A tumor-related lymphoid progenitor population supports hierarchical tumor organization in canine B-cell lymphoma

Daisuke Ito[1,2], **Melissa M. Endicott**[3], **Cristan M. Jubala**[4], **Karen M. Helm**[5], **Robert C. Burnett**[6,7], **Brian D. Husbands**[1,2], **Antonella Borgatti**[1,2], **Michael S. Henson**[1,2], **Kristine E. Burgess**[8], **Jerold S. Bell**[8], **William C. Kisseberth**[9,10], **Victor E. Valli**[11,†], **Gary R. Cutter**[12], **Anne C. Avery**[6,7], **Kevin A. Hahn**[3,*], **Timothy D. O'Brien**[2,13,14], and **Jaime F. Modiano**[1,2,4,5,14]

[1]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, University of Minnesota, St Paul, MN, USA

[2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN, USA

[3]Gulf Coast Veterinary Oncology, Houston, TX, USA

[4]Integrated Department of Immunology, School of Medicine, University of Colorado, Denver, CO, USA

[5]University of Colorado Cancer Center, Aurora, CO, USA

[6]Department of Microbiology, Immunology, and Pathology, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO, USA

[7]Animal Cancer Center, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, CO, USA

[8]Department of Clinical Sciences, Tufts Cummings School of Veterinary Medicine, North Grafton, MA, USA

[9]Department of Veterinary Clinical Sciences, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA

[10]Comprehensive Cancer Center and Solove Research Institute, The Ohio State University, Columbus OH, USA

[11]Department of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL, USA

[12]Department of Biostatistics, University of Alabama, Birmingham, AL, USA

[13]Department of Veterinary Population Medicine, College of Veterinary Medicine, University of Minnesota, St Paul, MN, USA

Corresponding author: Daisuke Ito, DVM, PhD, Masonic Cancer Center and Department of Veterinary Clinical Sciences, University of Minnesota, MCRB 530, 425 East River Road, Minneapolis, MN, USA 55455. Phone: 612-626-6890; Fax: 612-626-4915; itoxx018@umn.edu.
*Current address: Hill's Pet Nutrition, Inc., Topeka, KS, USA
†Current address: VDx Veterinary Diagnostics, Davis, CA, USA

[14]Stem Cell Institute, University of Minnesota, Minneapolis, MN, USA

Abstract

Background—Tumors have heterogeneous properties, which could be explained by the existence of hierarchically and biologically distinct tumor cells such as tumor-initiating cells (TICs). This model is clinically important, as TICs are promising targets for cancer therapies. However, TICs in spontaneous B-cell lymphoma have not been conclusively identified.

Hypothesis/Objectives—Tumor cells with a progenitor phenotype exist in B-cell lymphoma, reflecting a hierarchical organization.

Animals—Twenty-eight client-owned dogs with previously untreated B-cell lymphoma and six healthy dogs.

Methods—This was a prospective study. Flow cytometry was used to identify lymphoid progenitor cells (LPCs) that co-expressed hematopoietic progenitor antigens CD34, CD117 (KIT), and CD133, with lymphoid differentiation markers CD21 and/or CD22 in B-cell lymphoma. The polymerase chain reaction for antigen receptor rearrangements was used to analyze clonality and relatedness of tumor populations. A xenograft model using NOD/SCID/IL-2R$\gamma^{-/-}$ mice was adapted to expand and serially transplant primary canine B-cell lymphoma.

Results—LPCs were significantly expanded in lymph node samples from 28 dogs with B-cell lymphoma compared to six healthy dogs (p=0.0022). LPCs contained a clonal antigen receptor gene rearrangement identical to that of the bulk of tumor cells. Canine B-cell lymphoma xenografts in recipient mice that maintained LPCs in the tumors were recurrently observed.

Conclusions and clinical importance—These results suggest the presence of a hierarchy of tumor cells in canine B-cell lymphoma as has been demonstrated in other cancers. These findings have the potential to impact not only the understanding of lymphoma pathogenesis but also the development of lymphoma therapies by providing novel targets for therapy.

Keywords

Introduction

Lymphomas in dogs are a group of lymphoproliferative disorders originating from B-cells, T-cells, and NK cells. Lymphoma is the most common hematopoietic tumor in dogs, accounting for more than 20% of all canine tumors.[1] Approximately 85% of dogs with lymphoma respond to standard of care CHOP-based protocols; however, durable remissions for most B-cell lymphomas are not the norm, with median survival remaining at ~9–12 months.[2] A better understanding of the pathogenesis of lymphoma will assist in development of more effective treatment strategies.

It has long been known that cancer cells within tumors exhibit heterogeneity.[3, 4] Although individual tumor cells initially arise from a single cell and thus share common genetic aberrations reflecting their clonal origin, single-cell analysis has demonstrated variation

among tumor cells at the genetic, cellular, and molecular levels. According to the stochastic carcinogenesis model, all cells in a tumor are biologically equivalent and heterogeneity arises from random mutational or epigenetic events in individual cells. However, recent evidence supports a hierarchical model of tumor initiation where cells are not biologically equivalent, and which is the major source of tumor heterogeneity.[5, 6] According to this model (also known as the cancer stem cell model), tumors are organized hierarchically into a population of cancer stem cells or tumor-initiating cells (TICs) and a population of partially differentiated tumor cells, where the TICs are the only cells able to initiate and maintain tumors. In this model, TICs are singly responsible for resistance to therapy, relapse, and metastatic spread.[7, 8] A primitive population of tumor cells might be as important in lymphoma biology as it is in the biology of other hematopoietic tumors.[9–13] However, TICs have not been conclusively identified in naturally occurring lymphoma of any species. Since hierarchical organization may not be a feature of every tumor type,[14] it is possible that lymphoma conforms to the stochastic model. The implication that tumors organized into stochastic or hierarchical populations will have fundamentally different natural histories and response to treatment underscore the importance of this question.

In this study, we focused on B-cell lymphoma of dogs (which is more common than T-cell and NK cell lymphoma[2]) to test the hypothesis that lymphoid progenitor cells (LPCs) exist in lymph nodes (LNs) of dogs with B-cell lymphoma, reflecting a hierarchical organization. TICs have initially been defined in many tumors by the expression of certain lineage-specific and progenitor markers in subpopulations of enriched cells. Thus, we assessed the existence within B-cell lymphomas of cells that expressed hematopoietic progenitor markers, CD34, KIT, and CD133, in combination with lymphoid lineage markers.

Materials and Methods

Study design

This was a prospective study. The present study was designed to confirm the biological and clinical relevance of lymphoid progenitor cells (LPCs) found in lymph nodes from dogs with malignant B-cell lymphoma. LPCs, defined as cells that co-expressed CD34, Kit, and/or CD133 ("progenitor markers") with CD45 and lymphoid lineage markers, have been identified in lymph node (LN) samples from nine dogs with diverse types of malignant lymphoma (JFM, MME, and KAH, unpublished). Here, samples from an expanded cohort of dogs with B-cell lymphoma (cohort-II) were used to prospectively analyze expression of lymphoid lineage markers in Progenitor$^+$CD45$^+$ cells. The initial objective was to exclude the possibility that LPCs represented resident or mobilized hematopoietic progenitor cells (HPCs) in malignant LNs. Next, the percentage of LPCs in LNs from healthy dogs vs. LNs from dogs with B-cell lymphoma, as well as the clonal relationship between LPCs and the rest of the resident malignant population in the LNs were assessed. The side population (SP) assay to determine if LPCs had the capability to exclude vital dyes was used, as has been described for normal hematopoietic stem cells[15] and some cancer stem cells.[16] Finally, the persistence of LPCs in tumors after xenotransplantion was analyzed.

Lymph node samples and animals

Sterile biopsy or fine needle aspirate (FNA) samples were available from 28 dogs (four dogs from cohort-I and 24 dogs in cohort-II) with a diagnosis of B-cell lymphoma at participating veterinary hospitals with owner consent under protocols approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) and the Colorado Multiple Institutional Review Board. The 28 dogs were from 14 breeds, including six Golden Retrievers, four Labrador Retrievers, two Pembroke Welsh Corgis, two Standard Poodles, and one each of 10 other breeds, as well as four mixed breed dogs. The gender distribution was 50:50, and the average age was 8.6 years (median = 9 years, range 3 - 14). Normal tissue controls were obtained at the time of euthanasia from six healthy, purpose-bred, one-year-old female Beagle dogs that were part of an unrelated project. Collection of normal tissues was done under a protocol approved by the University of Minnesota IACUC. Sample preparation for histopathology, cytology, and flow cytometry was performed as described.[17] The final histopathological classification was determined by one author (VEV) with a consensus of three additional authors (DI, TDO, JFM) upon review of the specimens. Tumors were classified according to the modified WHO classification.[18] They included 13 diffuse large B-cell lymphomas, four Burkitt-like lymphomas, two marginal zone lymphomas, two low-grade B-cell lymphomas of intermediate centrocytic cells with cleaved nuclei, one follicular lymphoma, and one anaplastic large cell lymphoma. Sufficient sample material was not available for histopathological analysis of five cases. An uncommon phenotype of canine B-cell lymphomas that express CD34 was previously reported.[19] Because it is unclear if this represents a unique subtype with distinct biology, inclusion criteria for samples was set as <5% $CD34^+$ cells in the tumor populations to limit the confounding effects of this variable. NOD/SCID/IL-2R$\gamma^{-/-}$ (NSG) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained under specific pathogen-free conditions according to institutional guidelines. Lymph node collection from dogs and xenotransplantation of canine lymphoma cells into conditioned, immunocompromised (NSG) mice were conducted with approval of the University of Minnesota IACUC.

Flow cytometry

We used flow cytometry to prospectively analyze the existence of LPCs in single cell suspensions from lymphoma biopsies as described.[17] Primary antibodies are listed in Supplementary Table 1. LPCs were classified based on the expression of hematopoietic progenitor markers CD34, KIT, and CD133 (detected by a cocktail of antibodies against these antigens where the mix was designated as "Progenitor"), the common leukocyte antigen CD45, and lymphoid lineage antigens CD21 and CD22. The frequency of Progenitor$^+$ cells and Progenitor$^+$CD22$^+$ cells was normalized to the total number of $CD45^+$ cells. Samples also were stained with a separate antibody (labeled by different fluorescent) for each antigen to analyze the specific antigens expressed in Progenitor$^+$ cells. Side population (SP) assay was performed as described using Hoechst 33342 (Sigma-Aldrich, St. Louis, MO), except that exposure to Hoechst dye was limited to 30 minutes to minimize toxicity.[15] To set the SP gate, paired aliquots of cells were pre-incubated with or without verapamil (50 μM), which acts as an ABC transporter inhibitor, for 15 minutes at 37°C. Flow cytometry was performed using Coulter Cytomics FC500 (Beckman Coulter, Fullerton, CA), FACSCalibur, or LSRII cytometers (BD Immunocytometry Systems, San

Jose, CA), and results were analyzed using Winlist (Verity Software, Topsham, ME) and FlowJo (Tree Star, Ashland, OR).

Polymerase chain reaction for clonal antigen receptor rearrangement (PARR)

LPCs were enriched from lymphoma samples using immunomagnetic selection (EasySep PE selection kit; STEMCELL Technologies, Vancouver, Canada) with a cocktail of PE-conjugated "Progenitor" antibodies. DNA samples were prepared from enriched Progenitor$^+$ cells and from Progenitor-depleted cells using QIAamp DNA mini kit (Qiagen, Valencia, CA), and PCR was run to detect immunoreceptor gene rearrangements using primers for Ig heavy chain (IgH), T-cell receptor gamma chain (TCRγ), and the constant region of IgM (Cμ; positive control) as described.[20] IgH, TCRγ, and Cμ primers labeled with VIC, PET, and 6FAM dyes, respectively, were obtained from Applied Biosystems (Foster City, CA) and used for PCR reactions and capillary electrophoresis. The size of PCR products was analyzed with the GeneScan 600 LIZ size standard (Applied Biosystems) using the Genetic Analyzer 3130xl (Applied Biosystems). For gel electrophoresis analysis, PCR products were separated on 15% acrylamide gels, visualized by ethidium bromide staining using an AlphaImager HP imaging system (Alpha Innotech, San Leandro, CA). Semi-quantitative PARR with serial dilutions of input DNA from 1 to 0.01 ng was performed to confirm the assay did not simply detect clonal IgH gene rearrangement from residual Progenitor$^-$ cells in the "Progenitor$^+$" sample. This assay can be used to infer the relative number of clonal lymphoma cells in a population based on the relationship between the extinction slopes obtained in the serial dilution from antigen receptor and germline Cμ control reactions.

Xenotransplantation assay

A limitation in studies of primary B-cell lymphomas in dogs and humans is the absence of established methods to analyze the cells using *in vivo* models. The conditioned NSG mouse model, which is robust for hematopoietic cell transplantation,[21] was adapted to serially xenotransplant T-cell depleted canine B-cell tumors in vivo. Immunomagnetic depletion using a PE-conjugated anti-CD5 antibody was used to eliminate residual T-cells from primary B-cell lymphomas. Serially diluted malignant B-cells (3×10^7, 1×10^7, or 2×10^6) were transplanted intraperitoneally into conditioned NSG mice (by 200 cGy irradiation) using an X-RAD 320 (Precision X-Ray, North Branford, CT). Tumor growth was assessed daily, and tumor-bearing mice were sacrificed after 12–14 weeks. Tumor engraftment in LNs, spleen, lung, liver, and kidney was analyzed by histopathology. Single cell suspensions of spleen cells were prepared and blocked with dog IgG and 2.4G2 antibody (BD Biosciences, San Jose, CA). The phenotype of tumor cells and the presence of LPCs in spleen samples were analyzed using routine immunohistochemistry and flow cytometry as described above. Engrafted tumor cells were serially transplanted into conditioned NSG recipients twice more to confirm tumor propagation potential.

Statistical analysis

The Mann-Whitney Test (Prism 4, GraphPad Software, Inc., La Jolla, CA) was used to determine significance between LPC numbers in LNs from dogs with lymphoma and LNs from unaffected dogs.

Results

Lymphoid progenitor cells reside in lymph nodes of dogs with lymphoma

The first cohort included 4 dogs with B-cell lymphoma, each of which contained Progenitor^{+} cells in LN biopsies (0.11, 0.13, 0.29, and 1.51% of the total CD45^{+} cells, respectively); the second independent cohort included 24 dogs with B-cell lymphoma. Figure 1A and 1B show two-dimensional flow cytometry dot plots from a representative sample of cohort-II, showing >88% of Progenitor^{+} cells co-expressed CD45 and the B-cell marker, CD22. The percent of CD22^{+} cells in the total Progenitor^{+} population from the 24 samples in cohort-II was 90.8 ± 7.4% (mean, SD), and these Progenitor^{+}CD22^{+} cells comprised 0.16–2.10% (mean = 0.89%) of all LN lymphocytes (Fig. 1C). Two samples tested also showed co-expression of the B-cell antigen CD21 (data not shown). Most of the Progenitor^{+} cells in each sample CD34-single positive (0.63 ± 0.58%, mean, SD, n = 23), with fewer KIT-single positive (0.18 ± 0.12%, mean, SD, n = 22) and CD133-single positive cells (0.19 ± 0.21%, mean, SD, n =22). There were less than 0.05% CD34- and KIT-double-positive cells and CD34- and CD133-double-positive cells in all the samples. The percent Progenitor^{+}CD22^{+} cells in LNs from six healthy dogs (without lymphoma) ranged from 0.12 to 0.25% (mean=0.20%, Fig. 1C). The percentage of Progenitor^{+}CD22^{+} cells in B-cell lymphomas was statistically significantly different (p = 0.0022, two-tailed Mann-Whitney test) from that in the control group (Fig. 1C).

Lymphoid progenitor cells are related to lymphoma cells

Progenitor^{+} cells were enriched by ~2 orders of magnitude (from 0.86% to 71.8%) by immunomagnetic selection. Figure 2A shows the percentage of Progenitor^{+} cells in Progenitor-enriched (Progenitor[+]) and Progenitor-depleted (Progenitor[−]) fractions from a representative B-cell lymphoma sample as determined by flow cytometry. Clonal IgH gene rearrangements of the same size were found in the Progenitor[+] and Progenitor[−] populations (Fig. 2B). By semi-quantitative PARR, there was no difference in the extinction rate between Progenitor[+] and Progenitor[−] samples (Fig. 2C). Amplified IgH genes were further sequenced and confirmed to be identical between Progenitor[+] and Progenitor[−] samples (data not shown). A small number of verapamil-sensitive SP cells were detectable in B-cell lymphoma samples (Fig. 3), but these SP cells did not express Progenitor markers or CD22.

LPCs remain after xenotransplantation into immunocompromised laboratory mice

Three different primary canine B-lymphomas (of three attempted in this model) were successfully transplanted. Engrafted canine B-cell lymphoma cells became detectable within 12–14 weeks after transplant. Figure 4A shows diffuse infiltration of tumor cells in spleen in a representative recipient mouse. Lymphoma cells also were present in LNs, liver, kidneys, lungs, and pancreas (data not shown). The tumor cells expressed (canine) CD20 and CD79a, but not CD3 (Fig. 4A), and >90% of the spleen cells were found to be canine-derived large B-cells by flow cytometry (Fig. 4B). A population of Progenitor^{+} cells persisted recurrently in the tumor xenografts (Fig. 4B). The PARR analysis showed an identical IgH gene rearrangement in a biopsy sample and its associated xenograft (Fig. 4C). Success was not limited to primary transplants, as canine B-lymphoma cells derived from spleens of recipient

mice and containing the LPCs were serially transplanted twice more into NSG mice (data not shown).

Discussion

In this study, an expanded population of LPCs that expressed hematopoietic progenitor antigens and lymphoid markers was identified in primary LN samples of B-cell lymphoma. In addition, it was demonstrated that LPCs harbored tumor specific IgH gene rearrangements, which take place along a highly ordered B-cell developmental pathway, and thus they act as "genetic fingerprints" to identify clonally derived B-cells.[22] Because the PARR analysis might be sensitive enough to detect clonal IgH gene rearrangement in residual Progenitor− cells from the Progenitor+ sample,[20] the result was further confirmed using semi-quantitative PARR with serial dilutions of input DNA. There was no difference in the extinction rate of IgH normalized to the germline Cμ control between Progenitor+ and Progenitor− samples, suggesting the rearrangements were not present exclusively in residual Progenitor− cells. Taken together, these results suggest LPCs represent an atypical population that is expanded in malignant LNs of dogs with B-cell lymphoma. The LPCs were evident in different grades and types of canine B-cell lymphoma, suggesting they might be a common feature required to maintain and expand these various subtypes. In addition, these cells persist in serial xenotransplantation, suggesting they are pathogenetically involved in tumor formation.

To our knowledge, reports of resident LPCs in normal and malignant LNs are limited. There was an independent subpopulation of cells in malignant LNs that could differentiate to myeloid lineage cells in long-term in vitro cultures, but these cells were clonally unrelated to the tumor (DI and JFM, unpublished data). A population of cells that can exclude vital dyes (SP cells), which were similarly distinct from the LPCs, was also found in this study. These suggest that normal HPCs co-exist with LPCs in malignant LNs. Recent work reviewed in detail by Caligiuri identified a small population of CD34+ cells (<0.05% of total lymphoid cells) in normal human LNs.[23] This population is comprised almost entirely of lineage-committed NK cell progenitors. While the cells characterized by Caliguiri may retain multipotency, additional work will be necessary to document their significance in lymphoma.

Although there is anecdotal evidence of spontaneous regression of lymphoma in humans associated with immune responses against the causative Epstein-Barr virus,[24] lymphoma in dogs is not associated with a viral etiology.[2] Tumor reactive LPCs might give rise to expanded clones that mediate anti-tumor immunity elicited by active immunization through the use of idiotype vaccines, dendritic cell therapy, and other strategies,[25, 26] and infiltrating regulatory T cells have been shown to limit anti-tumor responses.[27] However, the peculiar tumor-like phenotype (i.e. CD22+ in the B-cell lymphomas) and the tumor-related immunoreceptor gene rearrangement of the Progenitor+CD45+ cells indicate these cells are probably not tumor-specific or tumor-infiltrating lymphocytes. Likewise, the absence of clonal TCR rearrangements excludes the possibility these are regulatory T-cells. We therefore conclude that the atypical, expanded population of LPCs represents a potential pathogenetically significant subset of cells in lymphoma.

It has been reported that tumor cells with progenitor phenotypes are prognostically significant in some types of cancer.[28, 29] While sample size in this study was large enough to establish the recurrent presence of LPCs, it was too small and too diverse to determine their prognostic significance. Additional work will be necessary to assess if LPCs have prognostic or predictive value.

In summary, there was an expanded population of LPCs related to malignant cells in LNs of dogs with B-cell lymphoma, suggesting that B-cell lymphoma in dogs might conform to the hierarchical progression model found in other types of cancer. Further, we have defined a reliable xenotransplantation model that can be used to test the biological behavior of these LPCs, as well as the potential benefits of treatment strategies targeting these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

List of abbreviations

NSG	NOD/SCID/IL-2R$\gamma^{-/-}$
TICs	tumor-initiating cells
LPCs	lymphoid progenitor cells
SP	Side population
PI	propidium iodide
IgH	immunoglobulin heavy chain
TCRγ	T-cell receptor gamma chain
Cμ	the constant region of IgM
HPCs	hematopoietic progenitor cells
PARR	polymerase chain reaction for clonal antigen receptor rearrangement

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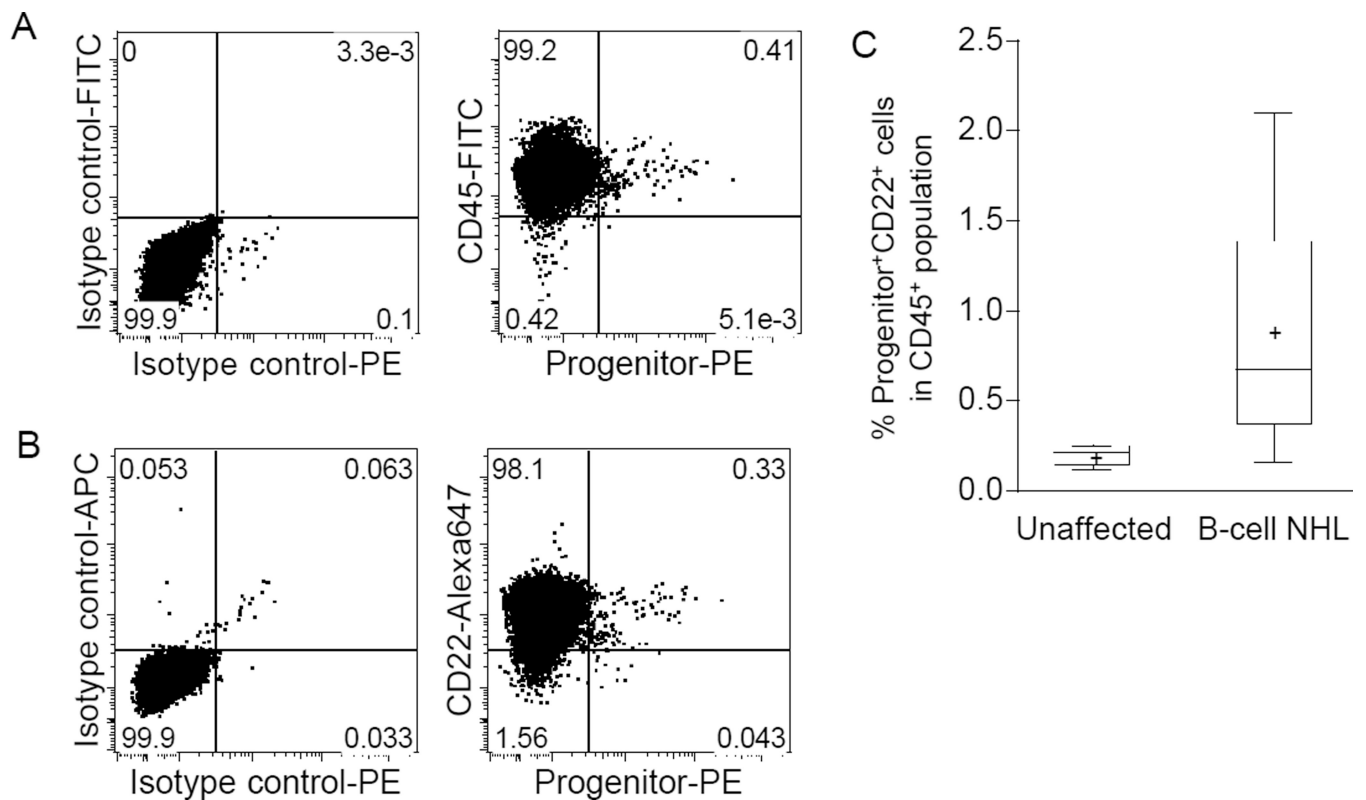
Figure 1.
Flow cytometry analysis of malignant LNs from dogs with B-cell lymphoma for LPCs. **(A)** A representative example of two-dimensional flow cytometry dot plots of tumor cells analyzed to identify hematopoietic progenitor cells. The left panel shows staining with isotype control antibodies. The right panel shows expression of "Progenitor" antigens CD34, KIT, and CD133 (using an antibody cocktail) on the X-axis and expression of CD45 on the Y-axis. **(B)** Analysis of the same sample showing expression of the lymphoid lineage commitment marker, CD22, in Progenitor+ cells. **(C)** Significantly greater numbers of Progenitor+CD22+ cells are detectable in LN samples from dogs with B-cell lymphoma as compared to LNs from healthy (unaffected control) dogs. The bottom and top edges of the boxes correspond to the sample 25th and 75th percentiles, and the box lengths represent one interquartile range.
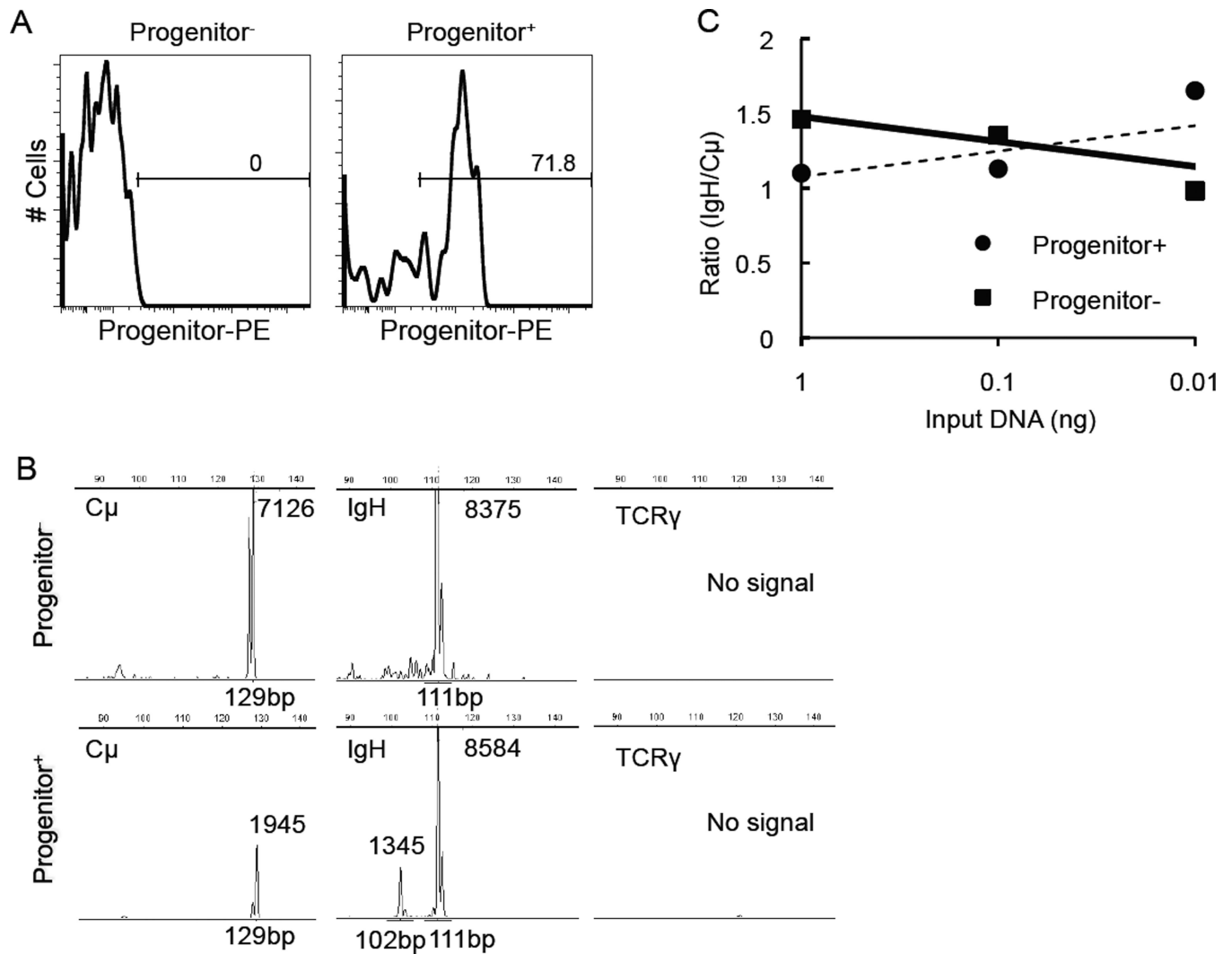
Figure 2.
Characterization of LPCs by PARR. **(A)** Flow cytometry analysis was used to determine the efficiency of Progenitor+ cell enrichment and depletion in a B-cell lymphoma sample after immunomagnetic separation. **(B)** PCR analysis of Progenitor+ and Progenitor− samples using primers for Cμ, IgH, and TCRγ primers. PCR product size was analyzed by capillary electrophoresis. The number next to the peak represents the fluorescence intensity. **(C)** Serial dilutions of Progenitor+ and Progenitor− cell DNA were used for PARR analysis. Data show the ratio of scanned pixel intensity from the IgH product (band) over the Cμ product. For DNA from LPC-enriched cells, the ratios of Cμ/IgH were 1.1, 1.1, and 1.6. For DNA from LPC-depleted cells, the values were 1.5, 1.4, and 1.0 (1 ng to 0.01 ng DNA input samples, respectively). Similar results were seen in two dogs.
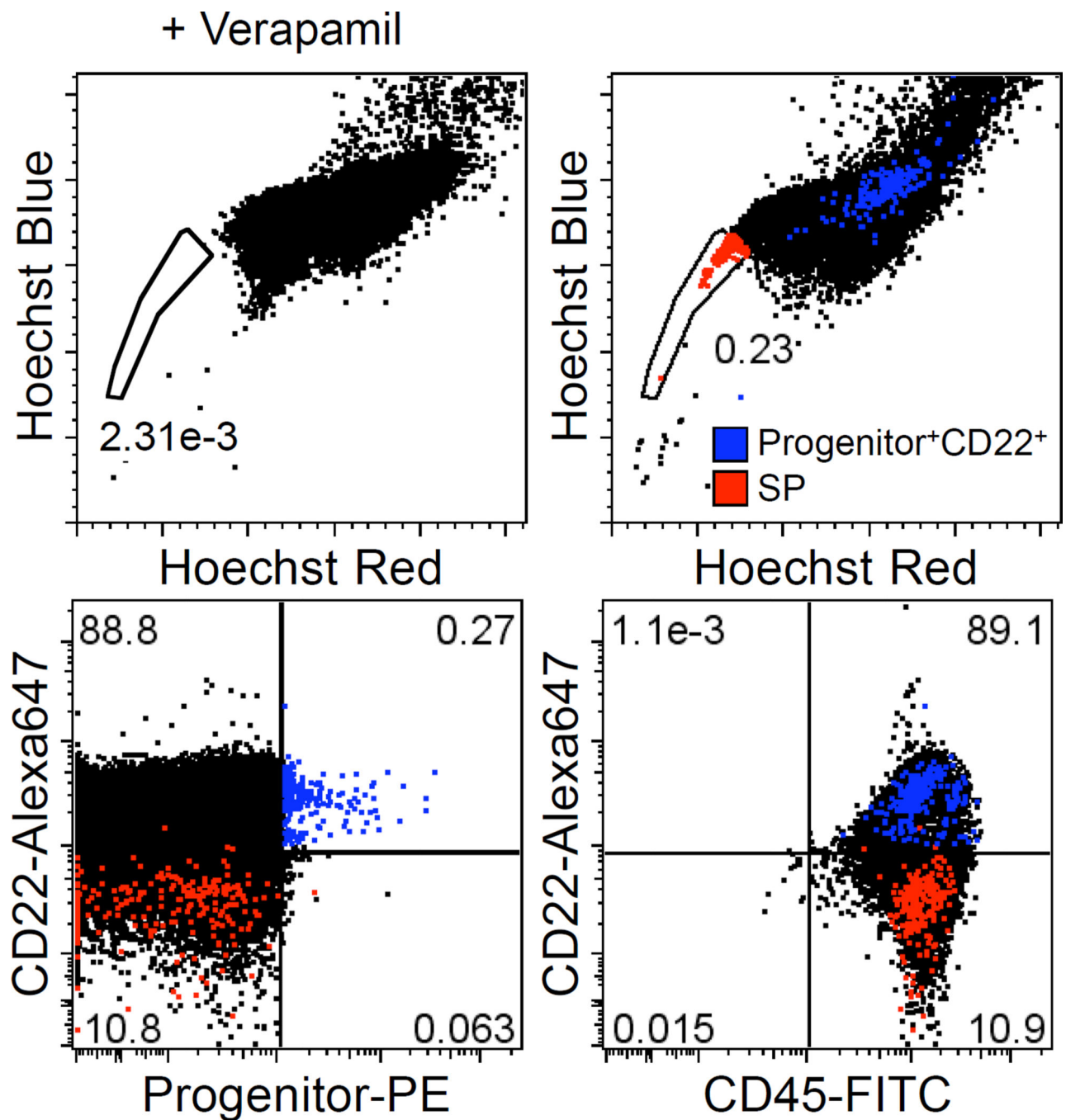
Figure 3.
SP assay of B-cell lymphoma cells. The SP assay was performed to detect cells with stem cell-like characteristics of high dye-efflux activity. B-cell lymphoma cells were incubated with Hoechst 33342 in the presence or absence of verapamil. Cells were stained with antibodies as indicated and analyzed on a LSRII flow cytometer. SP population is displayed in two-dimensional dot plots for Hoechst-blue versus Hoechst-red fluorescence. Red and blue dots represent SP cells and Progenitor^{+}CD22^{+} cells, respectively.
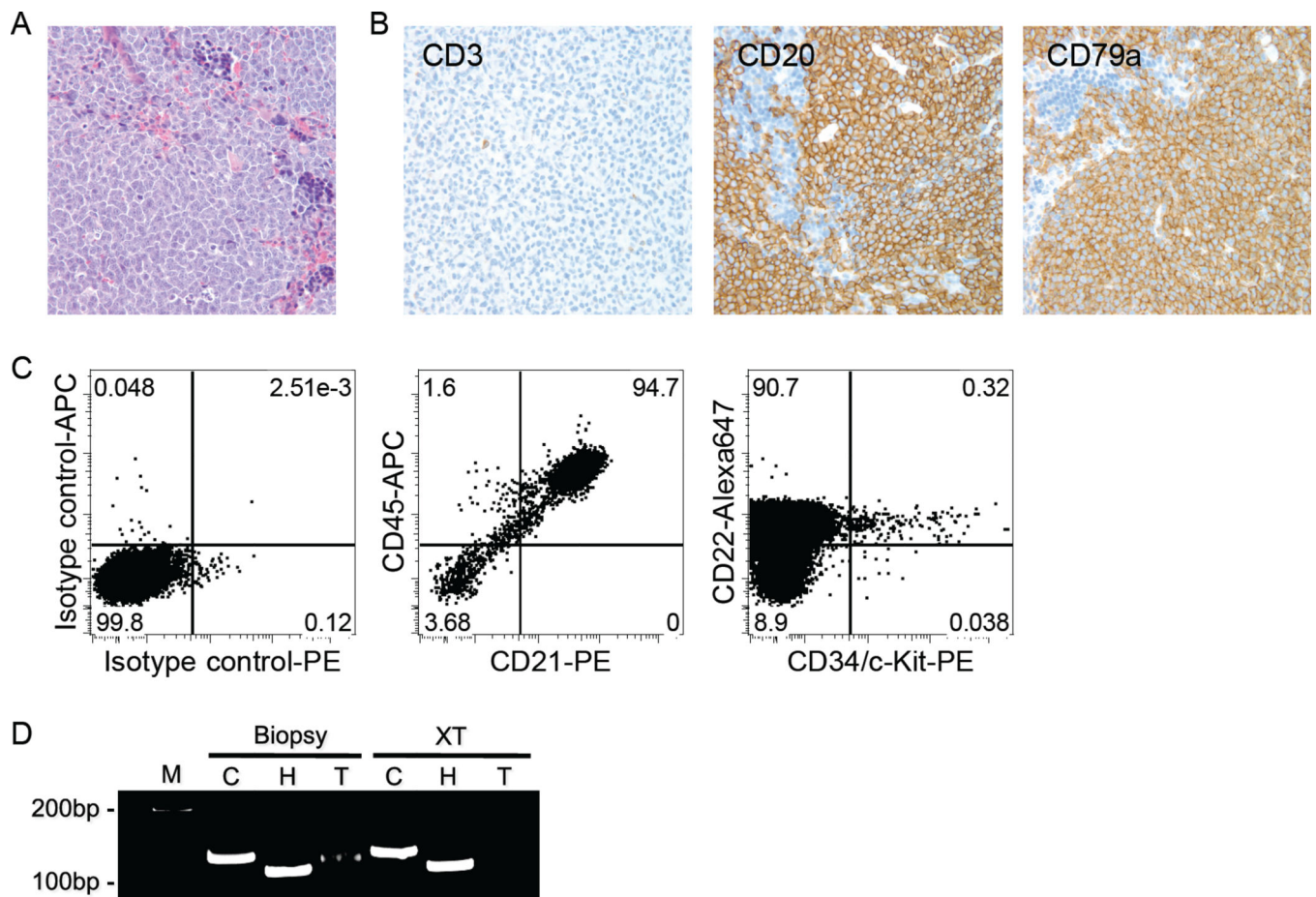
Figure 4.

Tumor engraftment of primary canine B-cell lymphoma in NSG mice. **(A)** Photomicrograph of diffuse infiltration of canine tumor cells in a recipient mouse spleen (hematoxylin-eosin (HE) stain, magnification ×200) and immunostaining of the donor (dog) cells for expression of canine CD3, CD20 and CD79a (magnification ×200). **(B)** Analysis of canine B-cell lymphoma in the spleen of a recipient mouse by multi-parameter flow cytometry. More than 94% of the cells were canine B-cells expressing CD21 and CD45. Similar data were obtained when cells were stained using anti-CD22 and anti-CD45. A small population of Progenitor+ cells was observed within the CD22+ population. **(C)** PARR performed for Cμ (C), IgH (H), and TCRγ (T) on genomic DNA from a biopsy sample of a dog LN and its corresponding xenograft in a NSG mouse spleen (XT). An identical clonal IgH gene rearrangement was present in both samples. M represents the molecular weight marker.