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Polyclonal B-cell lymphocytosis in English bulldogs

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Abstract

Background: 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.

Hypothesis: English bulldogs have a syndrome of nonneoplastic B-cell expansion.

Animals: Eighty-four English bulldogs with small-sized CD21+ B-cell lymphocytosis in the blood as determined by flow cytometry.

Methods: This is a retrospective study. We characterized this syndrome by assessing B-cell clonality, clinical presentation, flow cytometric features, and immunoglobulin gammopathy patterns. We identified 84 cases with CD21+ lymphocytosis among 195 English bulldogs with blood samples submitted to the Colorado State University-Clinical Immunology laboratory for immunophenotyping between 2010 and 2019. Flow cytometry features were compared to normal B-cells and BCLL cases. PCR for antigen receptor rearrangements (PARR) by 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.

Results: Seventy percent (58/83) 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 (range) lymphocyte count was 22 400/μL (2000-384 400/μL) and B-cells had low expression of class II MHC and CD25. Splenomegaly or splenic masses were detected in 57% (26/46) of cases and lymphadenopathy in 11% (7/61). Seventy-one percent (52/73) of cases had hyperglobulinemia and 77% (23/30) with globulin characterization had IgA ± IgM polyclonal or restricted polyclonal gammopathy patterns.

Conclusions and Clinical Importance: Polyclonal B-cell lymphocytosis in English bulldogs is characterized by low B-cell class II MHC and CD25 expression, splenomegaly

Abbreviations: BCLL, B-cell chronic lymphocytic leukemia; CSU-CI, Colorado State University-Clinical Immunology; IG, immunoglobulin; IGH, IG heavy chain; IGHV, IGH variable; IGH-VDJ, complete immunoglobulin heavy (IGH) chain variable (V)-diversity (D)-joining (J) gene rearrangement; IGL, IG lambda; IQR, interquartile range; Kde, kappa-deleting element; PARR, PCR for antigen receptor rearrangements; PBLEB, polyclonal B-cell lymphocytosis in English bulldogs; PCR, polymerase chain reaction; PE/IF, protein electrophoresis/immunofixation; PPBL, persistent polyclonal B-cell lymphocytosis; RALD, RAS-associated autoimmune lymphoproliferative disorder.

and hyperglobulinemia consisting of increased IgA ± IgM. We hypothesize that this syndrome has a genetic basis.

KEYWORDS

clonality, canine, clinical pathology, flow cytometry, hyperglobulinemia, IgA gammopathy, PCR for antigen receptor rearrangements

1 | INTRODUCTION

Lymphocytosis can 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.[1-5] Neoplastic lymphocytosis comprises clonally expanded lymphocytes and is a more common cause of persistent lymphocytosis in adult dogs.[6] 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 rearrangements.[7,8] Reactive or inflammatory processes might also cause increased production of polyclonal immunoglobulin proteins.[9,10] 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.[9,11]

B-cell chronic lymphocytic leukemia (BCLL) is a common hematopoietic neoplasm in dogs, defined by a clonal expansion of small-sized B-cells in the blood or bone marrow.[12,13] Our laboratory identifies BCLL based on inclusion criteria of >5000 lymphocytes/μL on CBC with small-sized CD21+ B-cells accounting for >60% of the lymphocyte population by flow cytometry. Small breed dogs have increased risk of developing BCLL.[13] English bulldogs have increased odds of developing BCLL, as defined in this study, but this breed had a unique presentation in being significantly younger at diagnosis compared to mixed breed dogs, having increased frequency of hyperglobulinemia, and their 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 might 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 by 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 with or without IgM, with normal to diminished IgG.

2 | MATERIALS AND METHODS

2.1 | 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 September 17, 2010 and August 31, 2019. Inclusion criteria included an expansion of the number 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 median forward scatter: neutrophil median forward scatter < 0.60.

The CSU-CI laboratory database query was also used to identify several control groups, which are as follows: (i) 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 and included 57% females and age ranged from 1.9 to 8.9 years old; (ii) 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 age ranged from 1 to 12 years old (median, 3.9 years old); (iii) 53 small breed BCLL cases from BCLL-predisposed breeds[13] with blood submitted for flow cytometry between 1 January 2015 and 10 January 2019 and clonal immunoglobulin (IG) rearrangements by PARR; and (iv) PARR results from 25 small breed BCLL cases 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.

2.2 | 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 by 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 as being present, or if the concentration of globulins was increased on a biochemistry panel or by PE. 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.

2.3 | Flow cytometry

Flow cytometry was performed on blood samples at the CSU-CI laboratory. Samples were collected, stored, and stained as previously described[14] by antibody panels listed in Supplemental Table 1. 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 was determined by median fluorescence intensity. Six English bulldog cases sampled before May 2012 did not have CD25 or class II MHC data available.

2.4 | PCR for antigen receptor rearrangements

The CSU-CI routine PARR assay was performed as previously described.[7,15] 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, whereas 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 Supplemental material. 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 because of 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 by the rubric provided in Table 1. 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 can rearrange 1 or both alleles of the IG locus resulting in up to 2 clonal peaks.[16] Representative tracings for the electrophoretic patterns are provided in Figure 1. DNA quantity and quality were determined to be sufficient based on PARR results, as previously described.[17] PARR results were independently interpreted by the authors (E.D. Rout, A.C. Avery), blinded to the signalment and flow cytometry data.

2.5 | Serum immunoglobulins

Agarose gel PE and IF were performed at Colorado State University to evaluate immunoglobulins, as previously described.[15,18] 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

TABLE 1 Immunoglobulin PCR for antigen receptor rearrangements (PARR) assay diagnostic criteria

Interpretation	Capillary electrophoretic pattern
IGH-VDJ rearrangements	
Clonal	1 or 2 tall narrow peak(s) >5000 in amplitude and >3 times 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 >2 times the height of the base peaks forming the polyclonal background
IGH-DJ, Kde, or IGL rearrangements	
Clonal	Tall narrow peak >8000 in amplitude and >3 × the height of the base peaks forming the polyclonal background
Polyclonal	Multiple peaks forming a Gaussian distribution

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

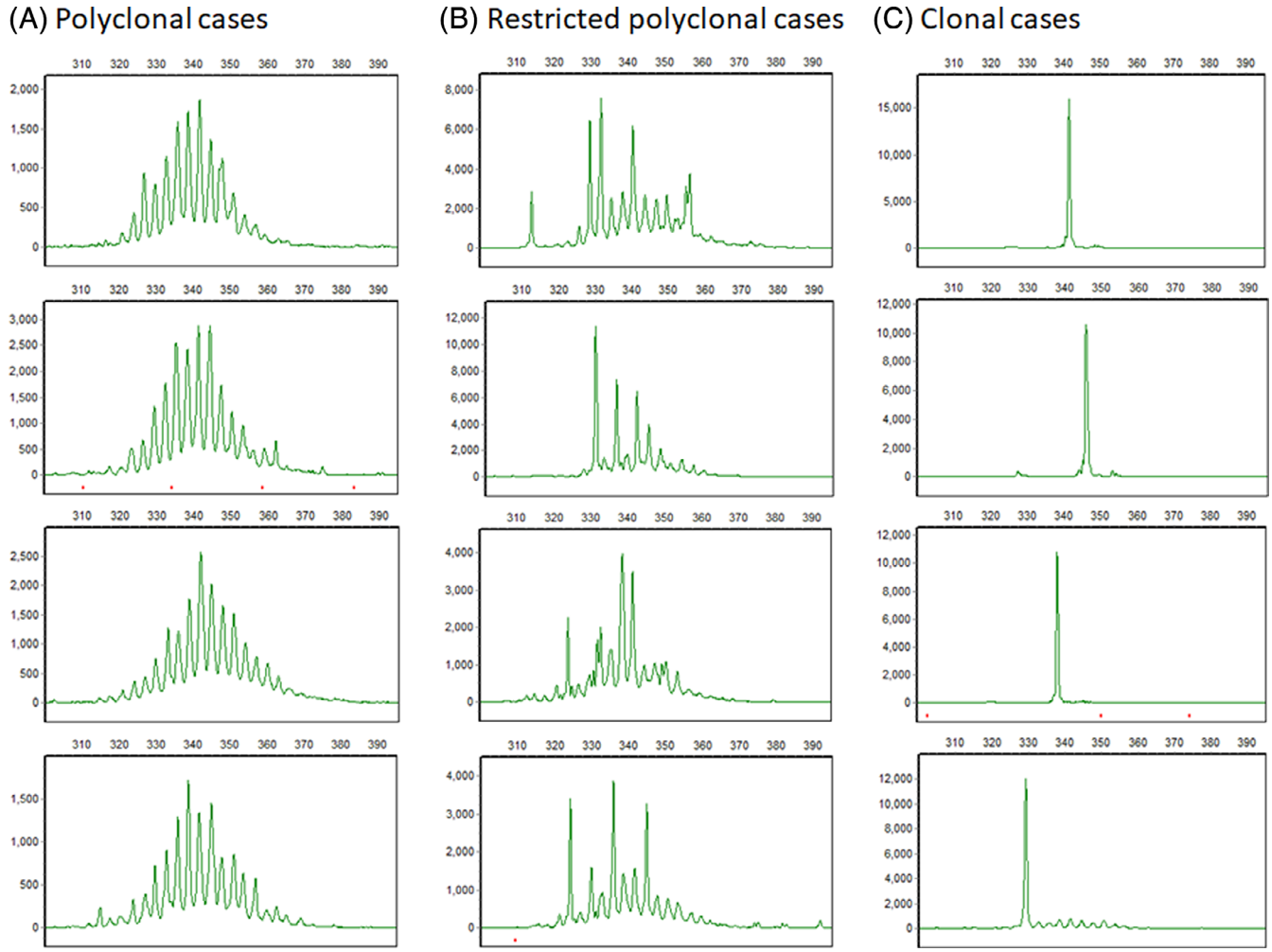

FIGURE 1 PCR for antigen receptor rearrangements (PARR) results for English bulldogs with B-cell lymphocytosis. GeneMarker tracings for complete immunoglobulin heavy (IGH) chain variable (V)-diversity (D)-joining (J) (IGH-VDJ) rearrangements are presented for 4 polyclonal cases (A), 4 restricted polyclonal 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 to 5 peaks >2000 in amplitude and 2 times the height of the polyclonal peaks forming the base. (C) Clonal cases have a peak >5000 in amplitude and >3 times the base height

without IF. The hemolysis and lipemia indices were determined for all samples (Cobas c501 Roche Diagnostics, Indianapolis, IN), as these factors can alter electrophoretogram morphology.[9,19] Total globulin concentration was calculated as the sum of all globulin fractions by PE to avoid overestimation of albumin in cases with a marked gammopathy.[20] Hyperglobulinemia was identified by PE if the total globulin concentration exceeded our internal upper reference limit 3.5 g/dL. PE/IF results were blindly and independently interpreted by the authors (E.D. Rout, P.R. Avery, A.R. Moore) using the rubric in Table 2. Representative results for the electrophoretic patterns are provided in Figure 2. Immunofixation results were interpreted to determine whether immunoglobulins were predominantly of IgG (detected by the IgG_{FC} antibody), IgA, or IgM subclass.[21] An IgA or IgM predominance was considered atypical, as normal dogs have predominantly IgG_{FC} labeling with no to faint IgA and IgM.[9]

Quantification of IgA, IgM, and IgG_{FC} proteins was performed with commercially available ELISA kits, as previously described.[18] 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.

2.6 | 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 by 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

TABLE 2 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 1 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

Abbreviation: IGH, immunoglobulin heavy chain.

comparisons. Immunoglobulin quantification was compared between controls and English bulldog cases with a Mann-Whitney test. For comparisons between clonal and nonclonal English bulldog cases, a Mann-Whitney test was calculated. A Fisher's 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 2-sided *P* value <.05 was considered significant.

3 | RESULTS

3.1 | Clinical presentation

Between September 17, 2010 and August 31, 2019, 84 of 195 English bulldogs with blood submitted to the CSU-CI laboratory for immunophenotyping by flow cytometry had an expanded number of small-sized CD21+ B lymphocytes. Signalment and laboratory data from these cases are summarized in Table 3. The median age at diagnosis was 6.8 years and there was a male predominance. The lymphocyte count was moderately increased in most cases, but a small number (16/84) of cases had >50 000 lymphocytes/μL (median, 22 400 lymphs/μL; interquartile range [IQR], 12 000-41 100 lymphs/μL; range, 2000-384 400 lymphs/μL). Anemia was present in 26/84 cases (31%) and mild to moderate in all affected cases (median hematocrit, 30%; IQR, 27-34%; range, 21-38%). The anemia was nonregenerative 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 all affected cases (median, 114 000 platelets/μL; IQR, 89500-152 000/μL; range, 38 000-198 000/μL). Hyperglobulinemia affected 65% of cases (45/69) with globulin data at diagnosis. An additional 7 cases had hyperglobulinemia detected after initial diagnosis, resulting in 71% of cases (52/73) having hyperglobulinemia at some point during the course of their syndrome. Splenomegaly or splenic masses or both were also common (26/46), but lymphadenopathy was rare (7/61).

Of 44 cases where lymphocyte morphology was evaluated by a clinical pathologist, 35/44 were described as having small-sized or small-to-intermediate-sized lymphocytes, 4 were described as having intermediate-sized lymphocytes, and 5 cases were described as having intermediate-to-large-sized lymphocytes. All cases met the flow cytometric inclusion criteria for small-sized lymphocytes. Chromatin was described as mature, clumped, or condensed in all cases evaluated, except in 1 where it was described as fine. Six cases were described as having few nucleoli. Ten cases with blood smears available at the Colorado State University Veterinary Diagnostic laboratory were reviewed by the authors (E.D. Rout, P.R. Avery). Lymphocytes were predominantly smaller than a neutrophil with a small round nucleus, condensed chromatin, and scant basophilic cytoplasm (Figure 3A). 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 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,

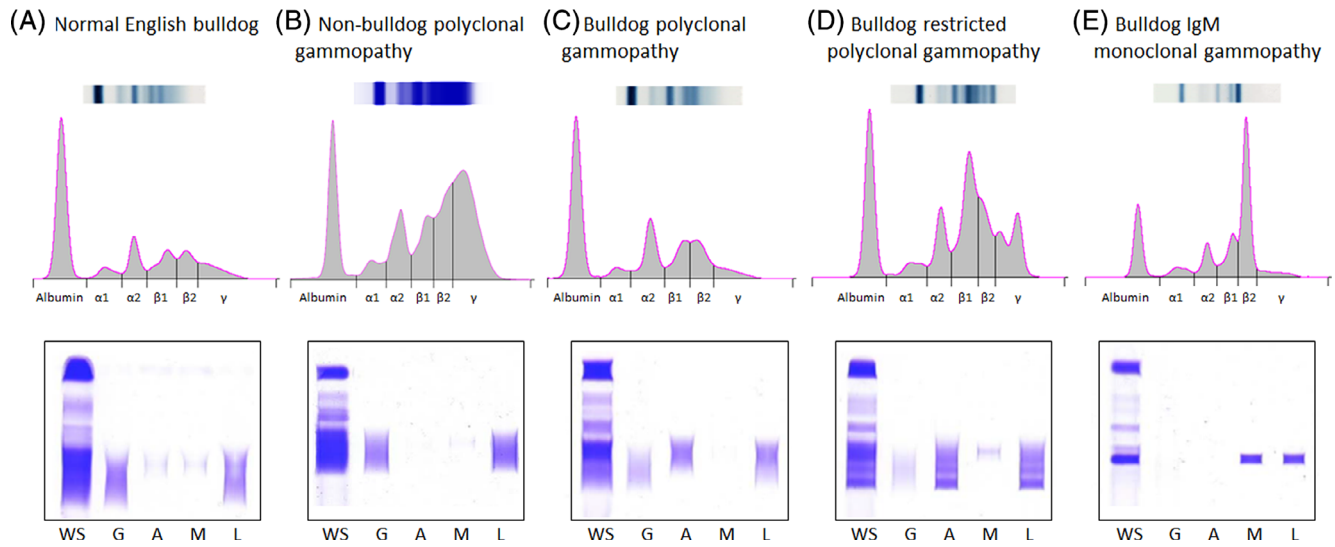

FIGURE 2 Protein electrophoresis (PE) and immunofixation (IF) results in control dogs and English bulldogs with B-cell lymphocytosis. An agarose gel protein electrophoresis gel and tracing (top) and immunofixation gel (bottom) are provided. Immunofixation gels are labeled with anti-whole serum (WS), 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 reveal 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

noncoalescing follicular structures to occasionally larger rarely coalescing follicles (Figure 3B). Follicles were composed of predominantly B-cells (Figure 3D) with scattered small T-cells often radiating around follicles and scattered plasma cells within sinusoids (not shown). 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 3C).

3.2 | Flow cytometry

Among English bulldog cases, the median CD21+ B-cell count was 20 606 cells/μL (IQR, 10461-42 682/μL; range, 1174-378 815/μL; reference interval, 0-724/μL). CD21+ B-cells accounted for 67%-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 (53/78) 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 ($P < .001$), clinically healthy English bulldog controls ($P < .001$) and small breed BCLL cases ($P < .001$) (Figure 4). B-cell class II MHC expression was also significantly lower in English bulldog cases compared to non-bulldog controls ($P < .001$), English bulldog controls ($P < .001$) and small breed BCLL cases ($P < .001$). English bulldog cases had significantly higher B-cell CD21 expression than non-bulldog controls ($P < .001$), English bulldog controls ($P < .001$) and small breed BCLL cases ($P < .001$). Comparing non-bulldog and English bulldog controls, English bulldog controls had significantly lower CD25 and CD21 expression ($P = .003$ and $P = .02$, respectively).

3.3 | 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 1 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 1. 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

TABLE 3 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	62 (74%)
Age, median (IQR; range), years	84	6.8 (5.2-9.0; 2.5-11.0)
Hematologic data		
Lymph count, median (IQR; range), $\times 10^3/\mu L$	84	22.4 (12.0-41.1; 2.0-384.4)
Anemia	84	26 (31%)
Thrombocytopenia	84	8 (10%)
Neutropenia	84	2 (2%)
Hyperglobulinemia	69	45 (65%)
Physical exam		
Splenomegaly/ splenic mass	46	26 (57%)
Peripheral lymphadenopathy	61	7 (11%)
Visceral lymphadenopathy	38	5 (13%)
Hepatomegaly/ hepatic mass	42	6 (14%)

Note: For variables besides age and lymphocyte count, the number of cases with available data, and 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.

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 4).

3.4 | Immunoglobulin protein analysis

Serum or plasma globulin concentration, electrophoretic morphology, and isotype location and distribution were evaluated in 30 English bulldog cases with PE/IF. Total globulin concentrations ranged from 3.3 to 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 (24/30) had hypoalbuminemia. Among hypoalbuminemic cases, 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). Approximately half of 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 2), and representative results are presented in Figure 2. Six cases had an IgA or IgM monoclonal gammopathy, and all 6 cases had clonal IG rearrangements by PARR (Table 4). 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 with or without increased IgM. Fifteen cases had restricted polyclonal gammopathy with increased IgA with or without increased 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 (23/30) were classified as polyclonal or restricted polyclonal with increased IgA with or without increased IgM. One of the cases 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 cases 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 concentrations seen on IF (Figure 5). 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 ($P < .001$) and IgM ($P = .012$), and significantly less IgG_{FC} than controls ($P = .004$). All 12 English bulldog cases had IgA values above the range seen in control cases, although 1 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

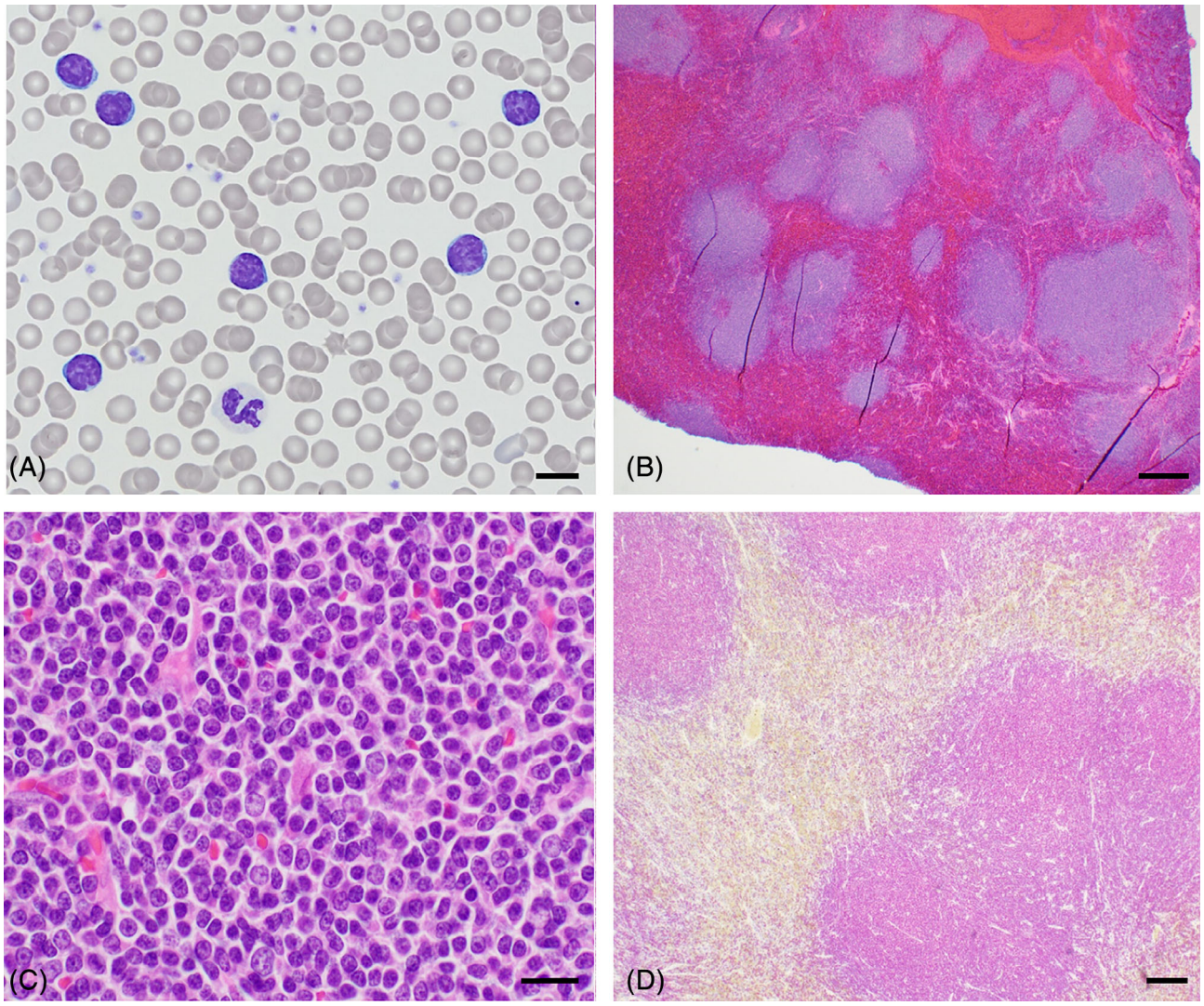

FIGURE 3 Blood smear and histopathology and immunohistochemistry of spleen from English bulldog cases with polyclonal B-cell lymphocytosis. (A) Peripheral blood film from an English bulldog with B-cell lymphocytosis and polyclonal immunoglobulin gene rearrangements. Lymphocytes are small with condensed chromatin and scant basophilic cytoplasm (Wright Giemsa, ×60 objective, 10 μm scale bar). (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, ×2 objective, 500 μm scale bar). 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, ×40 objective, 20 μm scale bar). Lymphocytes within the follicles are predominated by B-cells with strong nuclear immunoreactivity for PAX5 (D, PAX5, Fast Red chromogen, ×4 objective, 200 μm scale bar)

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 (n = 4) had markedly increased IgM.

3.5 | Clonal versus nonclonal English bulldog cases

We investigated clinical and immunophenotypic differences between clonal cases (n = 25) and nonclonal cases (n = 58). The nonclonal group included cases with polyclonal or restricted polyclonal IG rearrangements by PARR. Clonal cases were significantly older than nonclonal cases (P = .002) (Figure 6A). There were significantly more males in the nonclonal group (81%) compared to clonal cases (56%) (P = .03) (Figure 6B). The CD21+ B-cell count was not significantly different between clonal cases (median, 21 700/μL; IQR, 10900-73 500/μL; range, 1200-378 800/μL) and nonclonal cases (median, 19 700/μL; IQR, 9800-38 300/μL; range, 1400-285 000/μL) (P = .38). Class II MHC expression was not significantly different between groups (P = .12), but clonal cases had significantly lower expression of CD21 (P = .04, not shown) and significantly higher expression of CD25

(P = .003) (Figure 6C). The majority of nonclonal 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 nonclonal cases, although the type of

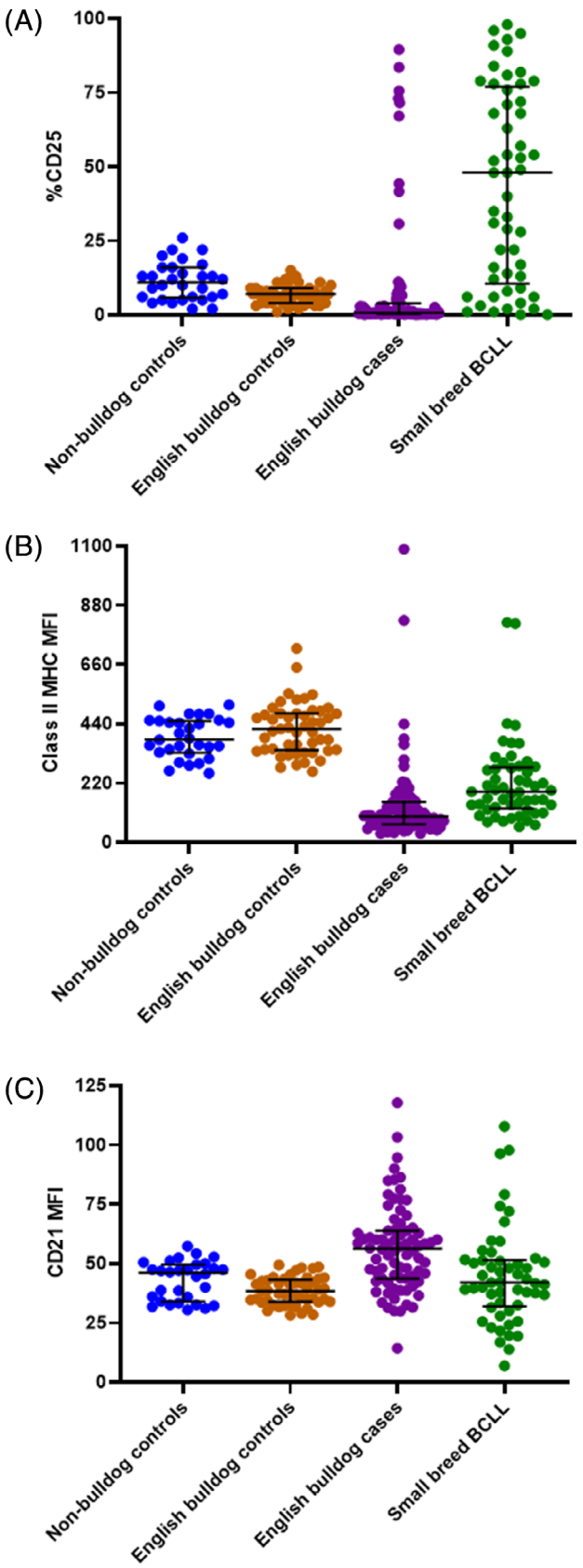

FIGURE 4 B-cell expression of CD25, class II MHC, and CD21 in English bulldog B-cell lymphocytosis cases compared to clinically healthy non-bulldog controls, English bulldog controls with normal B-cell counts, and small breed B-cell chronic lymphocytic leukemia (BCLL) cases. Expression of CD25 (A), class II MHC (B) and CD21 (C) by flow cytometry is plotted for individual cases. Lines depict the median and interquartile range for each group. 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

TABLE 4 Summary clonality results for immunoglobulin gene rearrangements and immunoglobulin proteins in English bulldog cases with B-cell lymphocytosis

Clonality result	Number of cases (%)	PE cases with clonal PARR result
Immunoglobulin gene rearrangements[a]		
Polyclonal	31/83 (37%)	NA
Restricted polyclonal	27/83 (33%)	NA
Clonal	25/83 (30%)	NA
Immunoglobulin proteins[b]		
IgG_{FC} polyclonal	1/30 (3%)	1/1
IgA polyclonal	7/30 (23%)	1/7
IgA + IgM polyclonal	1/30 (3%)	0/1
IgA restricted polyclonal	3/30 (10%)	0/3
IgA + IgM restricted polyclonal	12/30 (40%)	0/12
IgA monoclonal	2/30 (7%)	2/2
IgM monoclonal	4/30 (13%)	4/4

[a]Clonality of immunoglobulin (IG) 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 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.

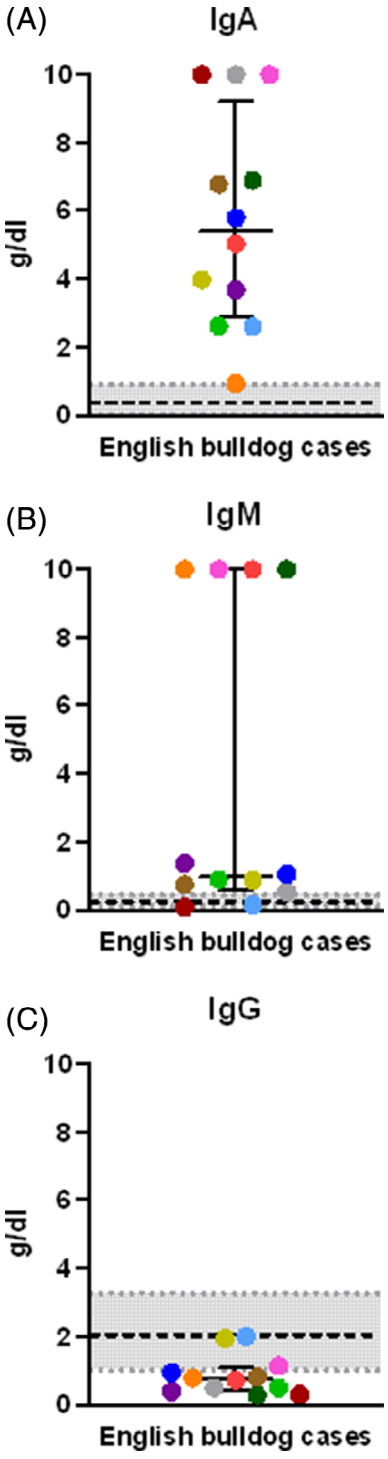

FIGURE 5 IgA, IgM, and IgG_{FC} protein quantification by ELISA in English bulldog cases with B-cell lymphocytosis. Each English bulldog case is colored consistently across the 3 graphs. Data points plotted at 10 g/dL were above the limits of quantification for the assay. Dotted lines represent the mean (black line) and range (gray lines) of values for 7 control dogs. English bulldog cases had significantly greater quantities of serum IgA ($P < .001$), and a subset of cases had increased IgM, compared to control dogs. English bulldogs had significantly less IgG_{FC} than controls ($P = .004$)

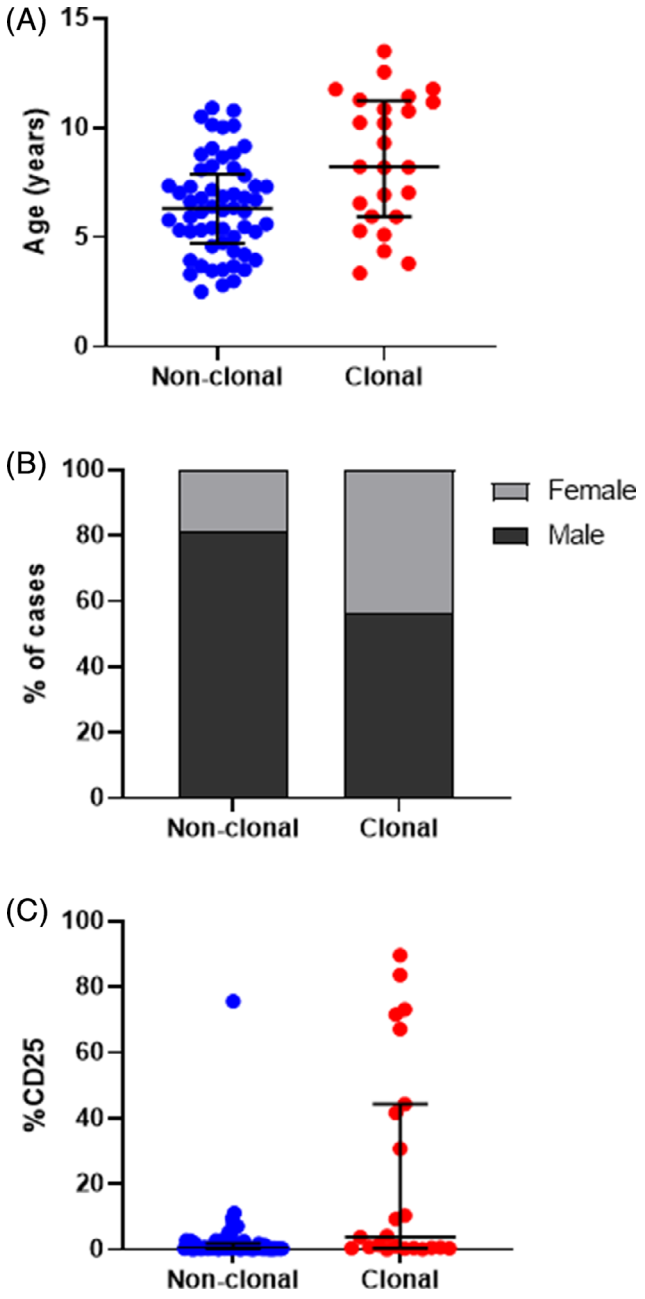

FIGURE 6 Signalment and B-cell CD25 expression in nonclonal and clonal English bulldog cases with B-cell lymphocytosis. (A) The age at diagnosis for nonclonal cases with polyclonal or restricted polyclonal immunoglobulin PARR results and clonal cases with clonal immunoglobulin PARR results. Clonal cases (median, 8.2 years old) were significantly older than nonclonal cases (median, 6.3 years old) ($P = .002$). (B) The percentage of males and females within nonclonal and clonal groups is presented. There were significantly more males in the nonclonal group ($P = .03$). (C) The percentage of B-cells expressing CD25 by flow cytometry in nonclonal and clonal cases. Nonclonal cases had significantly lower CD25 expression than clonal cases ($P = .003$)

gammopathy was different: in the 8 clonal cases with PE/IF, 6/8 cases had monoclonal IgA or IgM protein, and none of the nonclonal PARR cases had monoclonal PE/IF results. Peripheral lymphadenopathy was more common in clonal cases (32%) than nonclonal cases (2%) (P = .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 nonclonal cases.

3.6 | 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 to 5 sequential samples available for analysis. The time between initial diagnosis and the most recent sample varied from 2.6 to 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 7). In 1 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 7C). Of the 5 cases where PARR results changed over time, 2 restricted polyclonal cases became polyclonal (both were receiving oral chemotherapy and prednisone), 1 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.

Of 14 monitored cases with treatment data, 10 cases have been clinically stable over time with no treatment, and the B-cell count remained stable or decreased over time in 7/10 cases and increased in 3/10 cases though the count remained less than 50 000/μL. Four cases were managed with steroids alone or steroids and oral chemotherapy and these treated cases had reductions in the B-cell count. Two treated cases with sequential PE/IF analysis had reductions in globulin concentration, though atypical PE/IF patterns and IgA or IgA and IgM increases persisted. Treatment protocols were variable in these cases.

4 | DISCUSSION

Our study identified a syndrome in English bulldogs characterized by an expanded number of nonclonal B-cells in the blood, with polyclonal or restricted polyclonal IG rearrangements by PARR. This syndrome, termed polyclonal B-cell lymphocytosis of 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 with or without IgM.

Forty-three percent of English bulldogs with blood submitted to the CSU-CI laboratory for flow cytometry over a nearly 9-year period had a B-cell expansion. These cases 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 might have a neoplasm with somatic

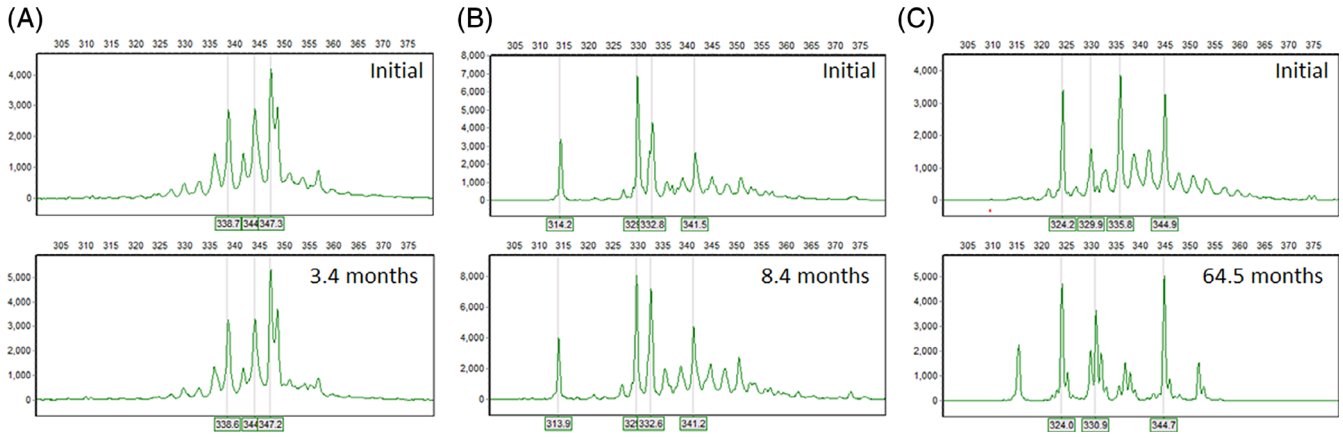

FIGURE 7 Sequential PCR for antigen receptor rearrangements (PARR) results with immunoglobulin primers for 3 English bulldog cases with B-cell lymphocytosis. For each case, immunoglobulin PARR results at initial presentation (top) and sequential PARR results (bottom) 3.4-64.5 months after diagnosis are 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 case that has been monitored over 5 years (C)

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.[16,17,22,23] Incomplete IGH-DJ and Kde rearrangements are less prone to somatic hypermutation, and analysis of the IG kappa locus is useful in detecting clonality in canine tumors with somatic hypermutation, such as plasmacytomas.[16,22] 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 nonclonal cases remained nonclonal 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 because cases have large quantities of immunoglobulin protein in the serum, suggesting that gene rearrangements are functional.[8] 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, but 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 can 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.[24] Patients are generally asymptomatic and a subset of cases have splenomegaly. Rare cases have massive splenomegaly, which might require splenectomy and mimic splenic lymphoma histologically.[25-27] Familial cases suggest a genetic predisposition and many cases have an isochromosome (3q) abnormality and chromosomal instability.[24,28] Most cases remain polyclonal and clinically stable, but there are rare reports of cases progressing to a clonal B-cell malignancy.[29] PPBL is hypothesized to result from hyperproliferation of B-cells with a marginal zone-like phenotype and altered CD40 signaling.[25,30,31] 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.[32] This syndrome is diagnosed at a young age and generally has an indolent clinical course, though rare cases can undergo malignant transformation. Sequencing in 1 case demonstrated a restricted B-cell receptor repertoire, with expansions of many different B-cell clones.[33] 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 1 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 might undergo malignant transformation over time. In human medicine, monoclonal gammopathy of undetermined significance is considered a premalignant disease that can undergo malignant transformation and progress to Waldenstrom macroglobulinemia or a plasma cell neoplasm.[34] Additionally, a subset of apparently healthy asymptomatic people with monoclonal B-cell lymphocytosis will progress to chronic lymphocytic leukemia/small lymphocytic lymphoma.[35] 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 might 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 could 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.

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 (data not provided).

Hyperglobulinemia and splenomegaly were common features in PBLEB cases. Globulin concentrations were often moderately to markedly increased. All cases tested had increased IgA, a subset of

cases 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 can promote B-cell proliferation and IgA class switching.[36] In T-cell-independent pathways, soluble factors such as BAFF and APRIL induce IgA class switching.[36] Several mechanisms might explain the constellation of findings in these dogs. PBLEB cases might have either increased factors that stimulate IgA class switching or a decrease in inhibitors of class switch recombination. For example, APRIL is hypothesized to cause hyperproduction of IgA in human IgA nephropathy and BAFF overexpression in mice resulted in a hyper-IgA syndrome with B-cell hyperplasia.[37,38] 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.[36,39] Many PBLEB cases had splenomegaly/splenic masses without lymphadenopathy. A subset of cases had massive splenomegaly and a few dogs were treated with splenectomy because of 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 could originate in the spleen, there could be stimulatory molecules in the spleen promoting proliferation and survival at this site, 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 might be involved. Examination of 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 these 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 rare 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.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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