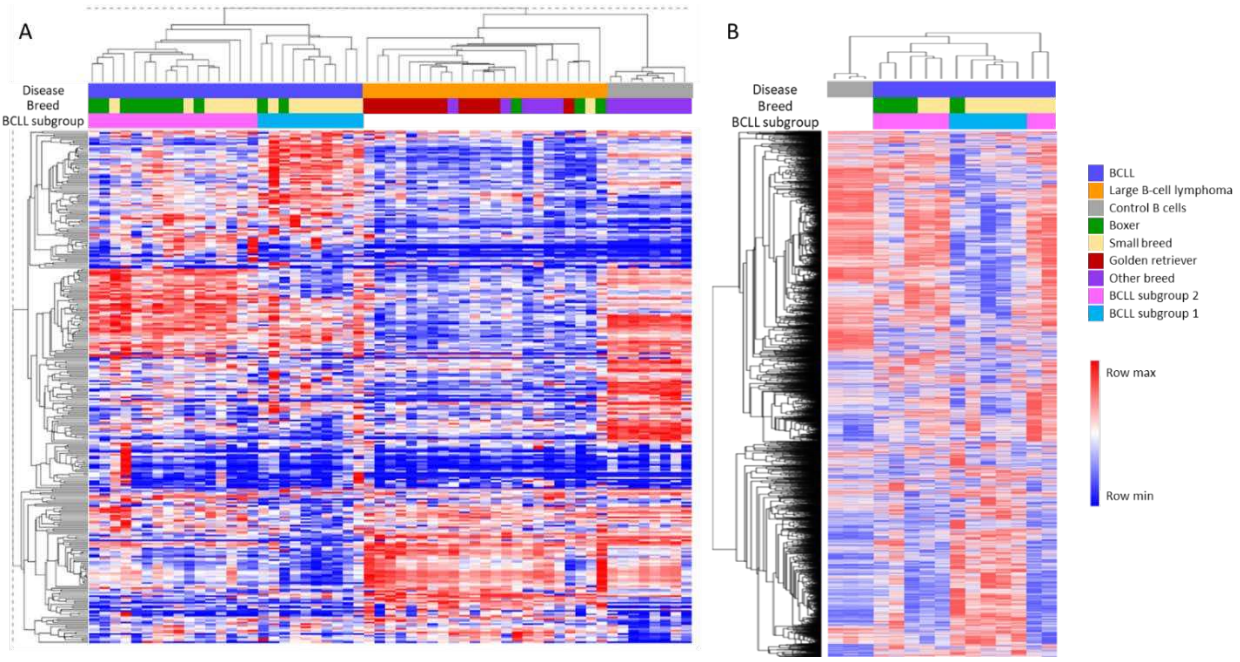

Figure 10. Hierarchical clustering analysis of BCLL cases, large B-cell lymphoma cases and control B cells. (A) Hierarchical clustering was performed using 312 genes in the NanoString experiment. The BCLL cases, large B-cell lymphoma cases, and normal B cells from healthy control lymph nodes separated clearly. The BCLL cases formed 2 main subgroups, and the majority of Boxer cases were in BCLL subgroup 2. (B) Hierarchical clustering was performed using FPKM values of 4,622 filtered genes in the RNA Seq experiment. Normal B cells from healthy controls formed a relatively homogeneous clade. The BCLL cases were more heterogeneous and formed 2 main subgroups. The 2 cases to the far right were grouped with BCLL subgroup 2 for further analysis.

In the NanoString dataset, we quantified the number of genes significantly differentially expressed in pairwise comparisons between normal B cells from control dog lymph nodes, BCLL cases (either all together or as BCLL subgroups 1 or 2), and large B-cell lymphoma cases (nSolver analysis, FDR <0.05) (Table 8). 175/327 (54%) genes in the NanoString experiment were differentially expressed between all BCLL samples and control B cells. 59 genes were differentially expressed between BCLL

subgroups 1 and 2. Using the RNA Seq dataset, we quantified the number of differentially expressed annotated genes in all BCLL samples vs. control B cells, BCLL subgroup 1 vs. control B cells, BCLL subgroup 2 vs. control B cells, and BCLL subgroup 1 vs. BCLL subgroup 2 (Table 9). 1,671 genes were differentially expressed between all BCLL samples and control B cells (p-adj <0.05). The top 500 transcriptional elements overexpressed or underexpressed in BCLL as determined by fold change are listed in Supplemental table S4. The vast majority (98%) of these elements are protein coding genes; non-coding RNAs may be underrepresented due to polyadenylated RNA enrichment methods, which can miss small non-coding RNAs like miRNAs, and a less well annotated canine non-coding RNA database compared to human. 1,086 genes that were differentially expressed between control B cells and BCLL samples were found in BCLL subgroup 1 vs. control and BCLL subgroup 2 vs. control comparisons, indicating that BCLL subgroups have a set of differentially expressed genes in common (Figure 11). Additionally, a large set of differentially expressed genes were identified in the BCLL subgroup 1.

Table 8. Number of genes differentially overexpressed or underexpressed (nSolver, FDR <0.05) between normal B cells from healthy controls, BCLL subgroups and large B-cell lymphoma cases by NanoString analysis.

NanoString sample group comparison	Differentially expressed genes (n)	Overexpressed genes (n)	Underexpressed genes (n)
All BCLL samples vs. control B cells	175	78	97
Large B-cell lymphoma vs. control B cells	201	59	142
Large B-cell lymphoma vs. all BCLL samples	205	56	149

BCLL subgroup 1 vs. control B cells	145	56	89
BCLL subgroup 2 vs. control B cells	146	64	82
BCLL subgroup 1 vs. BCLL subgroup 2	59	28	31

Table 9. Number of genes differentially overexpressed or underexpressed (DESeq2, p-adj <0.05) between normal B cells from healthy controls and BCLL subgroups by RNA Seq analysis.

RNA Seq sample group comparison	Differentially expressed genes (n)	Overexpressed genes (n)	Underexpressed genes (n)
All BCLL samples vs. control B cells	1671	1110	561
BCLL subgroup 1 vs. control B cells	4784	2670	2114
BCLL subgroup 2 vs. control B cells	1775	1150	625
BCLL subgroup 1 vs. BCLL subgroup 2	3541	1778	1763

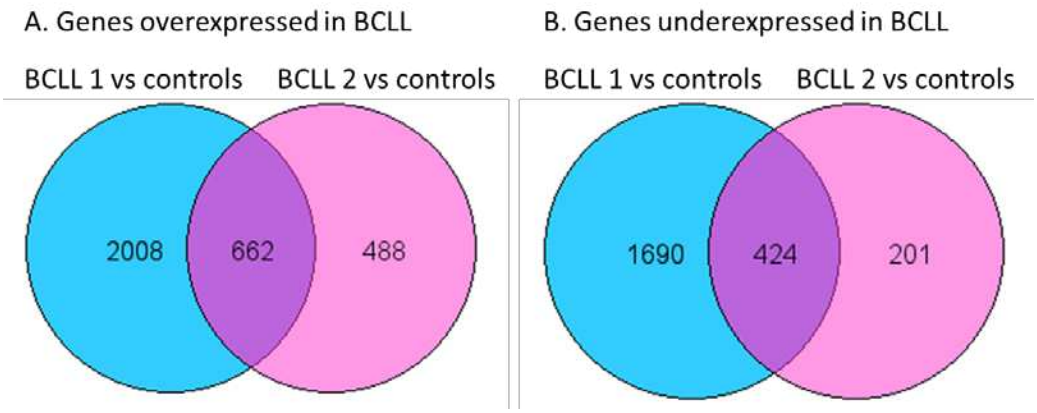

Figure 11. Venn diagram showing overlap in differentially expressed genes between BCLL subgroups by RNA Seq analysis. The number of significantly overexpressed (A) and underexpressed (B) genes in BCLL samples vs. control B cells are presented (DESeq2, p-adj <0.05). Gene lists were compared between BCLL subgroup 1 samples vs. control B cells and BCLL subgroup 2 samples vs. control B cells. A set of 1,086 differentially expressed genes were common among the gene lists, including 662 genes overexpressed in BCLL and 424 underexpressed in BCLL.

To validate the expression data, a few genes of interest were selected and the log transformed normalized counts were compared between the NanoString and RNA Seq analyses for the 10 BCLL samples that were included in both experiments (Table 10; Figure 12). There was high correlation between NanoString and RNA Seq expression for the genes evaluated.

Table 10. Pearson correlation of log transformed normalized counts between RNA Seq and NanoString methods for select genes of interest.

Gene	Pearson's r	95% confidence interval	p value
CD9	0.9795	0.9129 to0.9953	<0.0001
MKI67	0.9843	0.9329 to 0.9964	<0.0001
BCL2	0.9901	0.9572 to 0.9977	<0.0001
IL2RA	0.9621	0.8432 to 0.9913	<0.0001

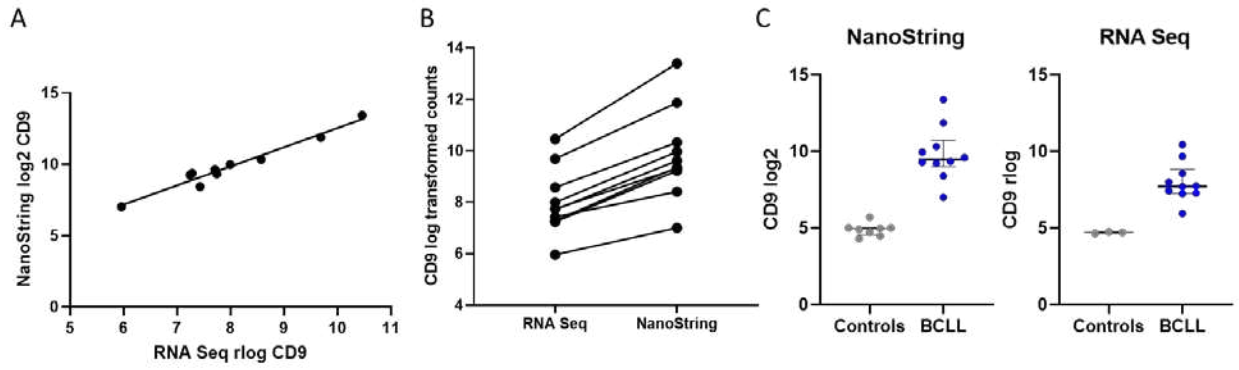

Figure 12. Comparison of CD9 expression between NanoString and RNA Seq experiments. Log transformed normalized CD9 counts from NanoString analysis or RNA Seq DESeq2 analysis are compared for 10 BCLL cases that were included in both experiments. (A) There was high correlation of CD9 expression between NanoString and RNA Seq methods (Pearson's r, 0.9795). (B) NanoString counts were relatively higher than RNA Seq counts, but increases were generally proportional across BCLL cases. (C) BCLL cases had significantly higher CD9 expression than control B cells in both experiments. Data points are shown for 10 BCLL cases included in both studies; the control samples were different in the NanoString and RNA Seq experiments.

Functional pathway analysis in canine BCLL

We used GSEA to predict biological functions and pathways underlying canine BCLL. Differentially expressed genes identified by RNA Seq between all BCLL samples and control B cells were analyzed. All enriched gene sets in the hallmarks gene set database (FDR <0.25) are listed in Table 11.[163] Genes involved in inflammatory responses, cytokine signaling pathways, KRAS signaling, and TNFA signaling via NF-κB were overexpressed in BCLL. Genes in cell cycle progression were underexpressed in BCLL compared to control lymph node B cells. This finding may suggest BCLL cells have a more quiescent phenotype as seen in human CLL, or this result may be attributed to a difference in tissue source, as BCLL samples were from blood and control B cells were collected from lymph nodes. Additionally, a BCR signaling gene set curated from the Staudt laboratory (published[164–166] and unpublished data, https://lymphochip.nih.gov/signaturedb/index.html) was enriched in the BCLL samples compared to controls (normalized enrichment score, 1.90; p=0.008; FDR=0.106).

Table 11. Summary of hallmark gene sets enriched between canine BCLL vs. control B cells.

Gene set	Enriched phenotype	NES	NOM p-val	FDR q-val
Inflammatory response	BCLL	3.05	<0.0001	<0.0001
KRAS signaling up	BCLL	2.86	<0.0001	<0.0001
IL2 STAT5 signaling	BCLL	2.74	<0.0001	<0.0001
Coagulation	BCLL	2.73	<0.0001	<0.0001
Epithelial mesenchymal transition	BCLL	2.42	<0.0001	0.0013
Complement	BCLL	2.32	0.0038	0.0032
TNFA signaling via NFKB	BCLL	2.24	<0.0001	0.0041
P53 pathway	BCLL	1.89	0.0214	0.0323
Apoptosis	BCLL	1.76	0.0261	0.0558
Hypoxia	BCLL	1.70	0.0303	0.0594
IL6 JAK STAT3 signaling	BCLL	1.71	0.0290	0.0613
Heme metabolism	BCLL	1.66	0.0241	0.0673
Interferon gamma response	BCLL	1.55	0.0659	0.0842
Cholesterol homeostasis	BCLL	1.59	0.0382	0.0859
Allograft rejection	BCLL	1.57	0.0447	0.0884
Glycolysis	BCLL	1.55	0.0500	0.0894
Xenobiotic metabolism	BCLL	1.29	0.1598	0.2359
PI3K AKT MTOR signaling	BCLL	1.27	0.1887	0.2442
E2F targets	Control B cells	-3.29	<0.0001	<0.0001
G2M checkpoint	Control B cells	-2.88	<0.0001	<0.0001
Oxidative phosphorylation	Control B cells	-1.91	0.0059	0.0206

Mitotic spindle	Control B cells	-1.95	0.0040	0.0215

NES, normalized enrichment score; NOM p-val, nominal p value; FDR q-val, false discovery rate.

We assessed whether the canine BCLL gene expression profile was enriched for human CLL gene sets by GSEA. Using the NanoString dataset, we tested whether the manually curated human CLL gene sets (CLL vs. normal B cells and CLL vs. DLBCL) were differentially expressed in canine BCLL samples vs. control B cells and BCLL samples vs. large B-cell lymphoma samples (Figure 13). The set of genes overexpressed in human CLL compared to normal B cells was significantly enriched in canine BCLL samples compared to control B cells (Figure 13B). Genes underexpressed in human CLL compared to normal B cells were not significantly enriched in the canine NanoString data. The set of genes overexpressed in human CLL compared to DLBCL was significantly enriched in canine BCLL samples compared to large B-cell lymphoma samples, though this gene set was relatively small.

A

Gene set (number of genes)	Enriched phenotype	NES	FDR q-val
Overexpressed in human CLL vs. normal B cells (n=96)	BCLL vs. control B cells	1.37	0.089
Underexpressed in human CLL vs. normal B cells (n=122)	Control B cells vs. BCLL	0.96	0.507
Overexpressed in human CLL vs. DLBCL (n=26)	BCLL vs. large B-cell lymphoma	1.39	0.059

B

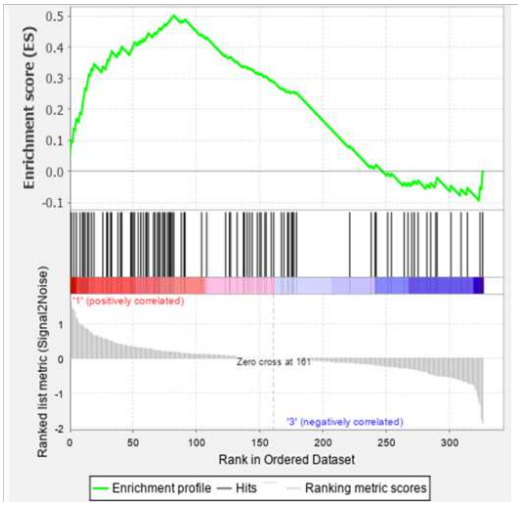

Figure 13. Comparison of human CLL gene sets and canine BCLL differentially expressed gene lists generated by NanoString analysis. (A) Results from GSEA testing whether sets of differentially expressed genes between human CLL vs. normal B cells and human CLL vs. DLBCL were enriched in canine BCLL samples, when compared to control B cells or large B-cell lymphoma samples, respectively. The normalized enrichment score (NES) reflects the degree to which the human gene set was

overrepresented in the canine phenotype of interest. A false discovery rate (FDR) <0.25 was considered significant. (B) GSEA enrichment score plot comparing the NanoString differentially expressed genes between canine BCLL and control B cells with the compiled list of genes overexpressed in human CLL vs. normal B cells. Differentially expressed genes in canine BCLL vs. control B cells are ranked with genes overexpressed in BCLL on the left. Vertical black lines represent genes in the human gene set that appear in the ranked list of genes from the canine NanoString analysis. An accumulation of lines on the left indicates that genes overexpressed in human CLL are overexpressed in canine BCLL.

We performed GSEA on the RNA Seq dataset to look for enrichment of human gene sets from CLL and other B-cell neoplasms that may have a leukemic component. Among CLL, mantle cell lymphoma, splenic marginal zone lymphoma, and follicular lymphoma gene sets, only the human CLL gene set was significantly differentially expressed in canine BCLL samples compared to controls. Genes overexpressed in human CLL vs. normal blood CD19+ B cells (Liao et al. data set[143]) were overexpressed in canine BCLL (NES, 5.44; FDR, <0.001) (Figure 14A), but genes downregulated in human CLL were not significantly underexpressed in canine BCLL. We tested BCLL subgroup 1 vs. control B cells and BCLL subgroup 2 vs. control B cells gene lists as well, to determine whether both BCLL subgroups were independently enriched for the human CLL gene set. Genes overexpressed in human CLL were overexpressed in both BCLL subgroup 1 (Figure 14B) and BCLL subgroup 2 (Figure 14C).

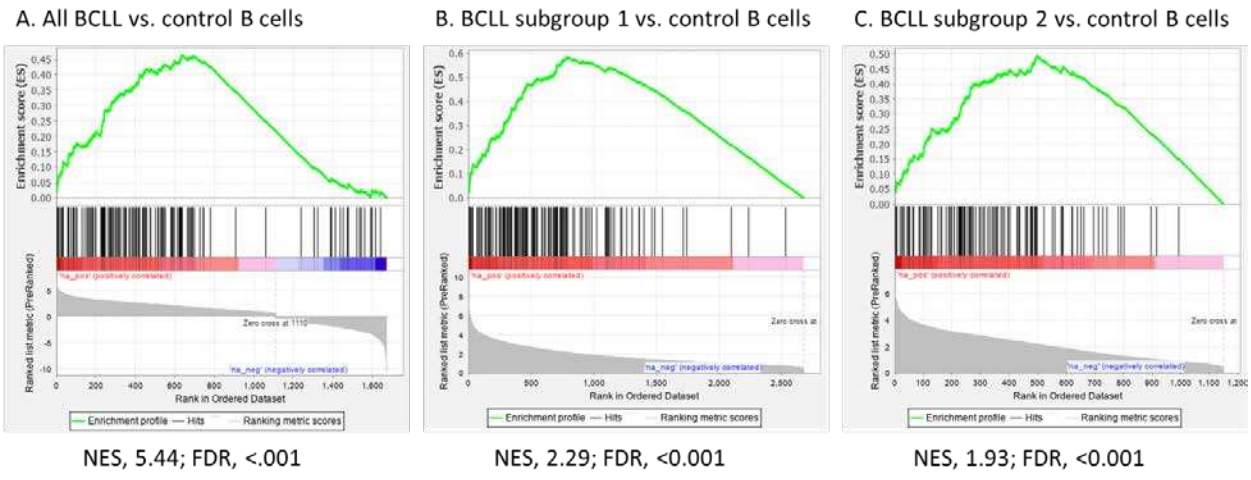

Figure 14. Enrichment of the human CLL gene set in canine BCLL subgroup gene expression profiles generated by RNA Seq. Enrichment plots from GSEA show significant enrichment of the human CLL gene

set in all BCLL samples vs. control B cells (A), BCLL subgroup 1 samples vs. control B cells (B), and BCLL subgroup 2 samples vs. control B cells (C). The human CLL gene set contains genes overexpressed in CLL tumor cells compared to normal blood CD19+ B cells.[143] Ranked gene lists from canine samples were obtained from differential gene expression analysis in DESeq2.

In summary, these results identified similarities in gene expression profiles between canine BCLL and human CLL by two different methods. And, both of the BCLL subgroups were independently enriched for the human CLL gene set, suggesting these BCLL molecular subtypes each share genes and pathways overexpressed in human CLL.

Comparison of BCLL subgroup transcriptomes

We compared the gene expression profiles of the two BCLL subgroups detected by hierarchical clustering in the NanoString and RNA Seq experiments. 3,541 annotated genes were differentially expressed between BCLL subgroups by RNA Seq (Figure 15A). Gene set enrichment analysis of genes differentially expressed in BCLL subgroup 2 vs. BCLL subgroup 1 found that BCLL subgroup 2 was significantly enriched for proliferation gene sets (Figure 15B), E2F targets, and glycolysis genes (Supplemental table S5). Top gene sets enriched in BCLL subgroup 1 included TNFA signaling via NF-κB (Figure 15C), unfolded protein response, and ribosome and spliceosome genes (Supplemental table S5). Given the increased expression of cell cycle progression genes in BCLL subgroup 2 samples, we hypothesized that these samples would have increased Ki67 expression. We compared normalized log transformed MKI67 counts for control B cells and BCLL samples and found that BCLL subgroup 2 samples had significantly higher MKI67 expression than BCLL subgroup 1 samples by RNA Seq ($p=0.0051$; Figure 15D) and NanoString ($p=0.0001$; Figure 15E) analyses.

We independently compared BCLL subgroup 1 samples and BCLL subgroup 2 samples to control B cells from healthy dog lymph nodes. A total of 4,784 annotated genes were differentially expressed between BCLL subgroup 1 samples and control B cells by DESeq2 analysis. A total of 1,775 annotated

genes were differentially expressed between BCLL subgroup 2 samples compared to control B cells.

There was overlap in the gene sets enriched in the BCLL subgroups compared to control B cells. Genes

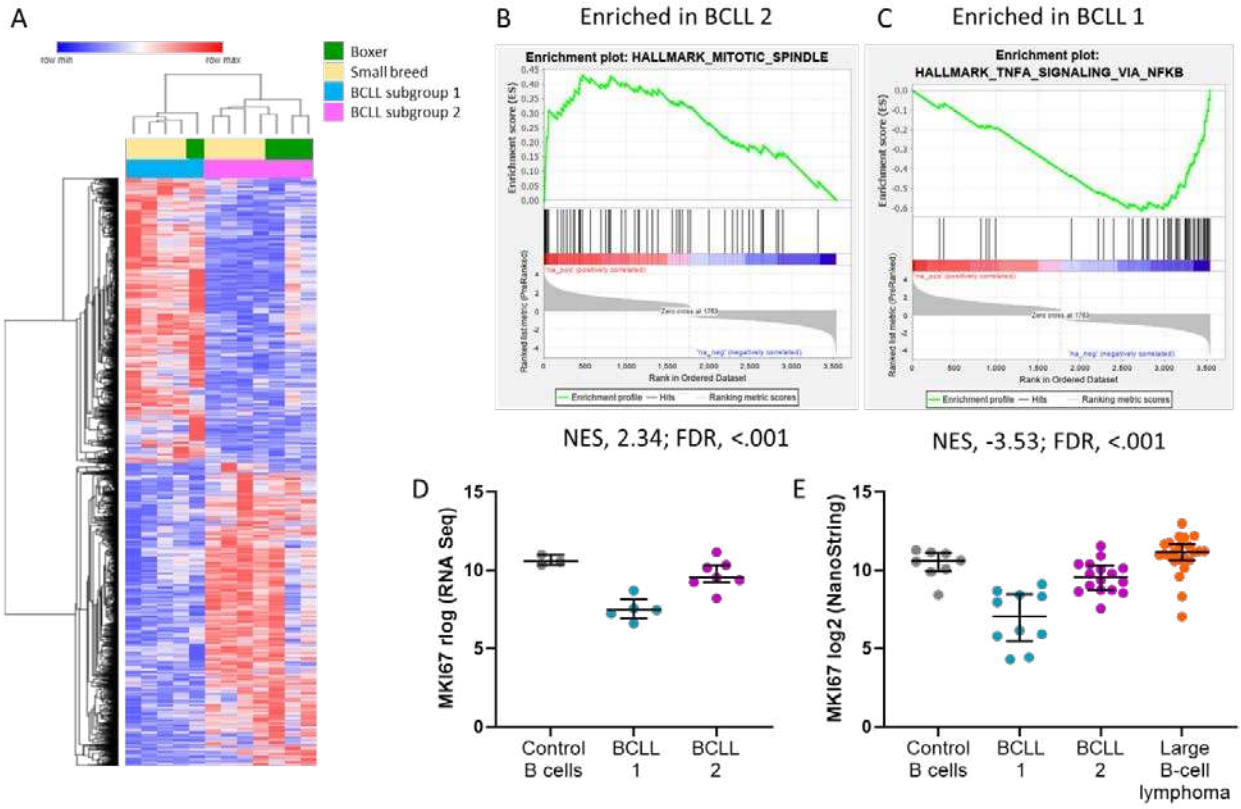

Figure 15. Transcriptome comparison between BCLL subgroups. Two major BCLL subgroups were identified by hierarchical clustering based on gene expression in the NanoString and RNA Seq experiments. (A) Unsupervised hierarchical clustering of BCLL samples using annotated genes differentially expressed in BCLL subgroup 2 vs. BCLL subgroup 1 by RNA Seq analysis (DESeq2, p-adj <0.05). A total of 3,541 genes were differentially expressed between BCLL subgroups. (B-C) GSEA enrichment plots comparing the ranked list of differentially expressed genes in BCLL subgroup 2 vs. BCLL subgroup 1. Vertical lines represent genes in the gene set that appear in the ranked list of differentially expressed BCLL genes. An accumulation of genes on the far left or far right indicate enrichment in the BCLL subgroup 2 or subgroup 1 phenotype, respectively. BCLL subgroup 2 samples were enriched for cell cycle progression gene sets, including mitotic spindle assembly (B). BCLL subgroup 1 samples were enriched for genes regulated by NF-κB in response to TNF (C). (D-E) RNA Seq normalized rlog MKI67 counts (D) and NanoString normalized $\log_2$ MKI67 counts (E) for control B cells, BCLL samples and large B-cell lymphoma samples. BCLL subgroup 2 samples had significantly higher MKI67 expression than subgroup 1 by RNA Seq (p=0.0051) and NanoString (p=0.0001).

overexpressed in BCLL subgroup 1 and 2 groups compared to control B cells were independently enriched in TNFA signaling by NF-κB, BCR signaling, inflammatory responses and cytokine signaling gene sets (Supplemental tables S6-S8). BCLL subgroup 1 samples were also enriched for NF-κB targets, TGFB signaling, ribosome and spliceosome KEGG gene sets, and splicing-related gene sets in the reactome database (Supplemental tables S6 and S7). Additionally, BCLL subgroup 2 samples had decreased expression of genes involved in DNA repair and TP53 activity compared to control B cells (Supplemental table S8).

Markers to distinguish BCLL subgroups

After identifying BCLL subgroups based on their transcriptome, we wanted to determine whether there was a targeted list of genes that could distinguish the subgroups in a larger number of cases. Of the 1,145 genes differentially expressed by >1.5 fold-change between RNA Seq BCLL subgroup 1 and BCLL subgroup 2 cases (n=12), 49 genes were represented in the NanoString probe set (Supplemental table S9). Expression of these 49 genes was examined in the larger set of BCLL cases (n=26) in the NanoString dataset (Figure 16). When these 49 genes were used to perform unsupervised hierarchical clustering of the NanoString BCLL cases, 23/26 cases were classified into their original BCLL subgroup. Three cases that were originally classified as BCLL subgroup 1 clustered with BCLL subgroup 2 cases using the refined gene list; these 3 cases are indicated with an asterisk in Figure 16A and an open blue circle in Figure 16B. Genes overexpressed in BCLL subgroup 1 included genes involved in cellular responses to TNF, apoptosis regulation and BCR, TLR and NF-κB signaling. Genes overexpressed in BCLL subgroup 2 included genes involved in cell cycle progression and proliferation, as well as the anti-apoptotic gene BCL2. A few of the genes differentially expressed between BCLL subgroups by RNA Seq and NanoString analysis, in addition to Ki67, include BCL2, CD72, RELA, IL10RA and BCL3 (Figure 16B). IL2RA (CD25) tended to have higher expression in BCLL subgroup 2 samples by NanoString analysis, but gene expression was not significantly different. By flow cytometry, a larger number of subgroup 2 cases

(63%) had high CD25 expression (>80% CD25+ B cells) compared to subgroup 1 cases (30%), but this trend was not significant. These comparisons identify a number of genes that could be tested in prospective studies for their ability to distinguish BCLL subgroups and predict prognosis.

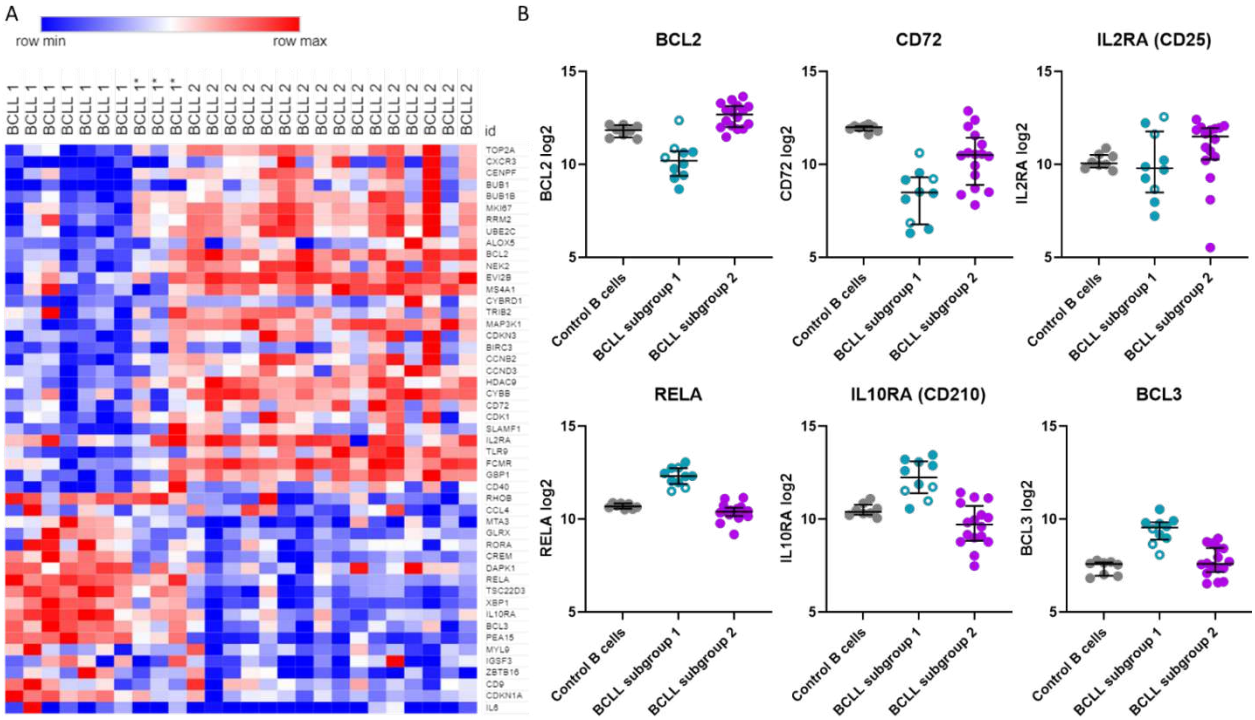

Figure 16. Expression of 49 genes differentially expressed between BCLL subgroups. Genes that were differentially expressed between RNA Seq BCLL subgroups (DESeq2 p-adj <0.05, fold change >1.5) and measured in the NanoString experiment were selected. (A) Heat map using normalized log_2 counts measured by NanoString technology in 26 BCLL cases. Unsupervised hierarchical clustering placed 3 cases originally classified as BCLL subgroup 1 into the BCLL subgroup 2 (asterisks). (B) Expression of select genes by NanoString in control B cells from healthy dog lymph nodes and BCLL subgroup cases, as originally classified in Figure 10A. The 3 BCLL subgroup 1 cases classified in subgroup 2 with the refined gene list are depicted as an open blue circle. BCLL subgroup 1 cases had significantly lower expression of BCL2 and CD72 ($p < 0.0001$, $p = 0.0023$, respectively) and significantly higher expression of RELA, IL10RA and BCL3 ($p < 0.0001$, for each gene) compared to BCLL subgroup 2 cases. There was a trend for BCLL subgroup 2 cases to have higher IL2RA expression, but there was significant overlap between BCLL subgroups ($p = 0.201$).

Discussion

This study evaluates the gene expression profile of canine BCLL and identifies genes differentially expressed between BCLL and normal B cells, compares canine BCLL and human CLL gene expression profiles, and identifies subgroups of BCLL cases based on differences in gene expression. This work utilized RNA Seq technology to evaluate the transcriptome globally and NanoString technology to measure expression of select genes in a larger number of cases.

There was clear separation of BCLL cases and control lymph node B cells from healthy dogs by RNA Seq and NanoString analysis. Additionally in the NanoString study, BCLL cases were compared to large B-cell lymphoma cases defined by flow cytometry. A previous study showed that the vast majority of large B-cell lymphoma cases with these flow cytometry features were DLBCL subtype by histology, which is the most common lymphoma subtype in the dog.[76,77,122,167] The set of genes measured in the NanoString study, which encompassed genes that define different normal B-cell subsets and/or are differentially expressed in human CLL, clearly separated BCLL and large B-cell lymphoma cases. A total of 1,671 genes were differentially expressed (p-adj <0.05) between all BCLL cases and control B cells by RNA Seq, and 856 genes were differentially expressed by >2 fold change. Some genes overexpressed in BCLL samples may be attributed to the blood tissue source compared to the lymph node source for control B cells; for example, apparent overexpression of platelet activation and coagulation cascade genes may be attributed to RNA released from platelets or platelet particles in the blood. Inflammatory response, cytokine signaling, KRAS signaling, NF-κB signaling, BCR signaling and JAK/STAT signaling were among the pathways enriched in BCLL. Cytokine-cytokine receptor signaling, NF-κB signaling, BCR signaling, and JAK/STAT signaling are among the mechanisms important for human CLL.[168] KRAS mutations, which are common in certain carcinomas, have been recognized more recently in CLL where they affect the Ras-MAPK pathway and are associated with shorter treatment free survival.[169]

We compared the canine BCLL gene expression profile to human CLL by NanoString analysis and RNA Seq analysis. For the NanoString comparison, we compiled a list of genes overexpressed or underexpressed in human CLL compared to normal B cells across several studies. We found significant enrichment of the human CLL upregulated gene set in the list of genes overexpressed in canine BCLL vs. control B cells. The list of genes underexpressed in canine BCLL was not significantly enriched for the human CLL downregulated gene set. For the RNA Seq comparison, we compared differentially expressed genes between canine BCLL and control lymph node B cells to differentially expressed genes identified by RNA Seq in a human CLL study by Liao et al.[143] Additionally we compared the canine BCLL transcriptome to other human B-cell tumor gene signatures, including mantle cell lymphoma, splenic marginal zone lymphoma and follicular lymphoma, but only the CLL gene set was significantly enriched in the canine BCLL phenotype. Similar to the NanoString study, RNA Seq found similarity between genes overexpressed in human CLL and canine BCLL, but the set of genes downregulated in human CLL was not significant in canine BCLL. A major limitation of these comparisons is the source of control B cells used for differential expression analysis. Our study used pooled CD21+ B cells from lymph nodes from healthy young dogs. These lymph nodes had prominent germinal centers by histology. We lack the antibodies in veterinary medicine to sort specific B-cell subsets so the control B-cell population in this study contained a mixture of B cells at different stages of differentiation. In contrast, many of the human CLL transcriptome studies use peripheral blood CD19+ B cells or sorted specific B-cell subsets as controls. In the future, normal canine blood B cells would be a useful control population for transcriptome comparisons. Additionally, as our ability to identify and sort specific canine B-cell subsets improves, it would be useful to compare canine BCLL to normal B-cell subsets to try and discern the cell of origin.

Gene expression analysis identified 2 major subgroups of BCLL cases, referred to as BCLL subgroup 1 and 2. BCLL subgroup 2 contained the majority of Boxer cases and had significantly higher MKI67 expression compared to subgroup 1, as determined by gene expression in the NanoString and

RNA Seq studies. The Boxer breed and high Ki67 expression were associated with poorer prognosis in our BCLL outcome study, suggesting BCLL subgroup 2 may represent more aggressive BCLL. Because the BCLL cases grouped together by hierarchical clustering and over one thousand differentially expressed genes were shared between BCLL subgroups when compared to control B cells, we hypothesize that BCLL subgroup 1 and 2 are manifestations of one tumor, rather than distinct neoplasms. When BCLL subgroups were independently compared to control B cells, there was overlap in the enriched pathways, including NF-κB signaling, KRAS signaling, and cytokine-JAK/STAT signaling, suggesting similar mechanisms may drive these tumors. Additionally, each BCLL subgroup was independently enriched for the human CLL gene set. However, there were a large number of genes differentially expressed between these subgroups as well. Compared to control B cells, BCLL subgroup 1 had upregulation of genes involved in TGFB signaling, PI3K AKT MTOR signaling, and misfolded protein responses, as well as ribosome and spliceosome genes. Among the genes differentially expressed between BCLL subgroups, BCLL subgroup 1 overexpressed genes included: RELA, involved in NF-κB heterodimer formation and survival of human CLL cells,[170] IL10RA, which has anti-inflammatory functions and is overexpressed in human CLL, mantle cell lymphoma, and Waldenström's macroglobulinemia,[171,172] and BCL3, which promotes NF-κB signaling and cell survival and is overexpressed in B-cell neoplasms including CLL.[173] Top genes overexpressed in BCLL subgroup 2 compared to subgroup 1 were primarily cell cycle and proliferation genes (e.g. CDK1, CCNB2, CCND3, BUB1, MKI67), including genes involved in B-cell proliferation, such as CD40 and TLR9. BCLL subgroup 2 had fewer genes differentially expressed when compared to control lymph node B cells, suggesting this subgroup was more similar to the lymph node B-cell population, which likely contained many proliferating and reactive B cells. Additional genes overexpressed in BCLL subgroup 2 compared to subgroup 1 included the anti-apoptotic gene BCL2, which is upregulated in human CLL, and CD72, which regulates BCR signaling. IL2RA (CD25) was significantly overexpressed in BCLL subgroup 2 cases compared to subgroup 1 by RNA Seq. In a larger

number of cases in the NanoString study, high IL2RA-expressing cases by gene expression or flow cytometry were more likely to be subgroup 2 cases, though there was overlap between subgroups. This marker may help distinguish subgroup cases when combined with other markers, and IL2RA (CD25) was increased in Boxers with BCLL and a poor prognostic indicator in our canine BCLL outcome study. The IGHV mutation status did not correlate with BCLL subgroup in this study, but few samples were examined. All BCLL subgroup 1 cases examined rearranged mutated IGHV genes, but subgroup 2 contained a mixture of mutated and unmutated IGHV cases. Previous human CLL studies have demonstrated large overlap between mutated and unmutated IGHV CLL transcriptomes, and an RNA Seq study by Ferreira et al. identified two distinct CLL subgroups independent of IGHV mutation status with significantly different clinical outcomes.[22,142,144] Our study suggests that there are distinct canine BCLL subgroups, and based on the prognostic significance of Ki67 expression, we hypothesize that these subgroups have different clinical outcomes. The next step is to perform a larger prospective study to correlate transcriptome profiles and outcome. This would allow us to determine the clinical significance of distinguishing BCLL subgroups. If these subgroups have different outcomes or therapeutic indications, designing a small panel of differentially expressed genes between subgroups, which could be measured by NanoString technology, would be useful for diagnostic purposes.

In summary, we identified a large number of genes and pathways differentially expressed between canine BCLL and control B cells for further study. To better determine the function of these pathways in the development and progression of BCLL, evaluating protein abundance and phosphorylation of these pathways and/or studying the effects of pathway inhibitors on BCLL cells is needed. Additional analyses to perform with this RNA seq dataset include identifying variants and fusion genes and assessing alterative splicing and transcriptional elements beyond protein-coding genes. Splicing alterations play an important role in human CLL, with recurrent mutations described in the splicing factor SF3B1 and splicing abnormalities identified in previous CLL RNA Seq studies.[143,144,174]

Future work is also aimed at characterizing the mutations and chromosomal abnormalities underlying canine BCLL and comparing them to human CLL. Investigation of the pathways dysregulated in canine BCLL, the cell of origin, the role of alternative splicing, and the genetic mutations will help further evaluate canine BCLL as a useful model for human CLL.

CHAPTER 4: Polyclonal B-cell lymphocytosis in English bulldogs

Summary

English bulldogs disproportionally develop an expansion of small B cells, which has been interpreted as B-cell chronic lymphocytic leukemia (BCLL). However, clonality testing in these cases has often not been supportive of neoplasia. We hypothesized that English bulldogs have a syndrome of non-neoplastic B-cell expansion. We characterized this syndrome by assessing B-cell clonality, clinical presentation, flow cytometric features and immunoglobulin gammopathy patterns. We evaluated 84 English bulldogs with small-sized CD21+ B-cell lymphocytosis in the blood as determined by flow cytometry. Flow cytometry features were compared to normal B cells and BCLL cases. PCR for antigen receptor rearrangements using multiple immunoglobulin primers was performed to assess B-cell clonality. A subset of cases with gammopathy were examined by protein electrophoresis, immunofixation and immunoglobulin subclass ELISA quantification. Seventy percent of cases had polyclonal or restricted polyclonal immunoglobulin gene rearrangements, suggesting nonmalignant B-cell expansion. The median age of all dogs in the study was 6.8 years and 74% were male. The median lymphocyte count was 22,400/µL and B cells expressed low class II MHC and CD25. Splenomegaly or splenic masses were detected in 57% of cases and lymphadenopathy was uncommon. Seventy-one percent of cases had hyperglobulinemia and the vast majority had IgA +/- IgM polyclonal or restricted polyclonal gammopathy patterns. Polyclonal B-cell lymphocytosis in English bulldogs (PBLEB) is characterized by low B-cell class II MHC and CD25 expression, splenomegaly and hyperglobulinemia consisting of elevated IgA +/- IgM. We hypothesize that this syndrome has a genetic basis.

Introduction

Lymphocytosis may be caused be a reactive or neoplastic process. In dogs, reactive expansions of nonneoplastic lymphocytes in the blood appear uncommon and are associated with only a small number of conditions, including *Ehrlichia canis* infection, hypoadrenocorticism and thymoma.[175–179] Neoplastic lymphocytosis is comprised of clonally expanded lymphocytes and is a more common cause of persistent lymphocytosis in adult dogs.[180] Clonality testing by PCR for antigen receptor rearrangements (PARR) can help differentiate a monoclonal population of neoplastic lymphocytes that have an identically-sized antigen receptor rearrangement from a polyclonal population of reactive lymphocytes which contains diverse antigen receptor rearrangments.[9,75] Reactive or inflammatory processes may also cause increased production of polyclonal immunoglobulin proteins.[181,182] Monoclonal immunoglobulin production is typically due to an immunoglobulin-secreting B-cell or plasma cell neoplasm, though rarely certain infectious or inflammatory conditions are associated with monoclonal gammopathy in dogs.[181,183]

B-cell chronic lymphocytic leukemia is a common hematopoietic neoplasm in dogs, defined by a clonal expansion of small-sized B cells in the blood or bone marrow.[62,84] Our laboratory identifies BCLL based on inclusion criteria of >5,000 lymphocytes/µL on CBC with small-sized CD21+ B cells accounting for >60% of the lymphocyte population by flow cytometry. We previously reported that small breed dogs had increased odds of developing BCLL.[62] English bulldogs also had increased odds of developing BCLL, as defined in this study, but this breed had a unique presentation: they were significantly younger at diagnosis compared to mixed breed dogs, had increased frequency of hyperglobulinemia, and their expanded B cells had decreased CD25 and class II MHC expression by flow cytometry. This unique

presentation raised the question of whether English bulldogs have a different form of BCLL, or a different B-cell disease entirely.

Since detecting this unique presentation in English bulldogs, our laboratory anecdotally found that English bulldogs with B-cell lymphocytosis frequently had polyclonal immunoglobulin gene rearrangements by PARR. These PARR findings suggested that the B-cell expansions in these dogs may be nonneoplastic, and that English bulldogs have a B-cell lymphocytosis syndrome separate from BCLL. The goal of this study was to identify English bulldogs with B-cell lymphocytosis, to evaluate clonality using PARR and protein electrophoresis/immunofixation (PE/IF) modalities, and to analyze the clinical features of the cases. Here we describe a syndrome of polyclonal B-cell expansion in English bulldogs characterized by substantial increases in IgA +/- IgM, with normal to diminished IgG.

Methods

Case selection

The Colorado State University-Clinical Immunology (CSU-CI) laboratory database was queried for English bulldog cases with blood submitted for immunophenotyping by flow cytometry between 9/17/2010 and 8/31/2019. Inclusion criteria included an expansion of small-sized CD21+ B cells exceeding the upper limit of the reference interval (724 CD21+ cells/µL) for canine blood samples at our institution. Small size was defined by a size ratio of CD21+ cell mean forward scatter:neutrophil mean forward scatter <0.60.

CSU-CI laboratory database query was also used to identify several control groups, including: 1) Blood from 30 clinically healthy non-English bulldogs without lymphocytosis or evidence of lymphoproliferative disease by flow cytometry and PARR, which were a range of breeds, included 57% females and ranged in age from 1.9-8.9 years old; 2) blood from 49 clinically healthy control English bulldogs with no suspicion of lymphoproliferative disease and a normal CD21+ B-cell count (<724/µL by

flow cytometry), which included 41% females and ranged in age from 1.0-12.0 years old (median, 3.9 years old); 3) 53 small breed BCLL cases from BCLL-predisposed breeds[62] with blood submitted for flow cytometry between 1/1/2015-10/1/2019 and clonal immunoglobulin (IG) rearrangements by PARR; and 4) PARR results from 25 small breed BCLL patients with a flow cytometry diagnosis and routine PARR performed on submitted blood.

Serum immunoglobulins were evaluated in 30 English bulldog cases, 6 control English bulldogs with no evidence of CD21+ lymphocytosis by flow cytometry, 15 non-bulldog BCLL cases with hyperglobulinemia, and 4 control non-bulldogs with polyclonal gammopathy and no lymphocytosis.

Clinical variables

Signalment, physical exam findings, and laboratory data were provided on the CSU-CI laboratory submission form. Hematology data were collected from the CBC performed at the time of flow cytometry submission. Anemia, thrombocytopenia and neutropenia were identified using the CBC laboratory's reference interval for hematocrit, platelet count and neutrophil count, respectively. Hyperglobulinemia was identified if indicated by the veterinarian on the submission form, or if globulins were elevated on a biochemistry panel or by protein electrophoresis. Physical exam findings, including lymphadenopathy, splenic abnormalities and hepatic abnormalities, were identified by palpation, ultrasound, radiographs or a combination of these tests. When available, blood films, bone marrow cytology reports and spleen histology samples were reviewed. Histology samples had immunohistochemistry performed for Pax5 expression (monoclonal mouse anti-human Pax5, DAK-Pax5 clone; Dako North America Inc., Carpinteria, CA), CD3 expression (monoclonal mouse anti-human CD3, LN10 clone; Leica Biosystems Newcastle Ltd., Newcastle Upon Tyne, UK) and MUM1 expression (monoclonal mouse anti-human MUM1, MUM1p clone; Dako North America Inc.). For cases with sequential samples, treatment information was collected from the CSU-CI laboratory submission form.

Flow cytometry

Flow cytometry was performed on blood samples at the CSU-CI laboratory. Samples were collected, stored, and stained as previously described,[65] using antibody panels listed in Chapter 2. Expression of CD25, class II MHC, and CD21 on B cells were determined in English bulldog cases, healthy control dogs and small breed BCLL cases. Because anti-CD25 is not in the same staining reaction as anti-CD21, the percentage of B cells expressing CD25 was determined by gating on lymphocytes in tube 2, excluding cells expressing CD3, CD4, CD5 and CD8, and calculating %CD25 on remaining cells. Class II MHC and CD21 expression were determined by median fluorescence intensity. Six English bulldog cases sampled prior to May 2012 did not have CD25 or class II MHC data available.

PCR for antigen receptor rearrangements

The CSU-CI routine PARR assay was performed as previously described.[75,123] This assay detects B-cell clonality by targeting both complete and incomplete IG gene rearrangements in the IG heavy chain (IGH) locus. Complete IGH-VDJ rearrangements consist of a V (variable), D (diversity) and J (joining) gene, while incomplete IGH-DJ rearrangements consist of a D and J gene.

An expanded PARR assay was developed to detect additional complete IGH-VDJ and incomplete IGH-DJ rearrangements not detected with routine PARR, as well as IG light chain rearrangements. Primers were designed and tested as described in the Appendix (Supplemental material for Chapter 4). The expanded assay for complete IGH-VDJ rearrangements targets IGH variable (IGHV) subgroup genes not detected by our routine PARR assay. Additionally, IGH-VDJ primers in the expanded assay target a different region of the IGHV gene, to increase detection of IGHV genes that did not amplify with routine PARR due to polymorphisms or somatic hypermutation in the primer binding region. Light chain primers target kappa deleting element (Kde) and IG lambda (IGL) rearrangements. The routine and expanded PARR assays were performed on all English bulldog cases with available sample.

PARR results were interpreted as clonal, polyclonal or restricted polyclonal using the rubric provided in Table 12. These criteria were internally validated and apply only to these primers and cycling conditions when analyzed by fragment analysis methods, and therefore, are not applicable for other assays and laboratories. Cases with 1 or 2 peaks reaching objective criteria for clonality were classified as clonal, as lymphocytes may rearrange one or both alleles of the IG locus resulting in up to 2 clonal peaks.[6] Representative tracings for the electrophoretic patterns are provided in Figure 17. DNA quantity and quality were determined to be sufficient based on PARR results, as previously described.[184] PARR results were independently interpreted by the authors (E Rout, A Avery), blinded to the signalment and flow cytometry data.

Table 12. Immunoglobulin PCR for antigen receptor rearrangements (PARR) assay diagnostic criteria.

Interpretation	Capillary electrophoretic pattern
IGH-VDJ, Kde or IGL rearrangements	
Clonal	1 or 2 tall narrow peak(s) >5000 in amplitude and >3x the height of the base peaks forming the polyclonal background
Polyclonal	Multiple peaks forming a Gaussian distribution
Restricted polyclonal	No peaks reach clonal criteria, but ≥1 peak is >2000 in amplitude and >2x the height of the base peaks forming the polyclonal background
IGH-DJ rearrangements	
Clonal	Tall narrow peak >8000 in amplitude and >3x the height of the base peaks forming the polyclonal background
Polyclonal	Multiple peaks forming a Gaussian distribution

IGH-VDJ, complete immunoglobulin heavy (IGH) chain variable (V)-diversity (D)-joining (J) gene rearrangement; IGH-DJ, incomplete immunoglobulin heavy chain diversity (D)-joining (J) gene rearrangement; Kde, kappa deleting element; IGL, immunoglobulin lambda.

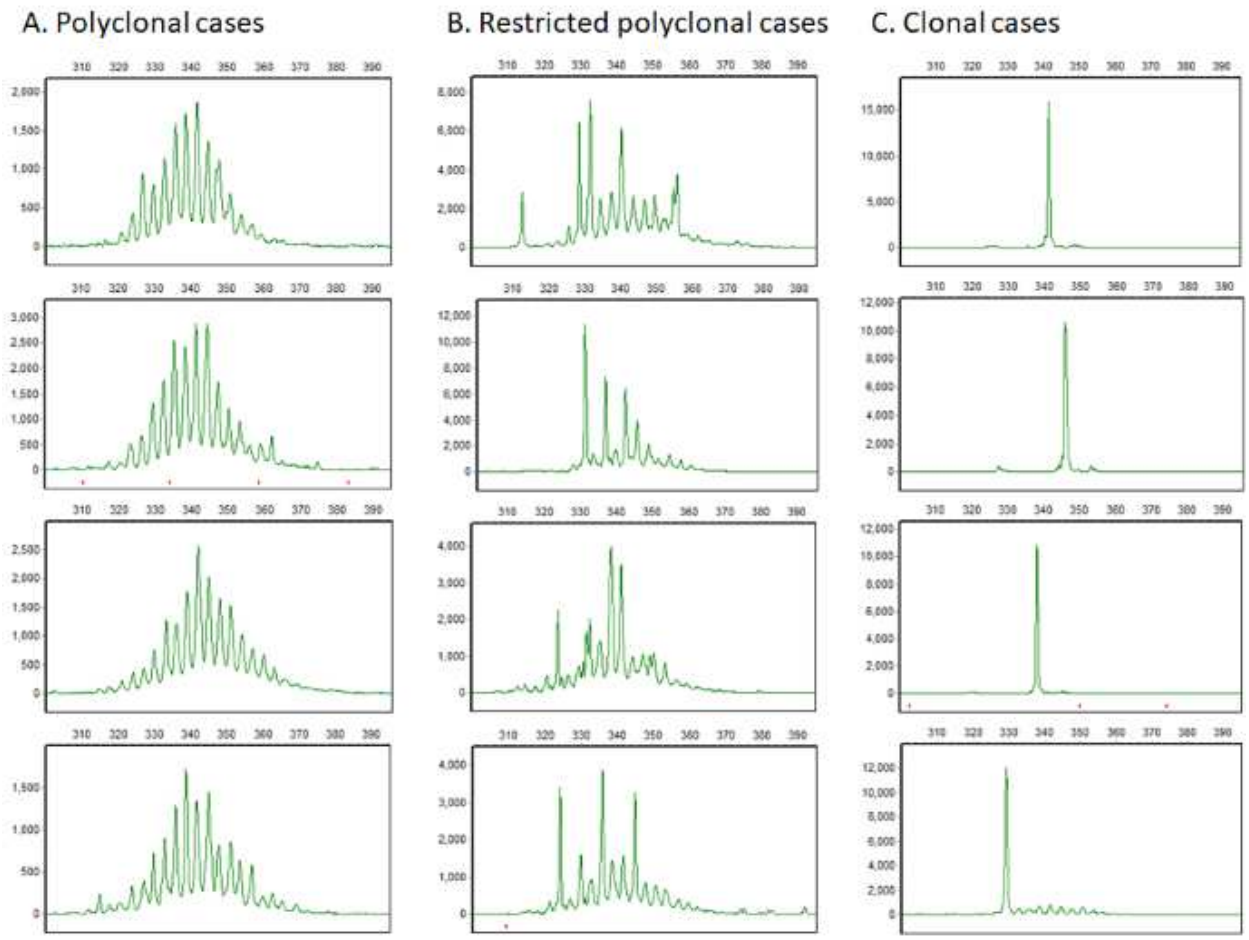

Figure 17. PCR for antigen receptor rearrangements (PARR) results for English bulldogs with B-cell lymphocytosis. GeneMarker tracings for complete immunoglobulin heavy (IGH) chain variable (V)-diversity (D)-joining (J) (IGH-VDJ) rearrangements are presented for 4 polyclonal cases (A), 4 restricted polyclonal cases (B), and 4 clonal cases (C). The size of the PARR amplicons is plotted on the horizontal axis and the abundance of amplicons is on the vertical axis. (A) Polyclonal cases have multiple peaks forming a Gaussian distribution. (B) Restricted polyclonal cases have 1-5 peaks >2000 in amplitude and 2x the height of the polyclonal peaks forming the base. (C) Clonal cases have a peak >5000 in amplitude and >3x the base height.

Serum immunoglobulins

Agarose gel PE and IF were performed at the CSU-Veterinary Diagnostic Laboratory to evaluate immunoglobulins, as previously described.[123,185] PE and IF were performed on serum or plasma samples from a subset of English bulldog cases (n=30), control English bulldogs (n=6), and non-bulldog BCLL cases

(n=10). Five additional BCLL cases had PE without IF. The hemolysis and lipemia indices were determined for all samples (Cobas c501 Roche Diagnostics, Indianapolis, IN), as these factors may alter electrophoretogram morphology.[181,186] Total globulin concentration was calculated as the sum of all globulin fractions by PE to avoid over-estimation of albumin in cases with a marked gammopathy.[187] Hyperglobulinemia was identified by PE if the total globulins exceeded our internal upper reference limit 3.5 g/dL. PE/IF results were blindly and independently interpreted by the authors (E Rout, P Avery, AR Moore) using the rubric in Table 13. Representative results for the electrophoretic patterns are provided in Figure 18. Immunofixation results were interpreted to determine whether immunoglobulins were predominantly of IgG_{FC}, IgA or IgM subclass.[188] An IgA or IgM predominance was considered atypical, as normal dogs have predominantly IgG_{FC} labeling with no to faint IgA and IgM.[181]

Table 13. Protein electrophoresis and immunofixation diagnostic criteria.

Interpretation	Electrophoretic pattern
Protein electrophoresis	
Normal	No protein increases or atypical restricted bands present for the sample type
Monoclonal	Single atypical restricted peak with no more than minimal polyclonal increases
Biclonal	Pair of closely associated peaks with no more than minimal surrounding polyclonal increases
Restricted polyclonal	≥1 atypical restricted band(s) on gel amid moderate polyclonal increases, irrespective of height of any peak
Polyclonal	Only diffuse/broad bands present
Immunofixation	
Normal	Moderate IgG_{FC} polyclonal labeling with no to faint polyclonal IgA and IgM

Monoclonal	Single restricted band of one IGH class with no more than minimal IgG_{FC} polyclonal increase
Biclonal	2 bands (same IGH class or different classes) with no more than minimal IgG_{FC} polyclonal increase
IgA/IgM restricted polyclonal	≥1 restricted bands of IgA and/or IgM amid moderate IgA and/or IgM polyclonal labeling and decreased IgG_{FC}
IgA/IgM polyclonal	Increased immunoglobulin labeling without restricted bands in IgA and/or IgM with decreased IgG_{FC}
IgG_{FC} polyclonal	Increased immunoglobulin labeling without restricted bands with predominantly IgG_{FC} labeling and no to faint IgA and IgM

IGH, immunoglobulin heavy chain.

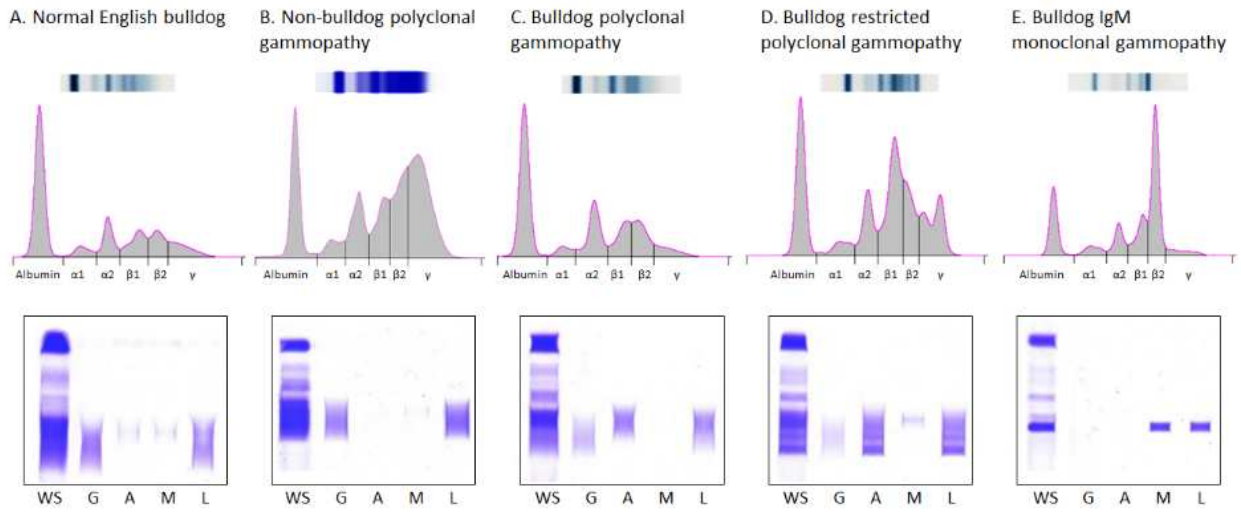

Figure 18. Protein electrophoresis (PE) and immunofixation (IF) results in control dogs and English bulldogs with B-cell lymphocytosis. An agarose gel protein electrophoresis gel and tracing (top) and immunofixation gel (bottom) are provided. Immunofixation gels are labeled with anti-whole serum (WS), anti-canine IgG_{FC} heavy chain (G), anti-canine IgA heavy chain (A), anti-canine IgM heavy chain (M) and anti-canine light chain (L) antibodies. (A) Serum PE and IF results from a healthy control English bulldog with no CD21+ B-cell lymphocytosis reveals polyclonal immunoglobulin proteins that

predominantly label with anti-IgG_{FC}. (B) Serum PE and IF results from a non-bulldog with polyclonal gammopathy. Immunoglobulin proteins form a broad smear and predominantly label with anti-IgG_{FC}. (C) Serum PE and IF results from an English bulldog B-cell lymphocytosis case diagnosed with polyclonal gammopathy, characterized by broad peaks in beta and gamma regions on PE, with no evidence of restricted bands by PE or IF. There is heavy labeling with anti-IgA by IF relative to IgG_{FC}, which is atypical as compared to the control (A), and a lack of a hypergammaglobulinemia, which is atypical for an IgG-centric polyclonal gammopathy. (D) Serum PE and IF results from an English bulldog B-cell lymphocytosis case diagnosed with restricted polyclonal gammopathy, characterized by multiple restricted peaks within a polyclonal background. By IF, the majority of protein labels with anti-IgA. (E) Plasma PE and IF results from an English bulldog B-cell lymphocytosis case with a clonal immunoglobulin gene rearrangement by PARR. There is a tall narrow peak in the beta 2 region on PE which labels with anti-IgM by IF, consistent with an IgM monoclonal gammopathy.

Quantification of IgA, IgM and IgG_{FC} proteins was performed using commercially available ELISA kits, as previously described.[185] Immunoglobulin proteins were measured in serum and plasma samples from a subset of English bulldog cases (n=12), 4 clinically healthy English bulldogs and 3 clinically healthy non-bulldogs.

Statistical analysis

For all English bulldog cases, signalment, physical exam findings, hematologic data and flow cytometry data were summarized, and descriptive statistics were calculated. For continuous variables, normality was assessed visually and using a Shapiro Wilk test. To compare continuous variables between English bulldog cases, healthy controls and BCLL cases, a Kruskal-Wallis test was calculated. Dunn's test for multiple comparisons was subsequently used for pairwise comparisons. Immunoglobulin quantification was compared between controls and English bulldog cases with a Mann-Whitney test. For comparisons between clonal and non-clonal English bulldog cases, a Mann-Whitney test was calculated. A Fishers exact test was used to compare categorical variables across groups. Cases with missing or unknown data on the submission form were censored for that data. Statistical analysis was performed in R version 3.5.2 and a two-sided p value <0.05 was considered significant.

Results

Clinical presentation

Between 9/17/2010 and 8/31/2019, 84 of 195 English bulldogs with blood submitted to the CSU-CI laboratory for immunophenotyping by flow cytometry had an expansion of small-sized CD21+ B lymphocytes. Signalment and laboratory data from these cases are summarized in Table 14. The median age at diagnosis was 6.8 years and there was a male predominance. The lymphocyte count was moderately elevated in most cases, but a small number of cases had >50,000 lymphocytes/µL (median, 22,400 lymphs/µL; interquartile range (IQR), 12,000-41,100 lymphs/µL; range, 2,000-384,400 lymphs/µL). Anemia was present in 26/84 cases (31%) and mild to moderate in most cases (median hematocrit, 30%; IQR, 27-34%; range, 21-38%). The anemia was non-regenerative in 14/17 cases with reticulocyte counts available, as defined by the laboratory's reticulocyte reference interval. Thrombocytopenia was uncommon, affecting 8/84 cases (10%), and mild to moderate in most affected cases (median, 114,000 platelets/µL; IQR, 89,500-152,000/µL; range, 38,000-198,000/µL). Hyperglobulinemia affected 65% of patients with globulin data at diagnosis. An additional 7 cases had hyperglobulinemia detected after initial diagnosis, resulting in 71% of cases having hyperglobulinemia at some point during the course of their syndrome. Splenomegaly or splenic masses or both were also common, but lymphadenopathy was rare.

Table 14. Summary signalment data, laboratory data and physical exam findings for 84 English bulldogs with B-cell lymphocytosis at the time of diagnosis.

	Number of cases with available data	Number affected (%) or median (IQR; range)
Signalment		
Male	84	74%
Age, median (IQR; range), yrs	84	6.8 (5.2-9.0; 2.5-11.0)

Hematologic data		
Lymph count, median (IQR; range), $x10^3/\mu L$	84	22.4 (12.0-41.1; 2.0-384.4)
Anemia	84	31%
Thrombocytopenia	84	10%
Neutropenia	84	2%
Hyperglobulinemia	69	65%
Physical exam		
Splenomegaly/splenic mass	46	57%
Peripheral lymphadenopathy	61	11%
Visceral lymphadenopathy	38	13%
Hepatomegaly/hepatic mass	42	14%

For variables besides age and lymphocyte count, the percentage of cases affected among those with available data are presented. For age and lymphocyte count, the median, interquartile range (IQR) and range of values are presented.

Of 44 cases where lymphocyte morphology was evaluated by a clinical pathologist, 35/44 were described as small or small to intermediate in size, 4 were described as intermediate-sized, and 5 cases were described as intermediate to large-sized. All cases met the flow cytometric small size inclusion criteria. Chromatin was described as mature, clumped or condensed in all cases evaluated, except in one where it was described as fine. Six cases were described as having few nucleoli. Ten cases with blood smears available at the CSU-Veterinary Diagnostic Laboratory were reviewed by the authors (E Rout, P Avery). Lymphocytes were predominantly smaller than a neutrophil with a small round nucleus, condensed chromatin and scant basophilic cytoplasm (Figure 19A). Rare cells were intermediate-sized with slightly expanded pale blue cytoplasm. When cases were blindly pooled with blood films from 10 Shih Tzu dogs with BCLL, the English bulldog cases could not be differentiated cytologically. Two English

bulldog cases had bone marrow aspirates performed. Megakaryocytes and erythroid and myeloid series were considered within normal limits and small mature lymphocytes accounted for 20% and 43% of nucleated cells.

Six cases had cytologic evaluation and 4 cases had histologic evaluation of the spleen. None of these samples were definitively diagnosed with lymphoma. Six cytology samples were diagnosed with lymphoid hyperplasia and 3 of those had a second diagnosis of possible lymphoma. One sample was minimally cellular, but remaining cases were described as moderately to highly cellular with a heterogeneous lymphoid population, consisting of predominantly small lymphocytes with condensed chromatin, with few large lymphocytes and plasma cells. Cytology report comments often indicated that the heterogeneity and predominance of small well-differentiated lymphocytes was consistent with lymphoid hyperplasia, but given the clinical history, a small cell lymphoma or BCLL infiltration into the spleen could not be ruled out. Histologically, there was expansion of the spleen with lymphoid hyperplasia of variable severity. Lymphoid hyperplasia ranged from mild expansion around periarteriolar sheaths to more marked nodular lymphoid hyperplasia. Nodular lymphoid hyperplasia formed small, non-coalescing follicular structures to occasionally larger rarely coalescing follicles (Figure 19B). Follicles were composed of predominantly B cells (Figure 19D) with scattered small T cells often radiating around follicles and scattered plasma cells within sinusoids. The B cells occupying the follicular structures were heterogeneous, composed of small and intermediate-sized lymphocytes with condensed chromatin or occasional marginal zone appearance with a prominent central nucleolus (Figure 19C).

Flow cytometry

Among English bulldog cases, the median CD21+ B-cell count was 20,606 cells/µL (IQR, 10,461-42,682/µL; range, 1,174-378,815/µL; reference interval, 0-724/µL). CD21+ B cells accounted for 67%-

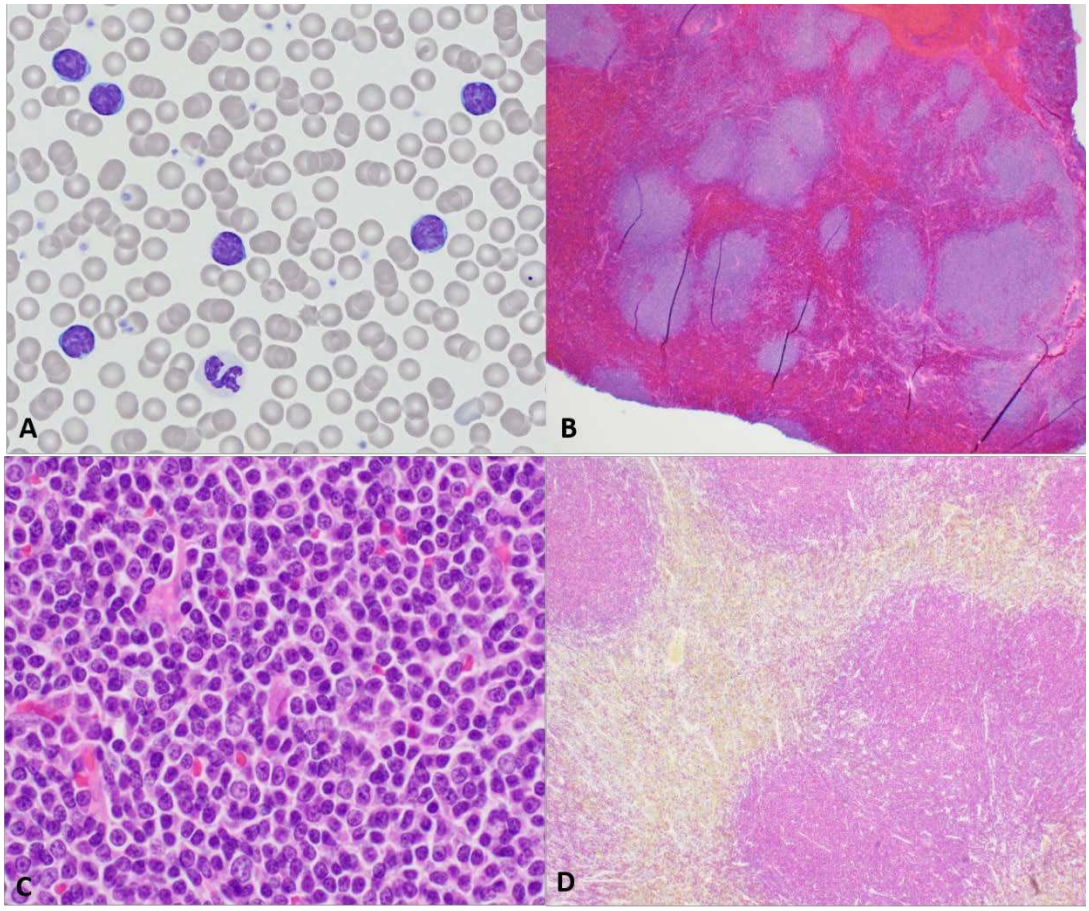

Figure 19. Blood smear and histopathology and immunohistochemistry of spleen from English bulldog cases with polyclonal B-cell lymphocytosis. (A) Peripheral blood film from an English bulldog with B-cell lymphocytosis and polyclonal immunoglobulin gene rearrangements. Lymphocytes are small with condensed chromatin and scant basophilic cytoplasm (Wright Giemsa, 60x objective). (B-D) Histopathology of spleen from a different English bulldog with B-cell lymphocytosis and polyclonal immunoglobulin gene rearrangements. There is lymphoid hyperplasia characterized by nodules of multifocal to rarely coalescing lymphoid follicular structures (B, H&E, 2x objective). The follicular structures are composed of primarily small lymphocytes with condensed chromatin with fewer intermediate-sized lymphocytes and scattered lymphocytes with marginal zone appearance with a single central prominent nucleolus (C, H&E, 40x objective). Lymphocytes within the follicles are predominated by B cells with strong nuclear immunoreactivity for PAX5 (D, PAX5, Fast Red chromogen, 4x objective).

99% of the lymphocyte population in the blood by flow cytometry. English bulldog cases were characterized by low expression of CD25 and class II MHC. Sixty-eight percent of cases had only 0-2% of B cells expressing CD25, and English bulldog cases had significantly lower CD25 expression than B cells

from clinically healthy non-bulldogs (p=0.001), clinically healthy English bulldog controls (p<0.001) and small breed BCLL cases (p<0.001) (Figure 20). B-cell class II MHC expression was also significantly lower in English bulldog cases compared to non-bulldog controls (p<0.001), English bulldog controls (p<0.001) and small breed BCLL cases (p<0.001). English bulldog cases had significantly higher B-cell CD21 expression than non-bulldog controls (p<0.001), English bulldog controls (p<0.001) and small breed BCLL cases (p<0.001). Comparing non-bulldog and English bulldog controls, English bulldog controls had significantly lower CD25 and CD21 expression (p=0.0024 and p=0.015, respectively).

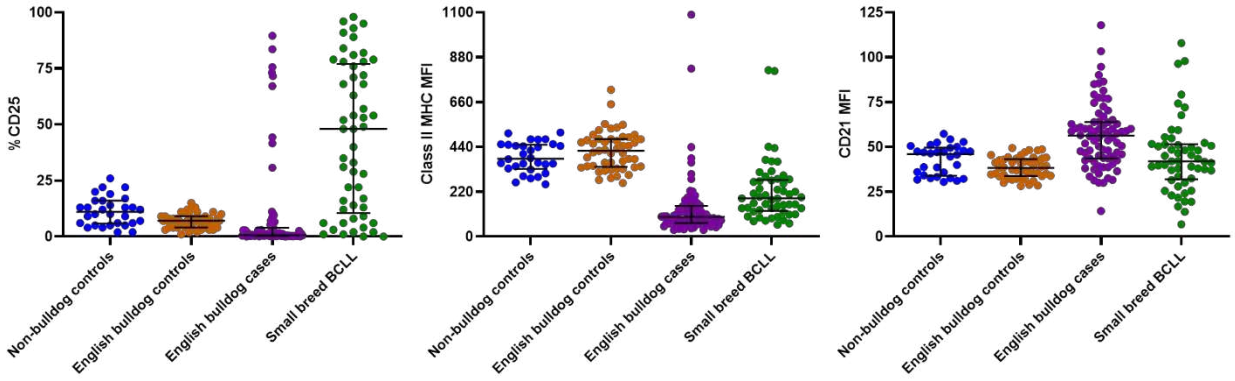

Figure 20. B-cell expression of CD25, class II MHC, and CD21 in English bulldog B-cell lymphocytosis cases compared to clinically healthy non-bulldog controls, English bulldog controls with normal B-cell counts, and small breed BCLL cases. Expression of CD25 (left), class II MHC (center) and CD21 (right) by flow cytometry is plotted for individual cases. Lines depict the median and interquartile range for each group. English bulldog cases had significantly lower expression of CD25 and class II MHC and significantly higher expression of CD21 compared to peripheral blood B cells from healthy non-bulldog and English bulldog controls and small breed BCLL cases.

Clonality

Eighty-three of 84 English bulldog cases had sample material available for routine PARR analysis. Eighty-one cases had PARR performed on the same blood sample that was submitted for flow cytometry. Two cases had tissue samples (spleen and bone marrow, respectively) submitted for PARR within one month of the blood flow cytometry analysis; PARR results from those sources were used for

analysis. Thirty-nine percent of cases had polyclonal IG rearrangements, 37% of cases had restricted polyclonal IG rearrangements, and 24% had clonal IG rearrangements by routine PARR. Representative PARR tracings for polyclonal, restricted polyclonal, and clonal cases are presented in Figure 17. Of the 25 cases which met inclusion criteria for small breed BCLL during an 18-month period, 100% of BCLL cases had clonal IG rearrangements by routine PARR analysis, with either complete IGH-VDJ primers alone (76%), incomplete IGH-DJ primers alone (12%), or both (12%).

Eighty-five percent of English bulldog cases (n=71) had sample available for PARR with expanded IG primers. Clonal results were detected with the expanded primer set in 5 cases that had been polyclonal by routine PARR. These 5 cases had polyclonal complete IGH-VDJ rearrangements by routine PARR, but clonal complete IGH-VDJ rearrangements with the expanded primers. When results from routine PARR and expanded PARR were combined, 37% of English bulldogs were polyclonal, 33% of cases had restricted polyclonal IG rearrangements, and 30% were clonal (Table 15).

Table 15. Summary clonality results for immunoglobulin gene rearrangements and immunoglobulin proteins in English bulldog cases with B-cell lymphocytosis.

Clonality result	Number of cases	Percent of cases	PE cases with clonal PARR result
Immunoglobulin gene rearrangements [a]			
Polyclonal	31	37%	NA
Restricted polyclonal	27	33%	NA
Clonal	25	30%	NA
Immunoglobulin proteins [b]			
IgG_{FC} polyclonal	1	3%	1/1
IgA polyclonal	7	23%	1/7
IgA + IgM polyclonal	1	3%	0/1

IgA restricted polyclonal	3	10%	0/3
IgA + IgM restricted polyclonal	12	40%	0/12
IgA monoclonal	2	7%	2/2
IgM monoclonal	4	13%	4/4

[a] Clonality of immunoglobulin gene rearrangements was determined by PCR for antigen receptor rearrangements (PARR) in 83 unique cases. NA, not applicable. [b] Clonality of immunoglobulin proteins was determined by protein electrophoresis (PE) and immunofixation in 30 unique cases. The number of cases with a clonal IG rearrangement by PARR is presented in the far-right column. All cases with monoclonal protein had clonal IG gene rearrangements. 22/24 cases with polyclonal or restricted polyclonal proteins had polyclonal or restricted polyclonal IG gene rearrangements.

Immunoglobulin protein analysis

Peripheral globulins were evaluated in 30 English bulldog cases with PE/IF. Total globulin concentrations ranged from 3.3-10.7 g/dL (median, 6.1 g/dL; IQR, 4.3-8.7 g/dL; reference interval, 2.2-3.5 g/dL). Eighty percent of cases had hypoalbuminemia. Among hypoalbuminemic patients, the median albumin concentration was 2.1 g/dL (IQR, 1.8-2.5 g/dL; range, 1.4-2.7 g/dL; reference interval, 2.8-3.7 g/dL). Many English bulldog samples had an atypical banding pattern, which did not fit a classic polyclonal or monoclonal pattern, so a rubric was developed to interpret these samples (Table 13) and representative results are presented in Figure 18. Six cases had an IgA or IgM monoclonal gammopathy, and all 6 cases had clonal IG rearrangements by PARR (Table 15). Remaining cases were classified as polyclonal (n=7) or restricted polyclonal (n=17) by PE alone. By IF, 1 case was classified as polyclonal with IgG_{FC} predominance; this case had a clonal IG rearrangement by PARR and mild hyperglobulinemia (3.95 g/dL). Eight cases had polyclonal gammopathy with increased IgA +/- IgM. Fifteen cases had restricted polyclonal gammopathy with increased IgA +/- IgM. All cases had an appropriate light chain pattern for all IG heavy chains present. Of 23 cases with IgA/IgM polyclonal or restricted polyclonal gammopathy, globulins were frequently increased in the beta region on PE and gamma globulins were

often within the reference interval. 2/23 (9%) cases had hypogammaglobulinemia and 15/23 (65%) cases had normogammaglobulinemia. In summary, 77% of cases were classified as polyclonal or restricted polyclonal with increased IgA +/- IgM. One of the patients with IgA/IgM polyclonal or restricted polyclonal IG protein had a clonal IG rearrangement by PARR, and the remaining cases had polyclonal or restricted polyclonal IG rearrangements.

English bulldog PE/IF results were compared to control English bulldogs without CD21+ B-cell lymphocytosis (n=6) and non-bulldog BCLL cases (n=15). Three of the control English bulldogs had normal PE/IF, and the other 3 dogs had mild hyperglobulinemia with mild polyclonal gammopathy on PE, labeling with predominantly IgG_{FC} heavy chain antibody. Two of these patients had increased acute phase proteins, suggesting underlying inflammation in these control dogs. Of 5 BCLL cases with PE only, 4 cases had a monoclonal protein in the beta 1-beta 2 region, and the fifth case had a suspicious band in the beta 2 region that could not be confirmed as M-protein without IF. Of 10 BCLL cases with PE and IF, 6 cases had monoclonal IgM gammopathy, 1 case had monoclonal IgA gammopathy, 1 case had biclonal IgA gammopathy, and 2 cases had polyclonal gammopathy with IgG_{FC} predominance.

Serum IgA, IgM and IgG_{FC} proteins were quantified by ELISA in a subset of English bulldog cases (n=12) to confirm the increases in IgA and IgM seen on IF (Figure 21). IgA, IgM and IgG_{FC} proteins were also measured in 3 non-bulldog healthy dogs and 4 healthy English bulldogs without CD21+ B-cell lymphocytosis. There were no significant differences in measurements between non-bulldog and bulldog control samples, so control values were combined. English bulldog cases with B-cell lymphocytosis had significantly higher quantities of IgA ($p<0.001$) and IgM ($p=0.012$), and significantly less IgG_{FC} than controls ($p=0.004$). All 12 English bulldog cases had IgA values above the range seen in control cases, although one case with marked IgM gammopathy had only a mild increase in IgA. Four cases had marked increases in IgM. All English bulldog cases had IgG_{FC} quantities below the mean concentration seen in controls and 9 cases (75%) had IgG_{FC} quantities below the minimum value seen in

controls, which correlates with the normogammaglobulinemia and hypogammaglobulinemia seen on PE/IF. The pattern of relative IG concentration by ELISA subjectively matched the pattern observed by IF, confirming that English bulldog cases had increased IgA and decreased IgG_{FC} concentrations, and a subset had increased IgM.

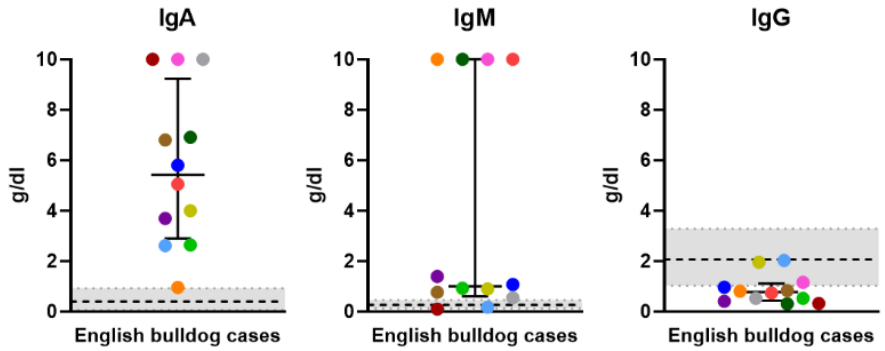

Figure 21. IgA, IgM and IgG_{FC} protein quantification by ELISA in English bulldog cases with B-cell lymphocytosis. Each English bulldog case is colored consistently across the three graphs. Data points plotted at 10 g/dL were above the limits of quantification for the assay. Dotted lines represent the mean (black line) and range (grey lines) of values for 7 control dogs. English bulldog cases had significantly greater quantities of serum IgA ($p<0.001$), and a subset of cases had increased IgM, compared to control dogs. English bulldogs had significantly less IgG_{FC} than controls ($p=0.004$).

Clonal versus non-clonal English bulldog cases

We investigated clinical and immunophenotypic differences between clonal cases (n=25) and non-clonal cases (n=58). The non-clonal group included cases with polyclonal or restricted polyclonal IG rearrangements by PARR. Clonal cases were significantly older than non-clonal cases (p=0.002) (Figure 22A). There were significantly more males in the non-clonal group (81%) compared to clonal cases (56%) (p=0.029) (Figure 22B). The CD21+ B-cell count was not significantly different between clonal cases (median, 21,700/µL; IQR, 10,900-73,500/µL; range, 1,200-378,800/µL) and non-clonal cases (median, 19,700/µL; IQR, 9,800-38,300/µL; range, 1,400-285,000/µL) (p=0.380). Class II MHC expression was not significantly different between groups (p=0.118), but clonal cases had significantly lower expression of

CD21 (p=0.040, not shown) and significantly higher expression of CD25 (p=0.003) (Figure 22C). The majority of non-clonal cases (76%) had <2% CD25-expressing B cells, while 48% of clonal cases had <2% CD25-expressing B cells. There were no significant differences in anemia, thrombocytopenia or splenomegaly/splenic mass between groups. The presence of hyperglobulinemia was not significantly different between clonal and non-clonal cases, although the type of gammopathy was different: in the 8 clonal cases with PE/IF, 6/8 cases had monoclonal IgA or IgM protein, and none of the non-clonal PARR cases had monoclonal PE/IF results. Peripheral lymphadenopathy was more common in clonal cases (32%) than non-clonal cases (2%) (p=0.003). Of the 6 clonal cases with peripheral lymphadenopathy, 5/6 were female, 5/6 were >6.8 years old, and 5/6 had high CD25 expression, in contrast to the young male signalment and low CD25 expression frequently seen in non-clonal cases.

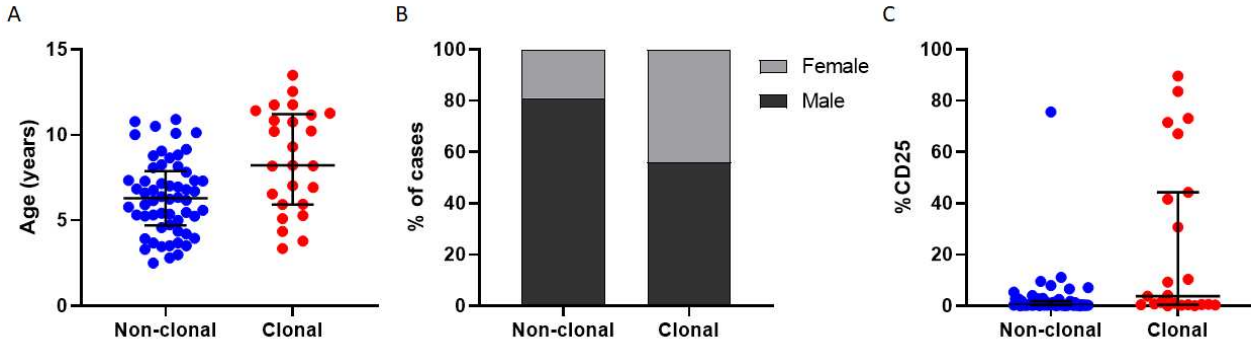

Figure 22. Signalment and B-cell CD25 expression in non-clonal and clonal English bulldog cases with B-cell lymphocytosis. (A) The age at diagnosis for non-clonal cases with polyclonal or restricted polyclonal immunoglobulin PARR results and clonal cases with clonal immunoglobulin PARR results. Clonal cases (median, 8.2 years old) were significantly older than non-clonal cases (median, 6.3 years old) (p=0.002). (B) The percentage of males and females within non-clonal and clonal groups is presented. There were significantly more males in the non-clonal group (p=0.029). (C) The percentage of B cells expressing CD25 by flow cytometry in non-clonal and clonal cases. Non-clonal cases had significantly lower CD25 expression than clonal cases (p=0.003).

Sequential sample analysis

Sequential PARR samples >1 month apart were available for 18 cases with polyclonal or restricted polyclonal IG rearrangements on initial presentation. Most cases had 1 sequential sample, but 6 cases had 2-5 sequential samples available for analysis. The time between initial diagnosis and the most recent sample varied from 2.6-64.5 months. The PARR results did not change over time for 13/18 cases. Cases with restricted polyclonal IG rearrangements had identically-sized peaks over time (Figure 23). In one case, which was followed for 64.5 months, the restricted peaks became more pronounced over time, resulting in several tall narrow peaks with minimal polyclonal background (Figure 23C). Of the 5 cases where PARR results changed over time, 2 restricted polyclonal cases became polyclonal (both were receiving oral chemotherapy and prednisone), one polyclonal case became restricted polyclonal, and 2 restricted polyclonal cases progressed to having clonal IG rearrangements. In both cases, a restricted peak initially identified increased in amplitude over time and reached clonal criteria in the sequential sample.

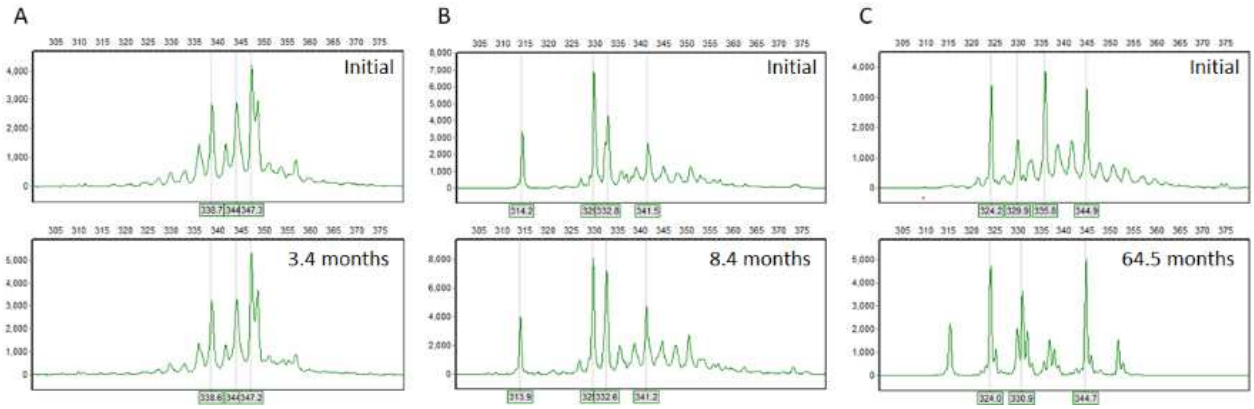

Figure 23. Sequential PCR for antigen receptor rearrangements (PARR) results using immunoglobulin primers for 3 English bulldog cases with B-cell lymphocytosis. For each case, immunoglobulin PARR results at initial presentation (top) and sequential PARR results (bottom) 3.4-64.5 months after diagnosis are presented. The size of the PCR amplicons is indicated along the horizontal axis. Restricted peaks persist over time and are identical in size to those present at initial presentation. The PARR tracings maintain a similar pattern over time in 2 cases (A, B), and become more restricted in a patient that has been monitored over 5 years (C).

A number of monitored cases have been clinically stable over time with no treatment, while others were managed with steroids alone or steroids and oral chemotherapy. In general, treated cases had reductions in the B-cell count, improvement of splenomegaly, and reductions in globulins, though atypical PE/IF patterns and IgA increases often persisted. Treatment protocols were variable in these cases and a larger outcome study is underway to evaluate indications to treat and response to treatment.

Discussion

Our study identified a syndrome in English bulldogs characterized by an expansion of non-clonal B cells in the blood, with polyclonal or restricted polyclonal IG rearrangements by PARR. This syndrome, termed polyclonal B-cell lymphocytosis in English bulldogs (PBLEB), frequently affects young males and is commonly associated with splenomegaly or splenic masses and hyperglobulinemia. The hyperglobulinemia is characterized by a polyclonal or restricted polyclonal pattern, with increased IgA +/- IgM.

Forty-three percent of English bulldogs with blood submitted to the CSU-CI laboratory for flow cytometry over a nearly nine-year period had a B-cell expansion. These patients generally have moderate lymphocytosis (median, 22,400 lymphocytes/μL). An expansion of a single lymphocyte subset of this magnitude typically corresponds with lymphoid neoplasia in dogs, which is consistent with the fact that 100% of the small breed dogs with B-cell lymphocytosis in this study had clonal IG rearrangements. However, the majority (70%) of English bulldogs with B-cell lymphocytosis did not have clonal IG rearrangements by PARR, suggesting a nonneoplastic process.

To help rule out a false negative PARR result, an expanded PARR assay was developed. The routine PARR assay has high sensitivity among traditional BCLL cases, but its sensitivity in detecting neoplasms with extensive somatic hypermutation is not known. We were concerned that the bulldogs

may have a neoplasm with somatic hypermutation, which affected PARR sensitivity. We developed an expanded PARR assay, which assessed clonality in additional IG loci, to test whether this assay would detect clonality in bulldog cases. Detection of incomplete IGH-DJ and IG light chain rearrangements increases sensitivity in detecting B-cell and plasma cell neoplasms in humans, cats and dogs, as compared to complete IGH-VDJ rearrangements alone.[6,184,189,190] Incomplete IGH-DJ and Kde rearrangements are less prone to somatic hypermutation, and analysis of the IG kappa locus was shown to be useful in detecting clonality in canine tumors with somatic hypermutation, such as plasmacytomas.[6,189] The expanded PARR assay targeting additional IGH-VDJ and IGH-DJ gene rearrangements and IG light chain rearrangements identified an additional 5 cases as clonal, but the majority of non-clonal cases remained non-clonal with the expanded PARR assay. False negative PARR results in the English bulldogs due to nonfunctional IG rearrangements, such as a J region deletion, are unlikely since patients have large quantities of immunoglobulin protein in the serum, suggesting gene rearrangements are functional.[9] While it is still possible that the expanded B cells in these English bulldogs are rearranging an unusual or highly variable IGHV gene not detected with these assays, the PARR testing with this extensive pool of primers suggests that most of these English bulldogs have a nonneoplastic or preneoplastic syndrome.

One third of the bulldog cases had an unusual restricted polyclonal IG PARR pattern defined by IG peaks which did not meet objective criteria for clonality, within a polyclonal background. Approximately 50% of cases had an unusual restricted polyclonal PE/IF pattern, where restricted bands of IG protein were present among a polyclonal background. These patterns suggest restricted IG diversity in the B-cell population, possibly attributed to proliferation of a restricted, but not necessarily neoplastic pool of B cells. The nature of this restricted pool was remarkably consistent over time. Identically-sized IG rearrangements were detected in subsequent samples, suggesting persistence of

expanded B-cell populations. The unusual restricted IG protein pattern seen on PE/IF also supports the hypothesis that there are restricted pools of IG-secreting B cells.

There are rare human disorders of nonmalignant polyclonal B cells, which may be associated with splenomegaly and hyperglobulinemia. Persistent polyclonal B-cell lymphocytosis (PPBL) is a benign disease characterized by expanded polyclonal B cells in the blood and increases in polyclonal serum IgM.[191] Patients are generally asymptomatic and a subset of cases have splenomegaly. Rare cases have massive splenomegaly, which may require splenectomy and mimic splenic lymphoma histologically.[192–194] Familial cases suggest a genetic predisposition and many cases have an isochromosome (3q) abnormality and chromosomal instability.[191,195] Most patients remain polyclonal and clinically stable, but there are rare reports of patients progressing to a clonal B-cell malignancy.[196] PPBL is hypothesized to result from hyperproliferation of B cells with a marginal zone-like phenotype and altered CD40 signaling.[192,197,198] Ras-associated autoimmune lymphoproliferative disorder (RALD) is a rare nonmalignant human syndrome caused by mutations in RAS genes and characterized by persistent monocytosis, polyclonal B-cell lymphocytosis, massive splenomegaly, lymphadenopathy, hypergammaglobulinemia, and autoimmunity.[199] This syndrome is diagnosed at a young age and generally has an indolent clinical course, though rare cases may undergo malignant transformation. Sequencing in one patient demonstrated a restricted B-cell receptor repertoire, with expansions of many different B-cell clones.[200] There are features of human PPBL and RALD that are not consistent with the canine PBLEB syndrome described here, including binucleate lymphocyte morphology and female predisposition in PPBL, and monocytosis, lymphadenopathy, hypergammaglobulinemia and IgG gammopathy in RALD. However, these human syndromes do highlight the possibility for an underlying mutation or chromosomal abnormality to cause altered lymphocyte homeostasis, resulting in polyclonal B-cell expansions, splenomegaly and gammopathy.

We hypothesize that this syndrome begins as a nonneoplastic condition, with a potential for malignant transformation over time. In PBLEB cases with sequential samples, we documented progression from polyclonal IG rearrangements to restricted polyclonal IG rearrangements in one case, and from restricted polyclonal IG rearrangements to clonal IG rearrangements in 2 cases. Additionally, clonal cases were significantly older than polyclonal cases. A subset of those clonal English bulldogs had features consistent with PBLEB, including splenomegaly without lymphadenopathy, hyperglobulinemia and low class II MHC and CD25 expression on B cells, raising the possibility that some PBLEB cases may undergo malignant transformation over time. In human medicine, monoclonal gammopathy of undetermined significance is considered a premalignant disease that may undergo malignant transformation and progress to Waldenstrom macroglobulinemia or a plasma cell neoplasm.[201] Additionally, a subset of apparently healthy asymptomatic people with monoclonal B-cell lymphocytosis will progress to chronic lymphocytic leukemia.[202] We hypothesize that bulldogs with PBLEB also have a spectrum of disease, and some bulldogs will maintain nonneoplastic polyclonal B-cell expansions while others progress to malignancy. Therefore, these bulldogs may require monitoring over time to identify progression.

A separate subset of clonal English bulldog cases had a different clinical and flow cytometry presentation than the PBLEB cases. These cases had a female predominance, lymphadenopathy and higher class II MHC and CD25 expression. We hypothesize these bulldogs have a different B-cell disease, such as conventional BCLL as seen in other breeds. It is unclear if these cases have a different clinical course, but this information would be useful. Though these cases and the PBLEB cases that progress may both meet the clinical definition of BCLL, we hypothesize that these are different entities with different underlying predisposition and different events leading to B-cell neoplasia.

A larger outcome study is underway to investigate the progression of the PBLEB syndrome, variables associated with clonality, and indications for and response to treatment in English bulldogs.

Limited data suggest that a subset of bulldogs maintain stable lymphocyte counts and globulin levels without a need for therapeutic intervention, but a subset of cases (including polyclonal cases) require treatment to control marked hyperglobulinemia and its sequela and massive splenomegaly.

Hyperglobulinemia and splenomegaly were common features in PBLEB cases. Globulins were often moderately to markedly increased. All cases tested had increased IgA, a subset of patients had concurrent increases in IgM, and IgG_{FC} was typically normal to decreased. In T cell-dependent IgA class switching pathways, cytokines in combination with CD40L may promote B-cell proliferation and IgA class switching.[203] In T cell-independent pathways, soluble factors such as BAFF and APRIL induce IgA class switching.[203] Several mechanisms may explain the constellation of findings in these dogs. PBLEB cases may have either increased factors which stimulate IgA class switching, or a decrease in inhibitors of class switch recombination. For example, APRIL is hypothesized to cause hyper-production of IgA in human IgA nephropathy and BAFF overexpression in mice resulted in a hyper-IgA syndrome with B-cell hyperplasia.[204,205] Alternatively, certain B-cell subsets, including splenic marginal zone B cells in mice, class switch to IgA more effectively than other subsets, and perhaps the expanded B cells in PBLEB are primed to readily/robustly switch to IgA when exposed to stimuli.[203,206] Many PBLEB cases had splenomegaly/splenic masses without lymphadenopathy. A subset of cases had massive splenomegaly and a few patients were treated with splenectomy due to patient discomfort. Histology was only available in a small number of cases, but splenic samples were expanded by enlarged lymphoid follicles consisting of heterogeneous B cells. The expanded B cells in this syndrome may originate in the spleen, there may be stimulatory molecules in the spleen promoting proliferation and survival at this site, and/or the expanded B cells express receptors promoting homing to the spleen rather than lymph node. A limitation of this study is that only 2 cases had bone marrow aspirates performed. In these cases, small, mature appearing lymphocytes comprised 20% and 43% of the bone marrow nucleated cells

suggesting that this compartment may be involved. Additional cases would be necessary to confirm this suspicion.

There appears to be a genetic predisposition in English bulldogs for this syndrome. English bulldog controls with normal B-cell counts had significantly lower CD25-expressing B cells than non-bulldog controls of various breeds. Because we do not have this data for a population of other purebred dogs, we do not know if this finding is specific to English bulldogs, nor if it is related to the syndrome being described. Future directions are aimed at performing genome sequencing to identify underlying mutations. We have documented a few cases of this syndrome in other breeds. These non-bulldog cases were identified because of their clinical presentation, including splenomegaly and hyperglobulinemia, and flow cytometric B-cell features, including low class II MHC and CD25 expression. Polyclonal IG gene rearrangements by PARR and polyclonal IgA gammopathies by PE/IF supported a diagnosis of this syndrome in these non-English Bulldog cases. Future work is aimed at determining the prevalence of this syndrome in other breeds.

In summary, we have identified a syndrome found in English bulldogs characterized by polyclonal B-cell expansion. This syndrome frequently affects young males and is often associated with IgA +/- IgM gammopathy and splenomegaly. We hypothesize that this syndrome represents a nonneoplastic or preneoplastic syndrome with an, as yet incompletely defined, subset of patients progressing to malignancy.

CONCLUSIONS

Canine neoplasms provide an opportunity to study genetic risk, investigate tumor pathogenesis and test novel therapies, which may translate to human medicine. B-cell chronic lymphocytic leukemia (BCLL) is a common neoplasm in dogs, though little was known about the clinical progression of this disease or the underlying mechanisms. Our goal was to further characterize this canine cancer, to improve the diagnosis and management of canine patients and evaluate BCLL as a model for human chronic lymphocytic leukemia (CLL).

Our collective results demonstrate that canine BCLL is a heterogeneous disease, as seen in human CLL. BCLL cases had a heterogeneous clinical outcome and gene expression profile. One subset of cases was defined by high tumor cell proliferation. These cases had increased expression of proliferation gene sets by gene expression profiling and significantly higher Ki67 expression compared to other BCLL cases. In the outcome study, cases with high Ki67 expression by flow cytometry had poorer outcomes. Though we didn't have outcome data on the cases in the gene expression study, we hypothesize that the proliferative cases, which were defined as BCLL subset 2, had more aggressive disease based on the prognostic significance of Ki67. Boxer dogs were overrepresented in the proliferative BCLL subset by gene expression profiling. In the outcome study, Boxers had significantly higher Ki67 expression and shorter survival than non-Boxers. We determined that Boxers with BCLL preferentially rearrange unmutated immunoglobulin heavy variable region (IGHV) genes. Unmutated IGHV CLL in people is associated with increased tumor cell proliferation and shorter survival times, similar to what we found in Boxers.[13,14,165] These results suggest that unmutated IGHV genes may play a role in the pathogenesis and clinical manifestation of BCLL, similar to human CLL. Importantly however, when we tested non-Boxers, we found that cases with poor prognosis +/- increased proliferation included a mixture of unmutated and mutated IGHV cases. Therefore, the role of IGHV mutation in canine BCLL needs further

investigation. Future directions are aimed at evaluating IGHV mutation status in a larger number of non-Boxers with outcome and Ki67 expression data.

This heterogeneity raised the question of whether these canine patient subsets had distinct neoplasms, or different manifestations of BCLL. This is a question that has been raised with several human lymphoma subtypes with a high degree of molecular and genetic heterogeneity, but early classification schemes concluded the most practical approach was to define entities that can easily be recognized with available morphologic, immunologic and genetic techniques, even if an entity encompasses distinctive clinical presentations.[207] Human CLL demonstrates striking clinical heterogeneity, but cases had a similar gene expression profile irrespective of IGHV mutation status, suggesting a common pathogenesis.[142] Human mantle cell lymphoma has two molecular subtypes that have different clinical behaviors, cells of origin and gene expression profiles, but they are characterized by the same translocation t(11;14) leading to CCND1 overexpression, and are thus considered two distinct subtypes of one disease.[208] Human diffuse large B-cell lymphoma (DLBCL) has a highly variable clinical course and was thought to include more than one disease entity, but subgroups could not be identified by morphology.[12,207] Gene expression profiling revealed that DLBCL encompasses two distinct diseases with largely non-overlapping transcriptomes and different cellular origins and activation states, but these diseases are morphologically indistinguishable and still fall within the DLBCL category.[12] Because we cannot differentiate canine BCLL subtypes by morphology or current immunophenotyping panels, we continue to classify these cases under the BCLL category. However, it is important to distinguish between subtypes for clinicians and clients, as these dogs have very different outcomes, with some dogs surviving weeks to months despite aggressive therapy, and others surviving years with no therapy. Future work is aimed at identifying antibodies or a panel of genes that could distinguish clinically relevant subgroups. For example, in human DLBCL, a gene expression signature that identifies a clinically and biologically distinct subgroup was translated into a NanoString assay.[209] A NanoString assay

detecting a panel of genes that are differentially expressed between distinct canine BCLL subgroups could be applied routinely to BCLL samples, allowing for further classification and better prognostic information. Future directions also aim to examine the cell of origin and genetic mutations for canine BCLL subtypes.

We investigated molecular and clinical similarities between canine BCLL and human CLL to evaluate the utility of canine BCLL as a translational model. These diseases share similarities in disease distribution and clinical presentation, including an expansion of small mature-appearing B cells in the blood, bone marrow and secondary lymphoid tissues, variable concurrent anemia and thrombocytopenia, older age at diagnosis, and inherited susceptibility/breed predisposition. Both diseases have marked clinical heterogeneity, with some patients having short survival despite aggressive therapy, and other patients having normal lifespans in which they may not need treatment and die of causes unrelated to BCLL/CLL. The canine BCLL gene expression profile was significantly enriched for human CLL gene sets by RNA Seq and NanoString analyses. Importantly, the gene expression profile of canine BCLL was not enriched for gene sets from other human B-cell neoplasms tested, including mantle cell lymphoma, follicular lymphoma and splenic marginal zone lymphoma. These results suggest that of the human B-cell neoplasms with a leukemic component, canine BCLL is most similar to human CLL. Numerous pathways are important for human CLL cell survival and proliferation, and we found that some of these pathways, including B-cell receptor signaling and NF-κB signaling, are altered in canine BCLL. Future work is aimed at evaluating these pathways functionally, to examine phosphorylation of signaling molecules and study effects of B-cell receptor activation and pathway inhibitors. We also aim to characterize the spectrum of genomic aberrations and somatic mutations in canine BCLL, to determine whether dogs have similar mutations to any of the human CLL subsets, which demonstrate a range of genomic aberrations and driver mutations. Other important aspects of human CLL biology that need to be evaluated in canine BCLL include the cell of origin, the epigenetic signature,

microenvironment interactions, microRNA dysregulation, splicing alterations and subclonal heterogeneity. As we explore these areas in the dog, we hope to learn more about this common canine neoplasm and uncover similarities to human CLL.

Breed predilections in canine BCLL may provide an opportunity to identify disease-risk alleles through genome-wide association studies (GWAS). The GWAS approach scans the genome with microarrays of single nucleotide polymorphisms (SNPs) to identify loci associated with a disease.[210] Because of linkage disequilibrium in the genome, alleles in different DNA segments (referred to as haplotype blocks) are associated with one another. A GWAS can identify SNPs in the region of the disease allele, and these discovered loci can then be finely mapped to identify disease-susceptibility variants. In humans, large sample sizes of cases and controls and large numbers of SNPs are needed to identify loci contributing to disease risk. Due to inbreeding and the resulting lack of genetic heterogeneity within pure dog breeds, canine GWAS are simpler and require fewer samples.[67,211] Dogs of a particular breed have larger haplotype blocks compared to humans, so fewer SNPs are needed to cover an inherited haplotype block.[67] Additionally, portions of haplotypes are shared across breeds, so genetic risk factors may be shared across breeds; for example, genetic risk factors for BCLL may be shared across small dog breeds.[67] A GWAS in small dog breeds with increased risk of developing BCLL may identify inherited risk loci associated with BCLL, which could potentially be of comparative value to human CLL. Additionally, a GWAS in Boxers, comparing BCLL cases and controls, may identify risk loci associated with more aggressive BCLL. We hypothesize that some of these loci identified in Boxers may be different than those identified in small breeds, and these results could perhaps inform studies of humans with aggressive unmutated IGHV CLL.

Finally, we discovered a novel syndrome in English bulldogs, defined by an expansion of polyclonal B cells in the blood. These cases have hyperglobulinemia, characterized by large amounts of polyclonal IgA +/- IgM, and normal to decreased amounts of IgG. Cases frequently have marked

splenomegaly, characterized by large expansions of polyclonal B cells. Future directions are aimed at examining the gene expression profile of these cases, to understand the signaling pathways driving non-malignant proliferation of B cells, IgA class switching and increased globulin secretion. The marked splenomegaly suggests that this syndrome may arise in the spleen, and future work is aimed at identifying the cell of origin. The predilection for this syndrome in young English bulldogs suggests a genetic predisposition in this breed, and we plan to pursue genomic sequencing to look for underlying mutations. We found that the diversity of immunoglobulin gene rearrangements in these cases becomes more restricted over time, and a subset of cases progress to a clonal malignancy. Future directions to monitor changes in gene expression and mutations over time would potentially identify mechanisms responsible for transformation from a pre-malignant reactive condition to a malignancy.

Overall, this work has helped improve our understanding of small-cell B-cell lymphocytosis diseases in dogs. As we continue to study the genetic risk, pathogenesis and outcomes of these diseases, we hope to improve care for our veterinary patients and contribute to the field of comparative oncology.

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APPENDIX

SUPPLEMENTARY MATERIAL FOR CHAPTER 1

Supplemental table S1. Canine IGHV genes, including the IMGT name, previous name, genomic location, and functionality information.

IMGT gene name and allele[a]	IMGT gene order	Previous gene name[b]	Functionality[c]	INIT-CODON position[d]	Open reading frame	OCTAMER sequence	INIT-CODON sequence	Splice site sequence	V-HEPTAMER sequence	V-NONAMER sequence	Slightly altered gene[e]	Highly altered gene[f]	New IGHV gene[g]
IGHV3-83*01	1	VH1-01P	P	74271225	no	ATGCAAAT	ATG	GT/AG	CACAGCG	ATAGAAACC	•		
IGHV3-82*01	2	VH1-02	F	74248215	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-81*01	3	VH1-03	F	74242927	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-80*01	4	VH1-04	F	74224404	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			

IGHV3-79*01	5	VH1-05P	P	74221247*	no	n/a	n/a	n/a	CACTGTG	ACACAAACC		•	
IGHV3-78*01	6	VH1-49P	P	74212847	no	ATGCAAAT	ATG	GT/AG	CACAGAG	ACAAACCTC	•		
IGHV3-77*01	7	VH1-07P	P	74196987*	no	n/a	n/a	TT/AG	CACAGTG	ACACAAACC		•	
IGHV3-76*01	8	n/a	F	74166350	yes	ATGCAAAT	ATG	GT/AG	CACCGTG	ATACACACC			•
IGHV3-75*01	9	VH1-08	F	74144648	yes	ATGCAAAG	ATG	GT/AG	CACAGTG	ATAGAAACC			
IGHV3-74*01	10	VH1-09P	P	74133429	no	AAGCAAAT	ATG	GT/AG	CACAATG	ACACAAACC	•		
IGHV3-73*01	11	VH1-10P	P	74108799	no	ATATAAAT	ATG	GT/AG	CACAGTG	ACACAAACA	•		
IGHV3-72*01	12	VH1-11P	P	74102005	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-71*01	13	n/a	P	74094207	no	ATGCAAAT	ATG	GT/AG	CACATTG	AGAAATCTC		•	•

IGHV3-70*01	14	VH1-12	F	74080028	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACT			
IGHV3-69*01	15	VH1-13	F	74068281	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-68*01	16	VH1-14P	P	74057772	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-67*01	17	VH1-15	F	74052503	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-66*01	18	VH1-16P	P	74045423	no	ATACAAAT	CTG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-65*01	19	VH1-17P	P	74036077	no	ATGAAAAT	ATG	GT/AG	CACTGTG	ACACAAACC		•	
IGHV3-64*01	20	VH1-18P	P	74028070	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC		•	
IGHV3-63*01	21	VH1-19P	P	74012210*	no	n/a	n/a	TT/AG	CACAGTG	ACACAAACC		•	
IGHV3-62*01	22	VH1-20P	P	73992957	no	ATACAAAA	ATG	GT/TG	CACAGTG	ACACAAACA	•		

IGHV3-61*01	23	VH1-21	F	73981005	yes	ATGCAAAT	ATG	GT/TG	CACATTG	ACACAAACC			
IGHV3-60*01	24	VH1-22P	P	73974531	no	ATGCAAAT	ATG	AT/AG	CACATTG	ACAGAAATC		•	
IGHV3-59*01	25	VH1-23P	P	73968132	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACT	•		
IGHV3-58*01	26	VH1-24	F	73956330	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACAGAAACC			
IGHV3-57*01	27	VH1-25P	P	73942869*	no	n/a	n/a	GT/AG	CAGAGTG	ACACAAACC		•	
IGHV3-56*01	28	VH1-26P	P	73939635	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC		•	
IGHV3-55*01	29	VH1-27P	P	73906008	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAAGC	•		
IGHV3-54*01	30	VH1-28	F	73886557	yes	ATGCAAAT	ATG	GT/AG	CACAATG	ACACAAACC			
IGHV3-53*01	31	VH1-29P	P	73865820*	no	n/a	n/a	GT/AG	CACAGTG	ACACAAACC		•	

IGHV3-52*01	32	VH1-30P	P	73851259	no	ATACAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-51*01	33	VH1-31P	P	73845797	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC		•	
IGHV3-50*01	34	VH1-32	F	73838094	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-49*01	35	VH1-33P	P	73829948	no	n/a	ATG	GT/AG	CACAGAG	ACACAAACC		•	
IGHV3-48*01	36	VH1-34P	P	73814217*	no	n/a	n/a	TT/AG	CACAGTG	ACACAAACC		•	
IGHV3-47-1*01	37	n/a	P	73774647	no	ATGCAAAT	ATG	GT/AG	CACCGTG	ATACACACC	•		•
IGHV3-47*01	38	VH1-35	F	73751519	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-46*01	39	VH1-36	F	73744495	yes	ATGCAAAT	ATG	GT/AG	CACAGTC	ACACAAACC			
IGHV3-45*01	40	VH1-37P	P	73734991*	no	n/a	n/a	GT/AG	TGCAGTG	ACAGAAACC		•	

IGHV3-44*01	41	VH1-38P	P	73729795	yes	ATGCAAAT	ATG	GA/AG	CACAGTG	ACACAAACC	•		
IGHV3-43*01	42	VH1-39P	P	73713601	no	ATGCAAAT	ATG	GT/AG	CACAATG	ACACAAACC	•		
IGHV3-42*01	43	VH1-40P	P	73702821	no	ATGCAAAT	ATG	GT/AG	CACAGAG	ACACAAACC		•	
IGHV3-41*01	44	VH1-41	F	73686189	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-40*01	45	VH1-42P	P	73663825	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACG	•		
IGHV3-39*01	46	VH1-43	F	73655490	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-38*01	47	VH1-44	F	73639568	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-37*01	48	VH1-45	F	73620033	yes	ATGCAAAT	ATG	GT/AG	CACAGTC	ACACAAACC			
IGHV3-36*01	49	VH1-46P	P	73600284	no	ATGCAAAT	ATG	GT/AG	TACAGTT	ACACAAACC	•		

IGHV3-35*01	50	VH1-47	F	73595945	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAAAC			
IGHV3-33*01	52	VH1-48	F	73580014	yes	ATGCAAAT	ATG	GT/AA	CACAGTG	ACACAAACC			
IGHV3-32*01	53	VH1-06	F	73576558	yes	ATGCAAAT	ATG	GT/AG	TACAGTG	ACACAAAAT			
IGHV3-31*01	54	VH1-50P	P	73565713	yes	ATGCAAAT	ATG	GT/AG	CACAATG	ACACAATCC	•		
IGHV1-30*01	55	VH2-51	F	73558100	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	TCAGAAACC			
IGHV3-29*01	56	VH1-52P	P	73547836	no	ATGCAAAT	ATG	GT/AG	CACAGTG	GCACAAACC	•		
IGHV3-28*01	57	VH1-53P	P	73534024	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-27*01	58	VH1-54P	P	73520719	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-26*01	59	VH1-55P	P	73509474	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		

IGHV3-25*01	60	VH1-56P	P	73487223	no	ATGCAAAC	ATG	GT/AG	CACAGTG	ATACAAACC	•		
IGHV3-24*01	61	VH1-57	F	73478222	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACTCACT			
IGHV3-23*01	62	VH1-58	F	73466463	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-22*01	63	VH1-59P	P	73450839	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-21-1*01	64	n/a	P	73442614*	no	n/a	n/a	AG	CACATTG	ACAGAAGTC		•	•
IGHV3-21*01	65	VH1-60P	P	73430644	no	ATGCAAAT	ATG	AT/AG	CACAATG	ACACAAACC	•		
IGHV3-20*01	66	VH1-61P	P	73412008	no	ACACAAAT	ATG	GT/AG	CAAAGTG	ACACAAACC		•	
IGHV3-19*01	67	VH1-62	F	73387704	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-18*01	68	VH1-63	F	73365401	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAAAC			

IGHV1-17*01	69	VH2-64P	P	73327998	no	n/a	ATG	GT/AG	GACAGTG	TCAGAAACC	•		
IGHV3-16*01	70	VH1-65	F	73306390	yes	ATGCAAAG	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV1-15*01	71	VH2-66P	P	73295459	no	ATGCAAAT	ATG	GT/AG	CACAGTG	TCAGAAACC	•		
IGHV3-14*01	72	VH1-67P	P	73272544	no	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC	•		
IGHV3-13*01	73	VH1-68	F	73267073	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-12*01	74	VH1-69P	P	73235778	no	ATGCAAAT	ATG	GT/AG	CACAATG	ACACAAACC	•		
IGHV3-11*01	75	VH1-70P	P	73216544	no	ATTCAAAT	ATG	GT/AC	CAGAGAG	AGACACAAA	•		
IGHV3-10*01	76	VH1-71	F	73195153	yes	ATGCAAAT	ATG	GT/AG	CACCGTG	ACACAAACC			
IGHV3-9*01	77	VH1-72	F	73185283	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			

IGHV3-8*01	78	VH1-73	F	73159977	yes	ATGCAAAT	ATG	GT/AG	CACAATG	ACACAAACC			
IGHV3-7*01	79	VH1-74	F	73153457	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-6*01	80	VH1-75	F	73137027	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			
IGHV3-5-1*01	81	VH1-76P	P	73123182	no	ATGCAAAT	ATG	GT/AG	CACAATG	ACACAAACC		•	
IGHV3-5*01	82	VH1-77	F	73109226	yes	ATGCAAAT	ATG	GT/AC	CACCGTG	ACACAAACC			
IGHV3-4*01	83	n/a	P	73068674	no	TTGCAAAT	ATG	GT/AC	CACAGTG	ACACAAACC	•		•
IGHV3-3*01	84	VH1-78	F	73056536	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACT			
IGHV3-2*01	85	VH1-79	F	73038393	yes	ATGCAAAT	ATG	GT/AG	CACAGTA	ACACAAACC			
IGHV4-1*01	86	VH3-80	F	73028843	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			

IGHV3-NL1*01	0	n/a	F	n/a	yes	ATGCAAAT	ATG	GT/AG	CACAGTG	ACACAAACC			•
IGHV3-NL2*01	0	n/a	P	n/a	no	ATACAAAG	ATG	AT/AG	CGCAGGG	ACGCAAACC		•	•

[a]The IMGT name is provided for the canine IGHV genes (http://www.imgt.org, release 201715-4 (13 April 2017)).
[b]The previous IGHV gene name from Bao et al. is listed.
[c]IGHV genes are designated as functional (F) or pseudogenes (P). The IMGT name is bolded for functional canine IGHV genes.
[d]The genomic location on chromosome 8 is identified for the start of the initiation codon, INIT-CODON (CanFam3.1, NCBI Accession NC_006590.3). Pseudogenes without an INIT-CODON are indicated with an asterisk, and the genomic location provided is the start of the V-HEPTAMER of the V-RS (recombination sequence).
The sequence for the following gene elements is provided: OCTAMER, INIT-CODON, ACCEPTOR-SPLICE AND DONOR-SPLICE sites, V-HEPTAMER and V-NONAMER. 'n/a' indicates that the gene element was not found.
[e]Pseudogenes that are slightly altered are identified with an '•.' These are pseudogenes in the reference Boxer genome, but appear to have the potential to be functional if there were minor changes/polymorphisms in the sequence.
[f]Pseudogenes that are highly altered are identified, indicating these genes have major alterations and do not appear to have the potential to be functional.
[g]Genes indicated with an '•' are newly identified IGHV genes, that were not previously reported in Bao et al.

SUPPLEMENTARY MATERIAL FOR CHAPTER 3

Supplemental table S2. RNA Seq quality control data and statistics for control B-cell and BCLL samples.

Sample	Raw reads	Clean reads	Clean bases	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
Control 1	77688718	73920248	11.1G	0.01	97.13	92.85	48.99
Control 2	75881332	72363692	10.9G	0.01	97.17	92.9	49.13
Control 3	54559714	52504426	7.9G	0.02	95.55	89.42	49.2
BCLL case 1	47531362	45433774	6.8G	0.02	96.52	91.49	49.5
BCLL case 2	40192404	39153634	5.9G	0.02	97.25	92.98	51.49
BCLL case 3	70326624	67326806	10.1G	0.02	97.23	92.85	49.23
BCLL case 4	49317490	47975092	7.2G	0.02	97.06	92.63	48.33
BCLL case 5	41326238	40378024	6.1G	0.02	97.12	92.78	48.64
BCLL case 6	51314044	49057702	7.4G	0.02	96.62	91.69	48.74
BCLL case 7	49739918	47767896	7.2G	0.02	96.8	92.07	48.53
BCLL case 8	50493844	48534680	7.3G	0.02	96.83	92.1	48.7
BCLL case 9	56455156	54476998	8.2G	0.02	97.14	92.67	47.89
BCLL case 10	49226940	47987090	7.2G	0.02	96.97	92.38	49.53
BCLL case 11	45011286	44037678	6.6G	0.02	97.16	92.78	49.74
BCLL case 12	49111508	47957864	7.2G	0.02	96.68	91.86	49.4

Raw Reads: the original sequencing reads counts; Clean Reads: number of reads after filtering; Clean Bases: clean reads number multiplied by read length; Error Rate: average sequencing error rate, which is calculated by Qphred=-10log10(e); Q20: percentage of bases whose correct base recognition rates are greater than 99% in total bases; Q30: percentage of bases whose correct base recognition rates are greater than 99.9% in total bases; GC content: percentage of G and C in total bases.

Supplemental table S3. RNA Seq mapping results for control B-cell and BCLL samples.

Sample	Total reads	Total mapped	Multiple mapped	Uniquely mapped
Control 1	73920248	62806568 (84.97%)	1472084 (1.99%)	61334484 (82.97%)
Control 2	72363692	62132754 (85.86%)	1303151 (1.8%)	60829603 (84.06%)
Control 3	52504426	42942469 (81.79%)	910145 (1.73%)	42032324 (80.05%)
BCLL case 1	45433774	38195608 (84.07%)	1008821 (2.22%)	37186787 (81.85%)
BCLL case 2	39153634	32661154 (83.42%)	1008805 (2.58%)	31652349 (80.84%)
BCLL case 3	67326806	58276073 (86.56%)	1609866 (2.39%)	56666207 (84.17%)
BCLL case 4	47975092	40848955 (85.15%)	1009378 (2.1%)	39839577 (83.04%)
BCLL case 5	40378024	34464840 (85.36%)	816139 (2.02%)	33648701 (83.33%)
BCLL case 6	49057702	42233186 (86.09%)	936891 (1.91%)	41296295 (84.18%)
BCLL case 7	47767896	41728332 (87.36%)	945811 (1.98%)	40782521 (85.38%)
BCLL case 8	48534680	42204240 (86.96%)	1130919 (2.33%)	41073321 (84.63%)
BCLL case 9	54476998	47781244 (87.71%)	1008555 (1.85%)	46772689 (85.86%)
BCLL case 10	47987090	41420202 (86.32%)	938795 (1.96%)	40481407 (84.36%)
BCLL case 11	44037678	37948814 (86.17%)	822888 (1.87%)	37125926 (84.3%)
BCLL case 12	47957864	40562951 (84.58%)	991397 (2.07%)	39571554 (82.51%)

Total reads: total number of filtered reads; Total mapped: total number of reads mapped to the reference genome; Multiple mapped: number of reads that mapped to multiple sites in the reference genome; Uniquely mapped: number of reads uniquely mapped to the reference genome.

Supplemental table S4. The top 250 genes overexpressed in all BCLL samples vs. control B cells (above) and the top 250 genes underexpressed in all BCLL samples vs. control B cells (below). Differentially expressed genes were identified RNA Seq DESeq2 analysis, p-adj <0.05. FC (fold change): log2(FC) BCLL vs. control B cells.

Genes overexpressed in all BCLL samples vs. control B cells

Gene name	FC
PPBP	7.95
ZNF503	6.02
SMOX	6.00
GNG11	5.94
TNS1	5.76
CD9	5.45
ANPEP	5.43
MGST1	5.43
GP1BB	5.32
PPIC	5.26
VCAN	5.24
PLAUR	5.17
CTSE	5.11
F13A1	5.08
PRSS57	5.01
VSTM1	4.93
TREM1	4.90
MPIG6B	4.86
MYCT1	4.82
THBS1	4.81
LRP1	4.78
SEMA3G	4.77
OLIG1	4.73
TMBIM1	4.70
RAB44	4.70
NRG1	4.67
GZMB	4.67
GUCY1A1	4.63
AHNAK	4.61
CCL14	4.56
VSIR	4.55
HSD17B14	4.47
ANXA1	4.45
FAM20C	4.44
NFE2	4.44
S100A8	4.43
EGF	4.38
GP6	4.38
C30H15orf48	4.34
FBLN2	4.29
GAS2L1	4.28
GPAT3	4.28
TBXAS1	4.28
KCNQ1	4.26
LPCAT2	4.26
ANXA2	4.24
TFPI	4.23
LZTS3	4.23
CD55	4.21
WLS	4.19
ST6GALNAC2	4.19
SERPINB10	4.17
HSF4	4.13
TRIM58	4.13
CAPN5	4.10
RAP1GAP2	4.09
CHRNB4	4.09
MMP1	4.08
SELP	4.07
LMO1	4.07
LCN2	4.06
TIMP2	4.05
CLEC1B	4.04
LGALS3	4.03
C5AR1	4.01
TMEM40	4.00
ITGA2B	4.00
SEPTIN5	3.99
TNS2	3.99
MARCO	3.97
ITGB3	3.96
LOC608987	3.96
SLC30A8	3.94
CDA	3.93
PDLIM1	3.93
CASS4	3.91
SH2D1B	3.90
FN1	3.89
BPI	3.85
PEAK3	3.85
HEPH	3.85
DPYS	3.84
CDKN1A	3.84
CLCF1	3.83
SLC16A3	3.82
ESRP1	3.82
MAFB	3.82
PLD1	3.78
SH3PXD2B	3.78
LATS2	3.77
DAGLA	3.75
VIM	3.74
B4GALNT1	3.73
ALDH3B1	3.73
SLCO4C1	3.71
ASAH1	3.71
CCR3	3.70
NFIL3	3.70
TNFRSF1A	3.70
YPEL4	3.69
HSD11B2	3.68
CMKLR1	3.67
PCDH19	3.66
MAPK13	3.66
SERPINA1	3.65
PLEC	3.65
HPGD	3.65
SLC49A3	3.65
SERPINE2	3.64
PPP1R3B	3.64
STOM	3.64
RBP4	3.63
PPARG	3.63
EHD2	3.63
NLRP1	3.62
ANKRD2	3.62
LIMS2	3.61
BCL2L15	3.59
CXCL6	3.59
CA6	3.59
PLCD1	3.57
PADI2	3.57
PALM	3.56
KLHL35	3.55
CDC42EP2	3.54
DGKG	3.54
MIOX	3.53
HBM	3.53
C5AR2	3.52
C20H19orf38	3.51
SULF2	3.48
DCSTAMP	3.48
COL6A5	3.47
CA8	3.47
PLA2G7	3.46
AQP1	3.46
RND3	3.46
DRAM1	3.46
RNF144B	3.45
CNKSR3	3.45
SMAD9	3.44
PYGL	3.43
SERPINB2	3.42
SH3BGRL2	3.41
ANKRD55	3.41
UNC5B	3.41
GPR84	3.41
GRAP2	3.40
RPS6KA2	3.40
REPS2	3.40
IL1R1	3.39
PTGIR	3.39
ARHGAP6	3.38
B3GALNT1	3.37
ADGRG3	3.37
RXRA	3.37
OSGIN1	3.35
TPPP	3.34
TGFBR3	3.34
ITGAM	3.34
SGSM1	3.34
TAL1	3.33
HK3	3.33
EPDR1	3.33
RCAN2	3.32
EPS8	3.32
MEFV	3.32
MYO1F	3.31
NDRG2	3.30
SYN3	3.30
COL8A2	3.27
RHOB	3.27
TSPAN13	3.26
TMEM240	3.25
NID1	3.25
LY6G6C	3.25
PRKAA2	3.24
MRVI1	3.24
TGFBI	3.24
HNMT	3.23
PRKN	3.22
MET	3.22
TCN1	3.22
EMP1	3.22
MIR29B2	3.22
CREB5	3.22
ZNF496	3.21
ABCA5	3.21
F5	3.20
PDE2A	3.19
PLEKHA5	3.18
DNAJA4	3.18
S100P	3.18
PIR	3.17
PKIB	3.16
RGS22	3.15
PTGER2	3.15
SNORD63	3.14
LMNA	3.13
SPECC1	3.13
CD226	3.12
MGAT3	3.12
LRMDA	3.12
KCNQ2	3.11
ESYT3	3.11
DLGAP3	3.11
MFAP3L	3.11
FGL2	3.11
TMEM74B	3.10
TCEA3	3.10
CRISP2	3.10
ADM	3.09
MAPK8IP1	3.09
CA2	3.09
GSDME	3.09
AIF1	3.09
CIDEA	3.09
CORO2A	3.09
GLRX	3.08
CEP295NL	3.08
ATF3	3.07
ESAM	3.07
cdkn2A	3.07
DGAT2	3.07
PTPRU	3.07
FBLIM1	3.06
LYZ	3.06
SYTL3	3.05
CKM	3.05
GALNT12	3.05
DPP4	3.04
CFP	3.04
ARHGEF10L	3.04
TNFRSF1B	3.04
GAB3	3.04
PNMA1	3.04
C3	3.04
CCDC3	3.03
SCARB2	3.02
KLF11	3.02
APBB1	3.02
LTBR	3.01
PLEKHG3	3.01
GRAMD1B	3.01
SRGN	3.00
NIBAN2	3.00
SCARF2	3.00
FRMD4B	3.00
IL1RN	2.98
PSCA	2.98

Genes underexpressed in all BCLL samples vs. control B cells

Gene name	FC	U3	-2.25	SPAG5	-2.64	NHS	-3.09	KCNF1	-4.07
CNTLN	-1.98	SPRED2	-2.25	EPHB3	-2.65	MEF2B	-3.10	TSHR	-4.08
THNSL1	-1.99	PIK3R3	-2.25	U6	-2.66	MPZL2	-3.10	KL	-4.10
HMCN2	-1.99	YPEL1	-2.26	AFF2	-2.66	PDGFRB	-3.10	SEMA6A	-4.14
PTPRB	-1.99	TPM2	-2.27	CKAP2L	-2.66	B3GALT1	-3.11	CDON	-4.22
KIF11	-2.00	KNTC1	-2.27	PTER	-2.68	TMTC2	-3.12	TNFRSF11B	-4.24
BPHL	-2.00	IGF2BP3	-2.28	TJP1	-2.68	ZC4H2	-3.14	VEGFC	-4.25
TASP1	-2.00	KCNK13	-2.28	LGALS12	-2.70	XIRP2	-3.14	YAP1	-4.29
TSPO	-2.02	CRIP3	-2.29	SLC26A5	-2.71	GADL1	-3.14	ZNF835	-4.31
HTR6	-2.02	TDRP	-2.29	CDO1	-2.72	MARCKSL1	-3.15	NRG3	-4.35
LPP	-2.03	ASNS	-2.29	LTBP1	-2.72	ABCC6	-3.15	POU2F3	-4.40
CBX2	-2.03	PCP4L1	-2.30	MEGF10	-2.74	NOVA1	-3.18	LYPD5	-4.44
ASIC1	-2.04	HSPG2	-2.31	SLC6A18	-2.75	ENAH	-3.18	DDIT4L	-4.51
ACKR2	-2.04	TMEM245	-2.31	RHOU	-2.75	ADGRV1	-3.19	NWD2	-4.54
PCLO	-2.05	VXN	-2.33	VPREB3	-2.75	IL21R	-3.19	ARHGEF9	-4.58
CACNA1I	-2.05	NR2F6	-2.33	RCAN1	-2.77	STK39	-3.20	ENPP6	-4.58
PLEKHA7	-2.06	IQGAP3	-2.33	OLFM4	-2.77	EGFL6	-3.20	CCL22	-4.59
RAPGEF5	-2.07	PNPLA5	-2.34	RIMKLB	-2.78	COL23A1	-3.21	NUGGC	-4.62
GCAT	-2.07	ZNF541	-2.35	GPC6	-2.78	PKP1	-3.25	CLCN4	-4.64
ARHGEF39	-2.08	C1QC	-2.35	PLPPR1	-2.78	FMNL3	-3.26	OAF	-4.75
SLCO5A1	-2.09	SSBP2	-2.35	SPRED1	-2.78	C1QB	-3.29	MBOAT2	-4.75
ZNF556	-2.12	IQCD	-2.36	CA9	-2.82	CCNB3	-3.31	LRAT	-4.79
WDR88	-2.12	MN1	-2.36	TROAP	-2.82	GTF2IRD1	-3.33	GALNT5	-4.89
CDC42BPA	-2.13	TOP2A	-2.36	SPIN4	-2.83	CCDC172	-3.35	ARRDC5	-4.97
ZNF551	-2.13	ME1	-2.36	SLAMF1	-2.84	AKAP12	-3.40	AMOTL1	-4.97
SNORD93	-2.14	CYSLTR2	-2.36	PLS1	-2.85	CLSTN3	-3.41	ZNF532	-5.03
CAPS2	-2.14	MMP11	-2.38	ZSCAN18	-2.86	MAL2	-3.42	PIMREG	-5.04
BCL6	-2.14	ENTPD3	-2.39	PTP4A3	-2.86	IGKC	-3.43	SPATA20	-5.09
SOX4	-2.14	GAS2L2	-2.39	IQSEC2	-2.86	GPR158	-3.52	HTR1F	-5.13
TACR1	-2.14	ZNF704	-2.40	NTSR1	-2.86	ZNF536	-3.56	ARPP21	-5.14
SIRT2	-2.14	KCNJ9	-2.42	CADPS2	-2.87	TOX	-3.57	FER1L5	-5.32
IGFBP4	-2.14	FMN1	-2.46	B3GALT2	-2.88	PLAC8L1	-3.57	ENO4	-5.35
GAS1	-2.15	BUB1B	-2.46	KLHL6	-2.88	MYBL1	-3.63	PROKR2	-5.40
FSTL4	-2.15	ST8SIA5	-2.46	ACTA2	-2.89	VCAM1	-3.63	UBE2QL1	-5.42
KIF22	-2.15	EXOC3L1	-2.49	RTKN2	-2.91	PBK	-3.63	DLGAP2	-5.54
MTUS2	-2.15	SHOX2	-2.51	LOC100682772	-2.91	BEX5	-3.68	MYH8	-5.59
ZNF571	-2.16	TLE2	-2.52	SOWAHA	-2.91	NHSL1	-3.69	C25H2orf72	-5.67
PLPP2	-2.16	ZNF550	-2.52	RASL10B	-2.92	CFAP300	-3.73	ARHGAP8	-5.72
PRAG1	-2.18	SEL1L3	-2.53	CTTN	-2.93	POU6F2	-3.76	ACTC1	-5.80
SYCE2	-2.19	NPR1	-2.54	MS4A13	-2.93	CD34	-3.78	INSYN1	-6.30
MEGF6	-2.21	LAMA3	-2.56	CES2	-2.94	TDRD1	-3.82	GCSAM	-6.63
EGR3	-2.21	ABCG2	-2.57	GRXCR2	-2.95	CACNA2D2	-3.82	GJD2	-6.83
IGSF8	-2.21	DZIP1	-2.57	TMEM108	-3.00	LRRC32	-3.84	TEKT3	-7.06
LRIG3	-2.21	PIF1	-2.58	FMO1	-3.01	ASIC4	-3.84	RBM46	-7.12
TMC3	-2.22	RARRES2	-2.58	PDE7B	-3.03	COL5A3	-3.86	CNTN5	-7.19
SNORD27	-2.22	UHRF1	-2.60	BCL7A	-3.04	PEG3	-3.88	AICDA	-7.79
BUB1	-2.22	CUBN	-2.60	CAB39L	-3.05	EFCAB6	-3.88	VPREB1	-10.46
FSCN1	-2.22	SPSB1	-2.60	PALM2AKAP2	-3.07	SMOC1	-3.93		
CHRNA5	-2.22	PAK3	-2.62	SLC6A12	-3.08	KIAA1755	-3.96		
ZHX3	-2.23	ADAMTS7	-2.63	SPO11	-3.09	DNMT3L	-4.03		
WIPF3	-2.23	FREM2	-2.63	RHEX	-3.09	COL9A2	-4.03		

Supplemental table S5. Summary of hallmark gene sets enriched between canine BCLL subgroup 1 and BCLL subgroup 2. Gene set enrichment analysis results using significantly differentially expressed genes in BCLL subgroup 2 vs. BCLL subgroup 1 (RNA Seq, DESeq2, p-adj <0.05). All gene sets with a false discovery rate <0.25 are listed.

Gene set	Enriched phenotype	NES	NOM p-val	FDR q-val
E2F targets	BCLL subgroup 2	3.15	<0.0001	<0.0001
Mitotic spindle	BCLL subgroup 2	2.11	<0.0001	0.0152
G2M checkpoint	BCLL subgroup 2	2.03	0.0042	0.0179
Glycolysis	BCLL subgroup 2	1.68	0.0305	0.1128
Spermatogenesis	BCLL subgroup 2	1.54	0.0535	0.1744
IL2 STAT5 signaling	BCLL subgroup 2	1.47	0.0963	0.2011
TNFA signaling via NFKB	BCLL subgroup 1	-4.80	<0.0001	<0.0001
Epithelial mesenchymal transition	BCLL subgroup 1	-3.25	<0.0001	<0.0001
Inflammatory response	BCLL subgroup 1	-2.53	<0.0001	<0.0001
Unfolded protein response	BCLL subgroup 1	-2.37	<0.0001	0.0018
Hypoxia	BCLL subgroup 1	-2.11	0.0020	0.0111
Myogenesis	BCLL subgroup 1	-2.05	0.0021	0.0141
KRAS signaling down	BCLL subgroup 1	-1.94	0.0100	0.0265
MYC targets	BCLL subgroup 1	-1.80	0.0123	0.0481
Oxidative phosphorylation	BCLL subgroup 1	-1.80	0.0102	0.0526
UV response up	BCLL subgroup 1	-1.60	0.0471	0.1091
KRAS signaling up	BCLL subgroup 1	-1.61	0.0452	0.1118
Coagulation	BCLL subgroup 1	-1.57	0.0463	0.1199
Apoptosis	BCLL subgroup 1	-1.47	0.0692	0.1670
DNA repair	BCLL subgroup 1	-1.38	0.1106	0.2260

Estrogen response early	BCLL subgroup 1	-1.36	0.1285	0.2307

NES, normalized enrichment score, NOM p-val, nominal p value; FDR q-val, false discovery rate.

Supplemental table S6. Summary of gene sets enriched between canine BCLL subgroup 1 and control B cells from healthy dog lymph nodes. Gene set enrichment analysis results using significantly differentially expressed genes in BCLL subgroup 1 vs. control B cells (RNA Seq, DESeq2, p-adj <0.05). All gene sets with a false discovery rate <0.25 are listed.

Gene set	Enriched phenotype	NES	NOM p-val	FDR q-val
Hallmarks gene sets[163]				
TNFA signaling via NFKB	BCLL subgroup 1	5.06	<0.0001	<0.0001
Inflammatory response	BCLL subgroup 1	4.10	<0.0001	<0.0001
Epithelial mesenchymal transition	BCLL subgroup 1	3.65	<0.0001	<0.0001
Coagulation	BCLL subgroup 1	3.64	<0.0001	<0.0001
KRAS signaling up	BCLL subgroup 1	3.10	<0.0001	<0.0001
Complement	BCLL subgroup 1	2.84	<0.0001	<0.0001
IL2 STAT5 signaling	BCLL subgroup 1	2.83	<0.0001	<0.0001
Apoptosis	BCLL subgroup 1	2.63	<0.0001	<0.0001
Hypoxia	BCLL subgroup 1	2.39	<0.0001	0.0015
Estrogen response early	BCLL subgroup 1	2.19	0.0020	0.0059
KRAS signaling down	BCLL subgroup 1	2.06	0.0063	0.0127
IL6 JAK STAT3 signaling	BCLL subgroup 1	2.01	0.0039	0.0151
UV response up	BCLL subgroup 1	1.97	0.0039	0.0171
Myogenesis	BCLL subgroup 1	1.83	0.0193	0.0354
Allograft rejection	BCLL subgroup 1	1.66	0.0240	0.0663

Unfolded protein response	BCLL subgroup 1	1.67	0.0444	0.0695
PI3K AKT MTOR signaling	BCLL subgroup 1	1.58	0.0501	0.0926
TGFB signaling	BCLL subgroup 1	1.54	0.0745	0.1080
UV response down	BCLL subgroup 1	1.50	0.0684	0.1255
Protein secretion	BCLL subgroup 1	1.47	0.0660	0.1369
p53 pathway	BCLL subgroup 1	1.44	0.0837	0.1457
Estrogen response late	BCLL subgroup 1	1.36	0.1319	0.1874
Heme metabolism	BCLL subgroup 1	1.36	0.1250	0.1950
Cholesterol homeostasis	BCLL subgroup 1	1.29	0.1728	0.2372
Adipogenesis	BCLL subgroup 1	1.27	0.1889	0.2381
E2F targets	Control B cells	-4.67	<0.0001	<0.0001
G2M checkpoint	Control B cells	-3.50	<0.0001	<0.0001
MYC targets	Control B cells	-2.31	<0.0001	0.0013
Mitotic spindle	Control B cells	-1.80	0.0155	0.0303
Oxidative phosphorylation	Control B cells	-1.66	0.0416	0.0523
Staudt laboratory gene sets (https://lymphochip.nih.gov/signaturedb/index.html)				
Quiescence	BCLL subgroup 1	2.06	0.0076	0.0272
BCR signaling up	BCLL subgroup 1	2.12	0.0060	0.0326
TGFB up	BCLL subgroup 1	1.75	0.0277	0.0928
IL4 up	BCLL subgroup 1	1.66	0.0437	0.1025
IL6 up	BCLL subgroup 1	1.57	0.0487	0.1082
KRAS up	BCLL subgroup 1	1.60	0.0282	0.1108
NFKB targets	BCLL subgroup 1	1.52	0.0719	0.1193

MYD88 up	BCLL subgroup 1	1.48	0.0629	0.1225
BCLL BCR signaling up	BCLL subgroup 1	1.45	0.0870	0.1267
KLF2 up	BCLL subgroup 1	1.30	0.1654	0.2079
JAK2 up	Control B cells	-2.63	<0.0001	<0.0001
MYC up	Control B cells	-2.13	0.0019	0.0199
E2F up	Control B cells	-2.01	0.0038	0.0305
MTOR down	Control B cells	-1.85	0.0173	0.0569
IRF4 down	Control B cells	-1.77	0.0204	0.0679
KEGG (Kyoto Encyclopedia of Genes and Genomes) gene sets				
Complement and coagulation cascades	BCLL subgroup 1	3.40	0.0000	0.0000
Huntingtons disease	BCLL subgroup 1	2.75	0.0000	0.0000
Ribosome	BCLL subgroup 1	2.67	0.0000	0.0004
Spliceosome	BCLL subgroup 1	2.52	0.0000	0.0031
Cell adhesion molecules cams	BCLL subgroup 1	2.46	0.0020	0.0033
Cytokine-cytokine receptor interaction	BCLL subgroup 1	2.48	0.0000	0.0035
Neuroactive ligand receptor interaction	BCLL subgroup 1	2.43	0.0000	0.0039
Parkinsons disease	BCLL subgroup 1	2.36	0.0000	0.0045
Hematopoietic cell lineage	BCLL subgroup 1	2.19	0.0020	0.0133
Oxidative phosphorylation	BCLL subgroup 1	2.05	0.0039	0.0267
Adipocytokine signaling pathway	BCLL subgroup 1	1.98	0.0135	0.0381

Alzheimers disease	BCLL subgroup 1	1.96	0.0078	0.0388
NOD like receptor signaling pathway	BCLL subgroup 1	1.92	0.0082	0.0407
ECM receptor interaction	BCLL subgroup 1	1.73	0.0165	0.0997
MAPK signaling pathway	BCLL subgroup 1	1.74	0.0246	0.1012
Gap junction	BCLL subgroup 1	1.69	0.0235	0.1091
Focal adhesion	BCLL subgroup 1	1.64	0.0407	0.1354
T cell receptor signaling pathway	BCLL subgroup 1	1.60	0.0356	0.1532
Prostate cancer	BCLL subgroup 1	1.57	0.0552	0.1682
Calcium signaling pathway	BCLL subgroup 1	1.51	0.0736	0.2048
Insulin signaling pathway	BCLL subgroup 1	1.47	0.0799	0.2429
Cell cycle	Control B cells	-2.14	0.0000	0.0296
DNA replication	Control B cells	-2.04	0.0000	0.0419
Pyrimidine metabolism	Control B cells	-2.14	0.0000	0.0592
Oocyte meiosis	Control B cells	-1.91	0.0079	0.0706
Peroxisome	Control B cells	-1.74	0.0259	0.0831
Valine leucine and isoleucine degradation	Control B cells	-1.77	0.0163	0.0837
Progesterone mediated oocyte maturation	Control B cells	-1.72	0.0297	0.0846
Purine metabolism	Control B cells	-1.78	0.0194	0.0915
Ubiquitin mediated proteolysis	Control B cells	-1.80	0.0081	0.1009
P53 signaling pathway	Control B cells	-1.50	0.0668	0.2106

NES, normalized enrichment score, NOM p-val, nominal p value; FDR q-val, false discovery rate.

Supplemental table S7. Summary of reactome gene sets enriched between canine BCLL subgroup 1 and control B cells from healthy dog lymph nodes. Gene set enrichment analysis results using significantly differentially expressed genes in BCLL subgroup 1 vs. control B cells (RNA Seq, DESeq2, p-adj <0.05). A total of 255 gene sets had a false discovery rate <0.25. Select gene sets with an FDR <0.1 are listed.

Gene set	Enriched phenotype	NES	NOM p-val	FDR q-val
Metabolism of RNA	BCLL subgroup 1	3.83	<0.0001	<0.0001
Cellular reponses to external stimuli	BCLL subgroup 1	3.72	<0.0001	<0.0001
GPCR ligand binding	BCLL subgroup 1	3.44	<0.0001	<0.0001
Processing of capped intron containing pre mRNA	BCLL subgroup 1	3.24	<0.0001	<0.0001
Eukaryotic translation elongation	BCLL subgroup 1	3.23	<0.0001	<0.0001
Signaling by GPCR	BCLL subgroup 1	3.23	<0.0001	<0.0001
Extracellular matrix organization	BCLL subgroup 1	3.06	<0.0001	<0.0001
mRNA splicing	BCLL subgroup 1	3.00	<0.0001	<0.0001
Post translational protein modification	BCLL subgroup 1	2.84	<0.0001	<0.0001
IL1 family signaling	BCLL subgroup 1	2.45	<0.0001	0.0022
Innate immune system	BCLL subgroup 1	2.44	<0.0001	0.0022
Autophagy	BCLL subgroup 1	2.45	<0.0001	0.0022
FCERI mediated NFKB activation	BCLL subgroup 1	2.29	0.0022	0.0053
Cell cell communication	BCLL subgroup 1	2.01	<0.0001	0.0216
Chromatin modifying enzymes	BCLL subgroup 1	2.00	0.0020	0.0219
Signaling by interleukins	BCLL subgroup 1	1.97	0.0117	0.0241
Cellular response to hypoxia	BCLL subgroup 1	1.95	0.0060	0.0267
IL4 and IL13 signaling	BCLL subgroup 1	1.92	0.0096	0.0305

Signaling by WNT	BCLL subgroup 1	1.91	0.0082	0.0317
Transport of mature transcript to cytoplasm	BCLL subgroup 1	1.87	0.0100	0.0379
Downstream signaling events of B-cell receptor	BCLL subgroup 1	1.86	0.0101	0.0387
Integrin cell surface interactions	BCLL subgroup 1	1.86	0.0136	0.0404
Signaling by FGFR2	BCLL subgroup 1	1.82	0.0226	0.0476
Signaling by ERBB2	BCLL subgroup 1	1.79	0.0099	0.0517
PTEN regulation	BCLL subgroup 1	1.79	0.0157	0.0520
Signaling by NOTCH4	BCLL subgroup 1	1.75	0.0211	0.0608
Cytokine signaling in immune system	BCLL subgroup 1	1.75	0.0171	0.0622
Regulation of PTEN stability and activity	BCLL subgroup 1	1.71	0.0151	0.0739
Death receptor signaling	BCLL subgroup 1	1.69	0.0287	0.0767
Antigen processing cross presentation	BCLL subgroup 1	1.68	0.0259	0.0808
MAPK6 MAPK4 signaling	BCLL subgroup 1	1.62	0.0339	0.0975
Cell cycle	Control B cells	-3.56	<0.0001	<0.0001
DNA repair	Control B cells	-3.09	<0.0001	<0.0001
Cell cycle checkpoints	Control B cells	-2.88	<0.0001	1.07E-04
Cell cycle mitotic	Control B cells	-2.93	<0.0001	1.28E-04
Homology directed repair	Control B cells	-3.00	<0.0001	1.60E-04
DNA double strand break repair	Control B cells	-3.01	<0.0001	2.13E-04
Chromosome maintenance	Control B cells	-2.70	<0.0001	6.66E-04
G2 M checkpoints	Control B cells	-2.41	<0.0001	0.0038
DNA replication	Control B cells	-2.22	<0.0001	0.0107
S phase	Control B cells	-2.19	0.0019	0.0108

Mitotic G1 phase and G1 S transition	Control B cells	-2.20	<0.0001	0.0109
Processing of DNA double strand break ends	Control B cells	-2.14	<0.0001	0.0129
M phase	Control B cells	-2.14	0.0020	0.0132
Base excision repair	Control B cells	-2.02	0.0084	0.0214
Telomere maintenance	Control B cells	-1.94	0.0077	0.0279
Transcriptional regulation by TP53	Control B cells	-1.94	0.0041	0.0284
Regulation of TP53 activity	Control B cells	-1.63	0.0377	0.0978

Supplemental table S8. Summary of gene sets enriched between canine BCLL subgroup 2 and control B cells from healthy dog lymph nodes. Gene set enrichment analysis results using significantly differentially expressed genes in BCLL subgroup 2 vs. control B cells (RNA Seq, DESeq2, p-adj <0.05). All gene sets with a false discovery rate <0.25 are listed.

Gene set	Enriched phenotype	NES	NOM p-val	FDR q-val
Hallmarks gene sets[163]				
Coagulation	BCLL subgroup 2	3.18	<0.0001	<0.0001
KRAS signaling up	BCLL subgroup 2	2.93	<0.0001	<0.0001
Complement	BCLL subgroup 2	2.90	<0.0001	<0.0001
Epithelial mesenchymal transition	BCLL subgroup 2	2.81	<0.0001	0.0004
Inflammatory response	BCLL subgroup 2	2.45	<0.0001	0.0019
IL2 STAT5 signaling	BCLL subgroup 2	2.40	0.0019	0.0021
Apoptosis	BCLL subgroup 2	2.15	0.0040	0.0079
TNFA signaling via NFKB	BCLL subgroup 2	2.11	<0.0001	0.0088
IL6 JAK STAT3 signaling	BCLL subgroup 2	2.00	0.0040	0.0156
Xenobiotic metabolism	BCLL subgroup 2	1.89	0.0082	0.0259
Hypoxia	BCLL subgroup 2	1.84	0.0060	0.0341

Cholesterol homeostasis	BCLL subgroup 2	1.79	0.0227	0.0428
Interferon gamma response	BCLL subgroup 2	1.72	0.0213	0.0513
Estrogen response early	BCLL subgroup 2	1.42	0.1098	0.1884
Heme metabolism	BCLL subgroup 2	1.32	0.1495	0.2121
Allograft rejection	BCLL subgroup 2	1.38	0.1051	0.2134
Glycolysis	BCLL subgroup 2	1.34	0.1199	0.2169
Estrogen response late	BCLL subgroup 2	1.35	0.1452	0.2204
Apical junction	BCLL subgroup 2	1.32	0.1549	0.2235
MTORC1 signaling	BCLL subgroup 2	1.28	0.1747	0.2350
E2F targets	Control B cells	-2.79	<0.0001	0.0003
Oxidative phosphorylation	Control B cells	-1.79	0.0081	0.0358
G2M checkpoint	Control B cells	-1.85	0.0203	0.0361
KRAS signaling down	Control B cells	-1.56	0.0469	0.0807
Staudt laboratory gene sets (https://lymphochip.nih.gov/signaturedb/index.html)				
Quiescence	BCLL subgroup 2	2.51	<0.0001	0.0027
IRF3 targets	BCLL subgroup 2	1.35	0.1295	0.2542
JAK2 up	Control B cells	-2.07	0.0020	0.0333
MTOR down	Control B cells	-1.94	0.0077	0.0463
MYC up	Control B cells	-1.74	0.0280	0.1004
MTOR up	Control B cells	-1.63	0.0507	0.1215
JAK targets	Control B cells	-1.54	0.0562	0.1428
KEGG (Kyoto Encyclopedia of Genes and Genomes) gene sets				
Hematopoietic cell lineage	BCLL subgroup 2	2.54	<0.0001	<0.0001

Regulation of actin cytoskeleton	BCLL subgroup 2	2.16	0.0021	0.0126
Cell adhesion molecules CAMS	BCLL subgroup 2	2.08	0.0041	0.0170
Complement and coagulation cascades	BCLL subgroup 2	1.90	0.0059	0.0341
Cytokine-cytokine receptor interaction	BCLL subgroup 2	1.96	0.0060	0.0346
Pathways in cancer	BCLL subgroup 2	1.92	0.0020	0.0354
Lysosome	BCLL subgroup 2	1.71	0.0241	0.0905
ECM receptor interaction	BCLL subgroup 2	1.68	0.0279	0.0917
Chemokine signaling pathway	BCLL subgroup 2	1.66	0.0401	0.0920
Focal adhesion	BCLL subgroup 2	1.59	0.0573	0.1191
Neuroactive ligand receptor interaction	BCLL subgroup 2	1.45	0.0758	0.2113
Ubiquitin mediated proteolysis	Control B cells	-2.28	0.0020	0.0068
Cell cycle	Control B cells	-1.55	0.0581	0.2454
Reactome gene set				
Neutrophil degranulation	BCLL subgroup 2	3.89	<0.0001	<0.0001
Innate immune system	BCLL subgroup 2	3.48	<0.0001	<0.0001
Hemostasis	BCLL subgroup 2	3.30	<0.0001	<0.0001
Extracellular matrix organization	BCLL subgroup 2	2.92	<0.0001	<0.0001
Cell surface interactions at the vascular wall	BCLL subgroup 2	2.67	<0.0001	0.0005
Leishmania infection	BCLL subgroup 2	2.57	<0.0001	0.0013

Platelet activation signaling and aggregation	BCLL subgroup 2	2.46	<0.0001	0.0024
GPCR ligand binding	BCLL subgroup 2	2.43	<0.0001	0.0026
Regulation of insulin like growth factor IGF transport and uptake by insulin like growth factor binding proteins IGFBPS	BCLL subgroup 2	2.44	<0.0001	0.0027
Degradation of the extracellular matrix	BCLL subgroup 2	2.34	0.0020	0.0044
Signaling by GPCR	BCLL subgroup 2	2.23	<0.0001	0.0091
Disease	BCLL subgroup 2	2.20	0.0020	0.0100
G alpha I signaling events	BCLL subgroup 2	2.07	0.0040	0.0219
Cell-cell communication	BCLL subgroup 2	1.98	0.0080	0.0285
Response to elevated platelet cytosolic Ca	BCLL subgroup 2	2.00	0.0061	0.0301
Class A 1 rhodopsin like receptors	BCLL subgroup 2	1.99	0.0078	0.0301
Metabolism of lipids	BCLL subgroup 2	1.93	0.0041	0.0353
Transport to the Golgi and subsequent modification	BCLL subgroup 2	1.83	0.0040	0.0559
Vesicle mediated transport	BCLL subgroup 2	1.82	0.0147	0.0581
Signaling by interleukins	BCLL subgroup 2	1.83	0.0157	0.0586
Membrane trafficking	BCLL subgroup 2	1.80	0.0181	0.0608

Anti-inflammatory response favoring leishmania parasite infection	BCLL subgroup 2	1.79	0.0132	0.0617
IL4 and IL13 signaling	BCLL subgroup 2	1.76	0.0183	0.0645
Cytokine signaling in immune system	BCLL subgroup 2	1.77	0.0346	0.0651
Post translational protein modification	BCLL subgroup 2	1.67	0.0350	0.0981
L1CAM interactions	BCLL subgroup 2	1.60	0.0562	0.1289
Fc epsilon receptor FCERI signaling	BCLL subgroup 2	1.59	0.0537	0.1291
Peptide ligand binding receptors	BCLL subgroup 2	1.56	0.0482	0.1438
G alpha 12 13 signaling events	BCLL subgroup 2	1.55	0.0569	0.1466
Fatty acid metabolism	BCLL subgroup 2	1.51	0.0651	0.1655
ADORA2B mediated anti-inflammatory cytokines production	BCLL subgroup 2	1.49	0.0660	0.1731
Signaling by VEGF	BCLL subgroup 2	1.41	0.1078	0.2321
Diseases of metabolism	BCLL subgroup 2	1.41	0.1111	0.2349
Signaling by WNT	BCLL subgroup 2	1.39	0.1019	0.2369
Signaling by nuclear receptors	BCLL subgroup 2	1.40	0.1091	0.2372
Infectious disease	BCLL subgroup 2	1.38	0.1333	0.2380
Asparagine N linked glycosylation	BCLL subgroup 2	1.38	0.1222	0.2397

Metabolism of RNA	Control B cells	-3.19	<0.0001	<0.0001
Cell cycle	Control B cells	-2.83	<0.0001	<0.0001
Cell cycle mitotic	Control B cells	-2.61	<0.0001	0.0012
RNA polymerase ii transcription	Control B cells	-2.37	<0.0001	0.0037
DNA repair	Control B cells	-2.27	<0.0001	0.0052
M phase	Control B cells	-2.29	<0.0001	0.0052
Mitotic G phase and G-S transition	Control B cells	-2.27	0.0020	00.0058
Cell cycle checkpoints	Control B cells	-2.31	0.0019	0.0061
S phase	Control B cells	-2.03	0.0061	0.0152
Separation of sister chromatids	Control B cells	-2.06	0.0062	0.0156
Transcriptional regulation by TP53	Control B cells	-2.04	0.0039	0.0159
Mitotic metaphase and anaphase	Control B cells	-2.05	<0.0001	0.0159
Neddylation	Control B cells	-1.93	0.0019	0.0256
Rho GTPases activate formins	Control B cells	-1.81	0.0257	0.0456
Antigen processing ubiquitination proteasome degradation	Control B cells	-1.78	0.0218	0.0516
HIV infection	Control B cells	-1.77	0.0150	0.0519
Regulation of TP53 activity	Control B cells	-1.75	0.0298	0.0532
Mitotic prometaphase	Control B cells	-1.72	0.0307	0.0572
Cellular responses to external stimuli	Control B cells	-1.65	0.0267	0.0808

Metabolism of carbohydrates	Control B cells	-1.48	0.0751	0.1629
TNFR2 non canonical NFKB pathway	Control B cells	-1.48	0.0731	0.1662
Cilium assembly	Control B cells	-1.41	0.0891	0.2074

NES, normalized enrichment score, NOM p-val, nominal p value; FDR q-val, false discovery rate.

Supplemental table S9. List of genes differentially expressed between BCLL subgroups by RNA Seq that were also measured in the NanoString experiment. The adjusted p value and fold change from the RNA Seq DESeq2 analysis is provided for each gene.

Genes overexpressed in BCLL subgroup 1

Gene name	DESeq2 p-adj	Fold change (BCLL2 vs. BCLL1)
IL6	6.40E-07	-4.46
CDKN1A	1.70E-06	-2.69
CD9	0.0011	-2.28
ZBTB16	0.0114	-2.17
IGSF3	0.0005	-2.12
MYL9	0.0014	-2.01
PEA15	4.04E-05	-1.95
BCL3	0.0002	-1.89
IL10RA	0.0030	-1.88
XBP1	0.0001	-1.85
TSC22D3	2.84E-06	-1.80
RELA	1.87E-07	-1.78
DAPK1	0.0032	-1.78
CREM	0.0001	-1.76
RORA	0.0404	-1.72
GLRX	0.0125	-1.72
MTA3	0.0362	-1.67
CCL4	0.0478	-1.65
RHOB	0.0028	-1.60

Genes overexpressed in BCLL subgroup 2

Gene name	DESeq2 p-adj	Fold change (BCLL2 vs. BCLL1)
TOP2A	9.32E-08	3.58
CXCR3	4.30E-05	3.52
CENPF	5.38E-06	3.47
BUB1	2.47E-07	3.36
BUB1B	9.55E-07	3.28
MKI67	2.57E-05	2.95
RRM2	9.63E-06	2.70
UBE2C	0.0001	2.53
ALOX5	0.0042	2.44
BCL2	9.21E-06	2.39
NEK2	0.0002	2.21
EVI2B	1.36E-05	2.15
MS4A1	5.91E-06	1.97
CYBRD1	0.0065	1.97
TRIB2	0.0023	1.92
MAP3K1	2.67E-05	1.91
CDKN3	0.0099	1.88
BIRC3	0.0030	1.88
CCNB2	0.0015	1.88
CCND3	0.0067	1.85
HDAC9	0.0015	1.83
CYBB	0.0024	1.82
CD72	0.0110	1.75
CDK1	0.0071	1.72
SLAMF1	0.0073	1.71
IL2RA	0.0113	1.65
TLR9	6.89E-06	1.64
FCMR	0.0009	1.64
GBP1	0.0031	1.63
CD40	0.0001	1.59

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

Routine and expanded immunoglobulin PCR for antigen receptor rearrangements (PARR) methods

Routine PARR primers

The Colorado State University-Clinical Immunology laboratory routine PARR assay detects complete immunoglobulin (IG) heavy chain rearrangements composed of a V (variable) gene, D (diversity) gene and J (joining) gene, and incomplete IG heavy chain rearrangements composed of a D gene and J gene. For complete IGH-VDJ rearrangement amplification in our routine PARR assay, forward primers bind the framework 1 region of immunoglobulin heavy variable (IGHV) genes in the predominant IGHV3 gene family. The majority of IGHV genes in the canine immunoglobulin locus belong to subgroup IGHV3 (previously the VH1 family), and four studies demonstrate that IGHV3 genes predominantly rearrange.[94,99,100,121] Two reverse primers target the three major immunoglobulin heavy joining (IGHJ) genes of the six IGHJ genes annotated in the locus.[98,121] For incomplete IGH-DJ rearrangements in the routine PARR assay, forward primers bind the most commonly rearranged immunoglobulin heavy diversity (IGHD) genes (IGHD2, IGHD3, and IGHD4) of the six IGHD genes annotated in the locus.[94,98,99] The forward IGHD primers are paired with the reverse IGHJ primers used to detect complete IGH-VDJ rearrangements. Primer sequences and amplification methods are described in the supplemental material in Colopy et al.[123]

Expanded immunoglobulin PARR reaction

An expanded primer set was designed to detect additional IG gene rearrangements in three separate reactions (Supplemental table S10). To detect additional complete IGH-VDJ rearrangements, IGHV3 subgroup primers binding framework region 3 were designed to target additional IGHV3 genes which do not have conserved homology in the framework 1 region. Two additional primers target

framework 2 of the functional gene in the IGHV1 subgroup (IGHV1-30) and the one functional gene in the IGHV4 subgroup (IGHV4-1), respectively, increasing the IGHV genes targeted. For incomplete IGH-DJ rearrangements, six IGHD primers were designed so that all six IGHD genes are targeted. To detect IG light chain rearrangements, primers were designed to amplify the kappa deleting element (Kde) and IG lambda (IGL) rearrangements. Five forward primers and a reverse primer were designed to bind a subset of IGLV and IGLJ genes.

For each of the three reactions in the expanded PARR assay, 100-500 ng of DNA was amplified with the QIAGEN Multiplex PCR Master Mix kit (QIAGEN, Hilden, Germany) in a 25 µL volume reaction. Primer sequences and primer concentrations are provided in Supplemental table S10. Primer sets were labeled with FAM fluorescent dyes. The PCR cycling conditions were identical to those used for routine canine PARR, and fragment analysis on an ABI 3130xl system (Applied Biosystems, Foster City, CA) and data analysis with GeneMarker software (Soft Genetics, State College, PA) were performed as previously described.[123]

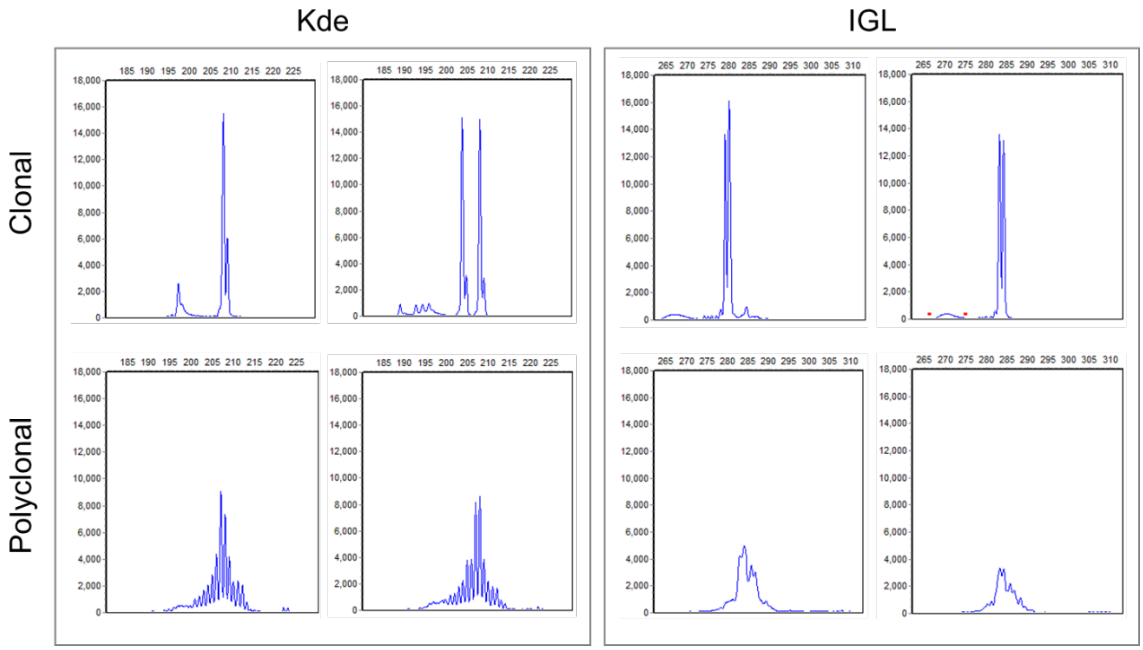
Supplemental table S10. PCR for antigen receptor rearrangements (PARR) primers for amplification of canine complete IGH-VDJ rearrangements, incomplete IGH-DJ rearrangements, Kde rearrangements and IGL rearrangements.

Primer name	Binding site	Fluorescent label	Sequence (5' – 3')	Primer concentration (nM)
Reaction 1: complete IGH-VDJ rearrangements				
267TAG	IGHV		CATAACCGATTCACCATCTCCAGAGA	400
193f	IGHV		AAGGGCCGATTCACCATCTC	400
V80_165	IGHV		TGGGAGGGGACTGGAATGGAT	100
V51_190	IGHV		CTGGGTACGACAGGCTYCAG	100

Brd3	IGHJ	FAM	ACCTGAGGAGACGGTGACC	250
Brd4	IGHJ	FAM	TGAGGACACGAAGAGTGAGG	250
Reaction 2: incomplete IGH-DJ rearrangements				
1pq4	IGHD		ACTGTGGTACTACTGTACTGATGAT	125
2pq1	IGHD		AAGGAACATTGCACTGTGCTACTA	125
3rb1-flap	IGHD		TCAAAGGGTGTCACACAGTGTATA	125
4pq2-flap	IGHD		CTGGTCTGTGTCACGGTGGTATAGTA	125
5pq3-flap	IGHD		AAGGCCTGAGGCAGTGTGAGTTCTA	125
6pq2-flap	IGHD		GTTTGGCCCAGGTAGGAACCACAGTGCTAAC	125
Brd3	IGHJ	FAM	ACCTGAGGAGACGGTGACC	250
Brd4	IGHJ	FAM	TGAGGACACGAAGAGTGAGG	250
Reaction 3: Kde and IGL rearrangements				
48F	Kde	FAM	TGGCGATCTTTTGTCAGGACTG	250
306R	Kde		TGCAAAAATGTCATGGGGCTCT	250
46F	IGLV		CAGAGGGTCACCATYTCCTGC	250
425F	IGLV		AGACTGTCACCATCTCCTGT	250
422F	IGLV		AGGGACAGTCACACTCACAT	250
420F	IGLV		CTGGAAGAGTCGGTCAAGC	250
426F	IGLV		AAGACAGCCACCATCACMTG	250
66R	IGLJ	FAM	CGAGGACGGTCAGDTGGG	250

Expanded immunoglobulin PARR analysis

Complete IGH-VDJ (reaction 1) and incomplete IGH-DJ (reaction 2) rearrangements amplified with expanded primers were interpreted with the same diagnostic criteria used for IGH-VDJ and IGH-DJ rearrangements in the routine assay, respectively. For IG light chain rearrangements (reaction 3), Kde and IGL reactions were defined as clonal by 1-2 tall narrow peak(s) >8,000 in amplitude in the absence of a robust polyclonal base. These loci have limited junctional diversity compared to complete V-D-J rearrangements from IG heavy chain loci, and they are more challenging to interpret.[9] For example, in this assay polyclonal Kde results frequently have 1-2 peaks which are 2x the height of the other peaks forming the base. Therefore, we use more stringent criteria to define clonality in these loci and only interpret a peak as clonal if there is minimal to no polyclonal base. Representative clonal and polyclonal results for Kde and IGL loci in the expanded PARR assay are shown in Supplemental figure S1.



Supplemental figure S1. PCR for antigen receptor rearrangements (PARR) clonal and polyclonal results for kappa deleting element (Kde) and immunoglobulin lambda (IGL) light chain reactions. The size of the PCR products is on the horizontal axis and abundance is on the vertical axis. Two clonal results and two polyclonal results are shown for each reaction. Clonal results (top) have 1-2 tall narrow peaks with a minimal to absent polyclonal base. Polyclonal results (bottom) have multiple peaks forming a Gaussian distribution.

Expanded immunoglobulin PARR primer testing

The expanded IG PARR primers were tested on 10 negative control and 10 positive control samples. For negative controls, routine PARR and PARR with expanded IG primers were performed on peripheral blood samples from ten healthy control dogs with no evidence of lymphoproliferative disease by flow cytometry. All three expanded IG reactions were polyclonal in all 10 cases. Ten randomly selected small breed BCLL patients that were clonal with routine PARR were tested with expanded IG primers. Results are presented in Supplemental table S11. All 10 cases were clonal with the expanded complete IGH-VDJ reaction (100%), and 7 cases were clonal with the light chain reaction (70%). Four cases were clonal with the expanded incomplete IGH-DJ reaction (40%). One of these cases had a clonal incomplete rearrangement on routine PARR, two cases had suspicious peaks that did not reach clonal criteria on routine PARR, and one case had polyclonal incomplete rearrangements on routine PARR. Of the six cases with polyclonal expanded incomplete IGH-DJ results, 1 was considered clonal with routine PARR IGH-DJ primers, 1 had a suspicious peak that did not reach clonal criteria, and 4 were polyclonal.

Supplemental table S11. PCR for antigen receptor rearrangements (PARR) results for 10 B-cell chronic lymphocytic leukemia cases, with routine primers and expanded assay primers for immunoglobulin loci.

BCLL case	IGH-VDJ (routine assay)	IGH-VDJ (expanded assay)	IGH-DJ (routine assay)	IGH-DJ (expanded assay)	Light chain loci (expanded assay)
1	Clonal	Clonal	Clonal	Clonal	Clonal
2	Clonal	Clonal	Clonal	Polyclonal	Clonal
3	Clonal	Clonal	Polyclonal	Clonal	Clonal
4	Clonal	Clonal	Polyclonal	Polyclonal	Polyclonal
5	Clonal	Clonal	Polyclonal	Polyclonal	Polyclonal
6	Clonal	Clonal	Polyclonal	Polyclonal	Clonal

7	Clonal	Clonal	Polyclonal	Polyclonal	Polyclonal
8	Clonal	Clonal	Possible clonal	Clonal	Clonal
9	Clonal	Clonal	Possible clonal	Clonal	Clonal
10	Clonal	Clonal	Possible clonal	Polyclonal	Clonal

LIST OF ABBREVIATIONS

AA, Amino acid

AIR, Airedale Terrier

BASS, Bassett hound

BCLL, B-cell chronic lymphocytic leukemia

BCR, B-cell receptor

BDC, Bearded Collie

BIC, Bichon frise

BOR, Border collie

BOST, Boston terrier

BOX, Boxer

CBC, Complete blood count

CCR, Chinese crested

CDR, Complementarity determining region

CDR3, Complementarity determining region 3

CDT, Coton de Tulear

CKP, Cockapoo

CLL, Chronic lymphocytic leukemia

CLL-IPI, Chronic lymphocytic leukemia international prognostic index

COC, Cocker spaniel

CRN, Cairn terrier

CSH, Chihuahua shorthair

CSU-CI, Colorado State University-Clinical Immunology

D, Diversity

DACH, Dachshund

DLBCL, Diffuse large B-cell lymphoma

F, Functional

FC, Fold change

FDR, False discovery rate

FPKM, Fragments per kilobase million

FR, framework region

FS, Forward scatter

GLDR, Golden retriever

GSEA, Gene set enrichment analysis

GWAS, Genome-wide association study

IF, Immunofixation

IG, Immunoglobulin

IGH, Immunoglobulin heavy chain

IGHD, Immunoglobulin heavy diversity

IGHJ, Immunoglobulin heavy joining

IGHM, Immunoglobulin heavy constant mu

IGHV, Immunoglobulin heavy variable

IGK, Immunoglobulin light chain kappa

IGL, Immunoglobulin light chain lambda

IMGT, International ImMunoGeneTics

IQR, Interquartile range

J, Joining

JRT, Jack Russell terrier

KDE, Kappa deleting element

LAB, Labrador retriever

LBD, Labradoodle

MAST, Mastiff

MFI, Median fluorescence intensity

MIX, Mixed breed

MLT, Maltese

MST, Median survival time

MTD, Maximum tolerated dose

N, Number

NA, Not applicable

NES, Normalized enrichment score

NEWFIE, Newfoundland

NHL, Non-Hodgkin lymphoma

P, Pseudogene

PARR, PCR for antigen receptor rearrangements

PBLEB, Polyclonal B-cell lymphocytosis in English bulldogs

PBS, Phosphate buffered saline

PE, Protein electrophoresis

PIT, Pit bull terrier

POM, Pomeranian

PPBL, Persistent polyclonal B-cell lymphocytosis

RALD, Ras-associated autoimmune lymphoproliferative disorder

RAT, Rat Terrier

RIN, RNA integrity number

RNA Seq, RNA Sequencing

RS, Recombination sequence

SHI, Shih Tzu

SLL, Small lymphocytic lymphoma

SNP, Single nucleotide polymorphism

STS, Schnauzer

TCR, T-cell receptor

TIBT, Tibetan terrier

TRB, T-cell receptor beta

TRG, T-cell receptor gamma

V, Variable

VSL, Vizsla

WET, Soft Coated Wheaten Terrier