THESIS

FLOW CYTOMETRY IN THE DIAGNOSIS AND PROGNOSIS OF CANINE LYMPHOMA AND LEUKEMIA

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2010

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ABSTRACT

FLOW CYTOMETRY IN THE DIAGNOSIS AND PROGNOSIS

OF CANINE LYMPHOMA AND LEUKEMIA

Chapter one explores new markers of prognosis for canine lymphoma by using flow cytometry of lymph node aspirates. Data from one hundred and sixty dogs with confirmed multicentric B-cell lymphoma were used to test the hypothesis that poor patient outcome could be predicted by tumor cells expressing a low level of class II major histocompatibility complex (MHC), a large cell size on flow cytometry, and expression of stem cell antigen CD34. Poor outcome was defined by a reduced first remission time (FRT) and/or overall survival time (ST). In this two-part retrospective study, two-thirds of patients (modeling group, n=106) were randomly selected to construct a multivariable statistical model to identify test variables which showed a significant association with poor patient outcome. The second part validated the statistical associations by using the model to predict outcome on the remaining one-third of patients (validation group, n=54), whose clinical outcomes were known. Results found that class II MHC expression was the most significant prognostic marker identified in both univariable and multivariable analysis. Treatment type also influenced probabilities of both relapse and mortality, while cell size and patient age only impacted mortality. CD34 expression did not influence outcome. Overall, the constructed model performed variably in predicting the

validation group's outcome at the 180 day post-treatment time point. Immunophenotyping of lymph node aspirates via flow cytometry is useful in the confirmation and prognosis of canine lymphoma. Reduced class II MHC expression is correlated with a reduced patient FRT and ST, and it may suggest a means by which tumor cells evade immune detection.

Chapter two describes a retrospective pilot study that assesses the immunologic, morphologic, and clinical features of canine CD34+ acute leukemias. Flow cytometry data, CBC and blood film evaluation from 11 dogs with CD34+ acute leukemia were compared to 11 dogs with CD34- acute leukemia in order to identify qualitative differences in terms of cellular morphology, degree of presenting cytopenias, patient signalment and survival. The CD34+ dogs were classified as follows: 8 had acute undifferentiated leukemia (AUL), 2 had undefined acute lymphoblastic leukemia (CD3-CD5+ undefined ALL) and 1 had acute myeloid leukemia (AML). CD34- dogs were classified as follows: 1 AUL, 1 CD3-CD5+ undefined ALL, 5 T-cell ALL, and 4 B-cell ALL. Most dogs were affected with single or bicytopenia, with both groups having a similar frequency of anemia and thrombocytopenia. CD34+ dogs had significantly more severe anemia and were significantly younger at time of diagnosis. The groups did not differ in total white blood count, blast cell count, flow cytometry cell size, or survival time. CD34+ and CD34- leukemic cells subjectively did not differ greatly in their morphologic appearances. Flow cytometry of peripheral blood enhances the traditional morphologic-based classification of acute leukemias, particularly when CD34 antigen is expressed. Some acute leukemias may not be accurately classified as such when only a morphologic evaluation of peripheral blood is performed.

ACKNOWLEDGEMENTS

In addition to the invaluable contributions made by the Committee members, I thank Anne Avery, Emily Marcus, and Sangeeta Rao for their significant contributions to Chapter 1 of this thesis. Anne Avery's flow cytometry database and helpful insight were also important to the work in Chapter 2. I also thank all the veterinarians, hospital staff and owners of dogs who were involved in these studies.

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INTRODUCTION

Lymphohematopoietic neoplasias represent some of the most common malignancies in both humans and veterinary species. Whereas lymphoma is a malignancy that usually arises from lymphoid organs such as lymph node or spleen, leukemia originates from bone marrow, less often from the spleen, and represents either a myeloid or lymphoid clonal proliferation. Regardless of its cell of origin, both lymphoma and leukemia can disseminate throughout the body, infiltrate any organ, and first be identified in peripheral blood or in lymph nodes. Both the diversity of disease manifestation and the shared characteristics of lymphohematopoietic malignancies create a diagnostic challenge for the medical team as to how best to diagnose, accurately classify and treat the lymphoma/leukemia patient.

Within that last two decades, advanced molecular diagnostics such as flow cytometry, immunohistochemistry, and polymerase chain reaction for antigen receptor rearrangements (PARR) have lead to better characterization and subclassification of lymphoma and leukemia.[1-7] These, in turn, have lead to a better understanding of the molecular interactions and pathophysiology involved in lymphoma/leukemia, which has even lead to new targets for cancer treatement.[8-10]

The body of work described in this thesis examines the role of flow cytometry in the diagnosis of canine lymphoma and leukemia. Flow cytometry evaluates cells in a fluid medium, most commonly in blood, and can provide an objective assessment about cell percentages, size, and their array of antigen expression. Together with its minimal invasiveness to the patient, ease of sample collection, and relative low cost, flow cytometry has become part of the gold-standard workup for lymphoma/leukemia

patients.[4-7,11-15] This thesis is comprised of two retrospective studies which explore whether our laboratory's flow cytometric panel of monoclonal antibodies can identify antigens that provide prognostically useful information in regard to canine multicentric lymphoma and canine leukemia.

The study aims for each of the following two chapters stem from our observation that a minority of canine lymphoma cases and many leukemia cases express the hematopoietic stem cell antigen, CD34. CD34 is generally accepted to be a marker of bone marrow-derived acute leukemia, and although its expression has also been reported in lymphoma,[11,14,16] its prognostic significance is not clear. Chapter one describes a study which evaluates the prognostic potential of CD34 expression along with two other flow cytometric variables in canine multicentric B-cell lymphoma. Chapter two is a pilot study that describes the immunophenotypic and morphologic features of canine CD34+ acute leukemias. Together, these studies help better characterize lymphohematopoietic neoplasias, clarify the significance of CD34 expression, and identify new and useful markers of prognosis.

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CHAPTER 1

NEW MARKERS OF PROGNOSIS FOR CANINE LYMPHOMA USING FLOW CYTOMETRY OF LYMPH NODE ASPIRATES

ABSTRACT

Hypothesis: Poor prognosis in canine B-cell lymphoma can be predicted by tumor cells expressing a low level of class II major histocompatibility complex (MHC), a large cell size on flow cytometry, and expression of the stem cell antigen CD34.

Methods: Two-part retrospective study involving 160 dogs with multicentric lymphoma that had peripheral lymph node aspirates evaluated by flow cytometry at initial diagnosis. Two-thirds of patients (modeling group; n=106) were randomly selected to construct a multivariable statistical model to identify test variables which showed a significant association with poor patient outcome. The second part validated the statistical associations by using the model to predict outcome on the remaining one-third of patients (validation group, n=54), whose clinical outcomes were known.

Results: Class II MHC expression was the most significant prognostic marker identified. Treatment type also influenced probabilities of both relapse and mortality, while cell size and patient age only impacted mortality. CD34 expression did not influence outcome. Overall, the constructed model performed variably in predicting the validation group's outcome at the 180 day post-treatment time point.

Conclusions and Clinical Importance: Immunophenotyping of lymph node aspirates via flow cytometry is useful in the confirmation and prognosis of canine lymphoma. Reduced class II MHC expression is correlated with a poor patient outcome, and it may suggest a means by which tumor cells evade immune detection.

INTRODUCTION

Lymphoma is a cancer arising from lymphoid tissues, and in the dog, it accounts for 7-24% of all neoplasia and 83% of all hematopoietic malignancy.[1] In such cases, fine needle aspiration of enlarged peripheral lymph nodes is the primary test method used to confirm a diagnosis. Various clinical parameters and immunologic measures are now commonplace in the characterization of lymphoma and provide prognostic information. For instance, clinical stage, clinical substage, and immunophenotype are recognized as reliable predictors of patient survival, where B-cell lymphoma (which accounts for ~75% of the canine multicentric form) tends to have a better prognosis than the T-cell immunophenotype.[1-6] Other outcome predictors include the presence of anemia,[7] argyrophilic nucleolar organizer regions (AgNor) staining characteristics,[8,9] and histological characterization.[4,6]

Flow cytometry of lymph node fine needle aspirates (FNA-FC) is a validated method[8,10-13] and logical supplement to the cytologic diagnosis of lymphoma because it provides the immunophenotype and yields a great potential for additional prognostic markers. Our laboratory previously showed that in dogs with peripheral blood lymphocytosis, representing either Stage V lymphoma or lymphocytic leukemia, larger-sized circulating B-lymphocytes carried a worse prognosis over those cases with smaller B-cells.[14] In addition, we found that expression of hematopoietic stem cell antigen CD34 had the poorest patient outcome with only a 16-day median survival time. In a similar fashion, this study utilizes lymph node FNA-FC to investigate new markers of prognosis for canine multicentric B-cell lymphoma.

Alongside CD34 expression and B-cell size, we have also chosen to investigate class II major histocompatibility complex (MHC), since its decreased expression on neoplastic B-cells has recently been shown to predict poor patient outcome in people with diffuse large B-cell lymphoma, the most analogous subtype to the canine multicentric form.[15-19] Class II MHC is a cell-surface protein on B-lymphocytes, monocytes, macrophages and dendritic cells. They typically present "foreign" antigen to CD4+ lymphocytes, which elicits a cell-mediated immune response. Although the precise mechanism for the poor patient outcome is not completely understood, several studies have shown that the nature of the immune response to the tumor may be prognostically more important than the tumor itself.[15,18,20]

We designed this retrospective study with the goal of identifying new markers of prognosis for canine multicentric B-cell lymphoma. Our hypothesis was that decreased expression of class II MHC, large B-lymphocyte cell size, and CD34 expression would all predict a poor patient outcome; specifically, a reduced first remission time (FRT) and overall survival time (ST). In the first part of our study, we constructed a multivariable statistical model to identify test variables which showed a significant association with poor patient outcome. The second part validated the statistical associations by using the model to predict outcome on a validation group of patients, whose clinical outcomes were known.

MATERIALS AND METHODS

Study Subjects

Patients were enrolled in two phases. The first phase of patients was selected from all dogs presenting to the Veterinary Teaching Hospital at Colorado State

University (CSU-VTH) between 1/1/2006 and 4/30/2009 on which flow cytometry of a lymph node aspirate was performed. Inclusion criteria were: 1) the flow cytometry panel revealed that greater than 60% of the large cells in the fine needle aspirate expressed B-cell marker CD21; 2) the patient had a definitive cytologic or histologic diagnosis of lymphoma; 3) there was no treatment for lymphoma prior to diagnosis, but the dog subsequently underwent chemotherapy with either a multi-agent (usually a CHOP-based) protocol, single-agent doxorubicin (+/- prednisone), or prednisone-only treatment; and 4) the medical record was available for review with at least one month follow-up post diagnosis.

In this first enrollment only a single dog had a CD34+ lymphoma. Therefore, a second enrollment phase was conducted using submissions from around the country. For each CD34+ dog in enrollment phase 2, a control CD34- B-cell lymphoma dog was enrolled from the same submitting hospital as long as both dogs had been diagnosed within 4 months of each other.

Flow Cytometry

Fine needle lymph node aspirates were performed on a peripheral lymph node by the attending clinicians. The aspirates were transferred into media consisting of RPMI 1640 with 5% fetal bovine serum (FBS) if at CSU-VTH. Referring hospitals transferred the aspirate into a sterile red-topped tube containing a mixture of 0.9 ml physiologic saline and 0.1ml canine serum (either from the patient or from another dog). Samples were express shipped with cooling packs, and flow cytometry was conducted within 72 hours from collection.

Flow cytometry was carried out as described by Lana et al.[11] Briefly, samples were pelleted and washed twice with an erythrocyte lysis buffer, then resuspended in 300µl phosphate buffered saline (PBS) with 2% FBS. 25µl of the final cell suspension was incubated with 25µl of antibody mixture according as listed in **Table 1.1**. All antibodies were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE). All antibodies were purchased from AbD-Serotec. After a 20 minute incubation at room temperature, the samples were washed twice in PBS-2% FBS, resuspended, and 0.01% propidium iodide added to stain for and exclude dead cells. Samples were then immediately analyzed on a Coulter XL flow cytometer. When possible, 5000 cells in the lymphocyte gate were collected after dead cell exclusion. As a guideline to determine the percentage of cells stained with each antibody, gates were set based on the isotype controls (supplied by the same manufacturer) such that <2% of cells were positive.

Data analysis was carried out with FCS Express (De Novo Software, Ontario, Canada). Size classification was based on the forward light scatter of CD21 gated cells measured on a linear scale. All cases of B-cell lymphoma in the study were larger than peripheral blood lymphocytes analyzed on the same day (generally from a different patient), and larger than CD5+ T-cells in the same node. Therefore, we classified these cells as either medium or large lymphocytes. All cases in which CD21+ lymphocytes had a median forward scatter greater than 720 units were assigned to cell size category "large," while the remaining cases were categorized as "medium." In order to translate this size value to other flow cytometers, cells classified as "large" had a median forward

scatter value greater than 1.6x the size of the CD5+ T cells detected in the same lymph node and the same staining tube.

The expression of a given antigen in human lymphoma and leukemia is reported as "dim or low" or "bright or high" or "partially expressed."[21] These adjectives are assigned based on comparison with the antigen's expression on the normal cellular counterpart and an isotype control. Accordingly, for this study we retrospectively assigned a patient's class II MHC expression as low class II MHC if the neoplastic population expressed a median fluorescence intensity (MFI) below 226 units on a log scale (**Figure 1.1**). In order to translate this for use in other laboratories, this cutoff represented the bottom 15th percentile of all class II MFI for all patients in the study. Normal lymphocytes usually have an MFI between 400 and 600 units on our flow cytometer, but we classified patients into the high class II MHC category if their cells had an MFI >228 units.

CD34 expression, unlike class II MHC expression, was a dichotomous rather than a continuous variable because either the cell population had positive expression or none at all (**Figure 1.2**). Therefore, cases in which greater than 5% of B cells stained for CD34 above background isotype control were considered CD34+.

Data Analysis

For all data, descriptive statistics were calculated and variables were summarized graphically. Patient outcome was defined by survival time (ST), which was the time interval post-onset of treatment to patient death, and first remission time (FRT), which was the time interval post-onset of treatment to date of first confirmed recurrence of

cancer, as measured by recurrence of lymphadenopathy or extranodal mass assessed by a veterinarian.

The independent variables evaluated were: patient age (≤7 yrs, >7 yrs), gender (F, M), treatment type [prednisone-only, single-agent doxorubricin (plus or minus prednisone), or multi-agent (CHOP based regimen)], cell size (medium, large), CD34 expression (negative, positive), MHC II expression (low, high), CD5 expression (low, high), and CD21 expression (low, high). Only two breeds (Golden retrievers and Labrador retrievers) had sufficient cases to be analyzed separately. The remaining breeds were grouped into the "other" category, and dogs designated as mixed or cross breed comprised the fourth category.

Data from patients enrolled in the different phases were compared statistically using univariable Cox proportional hazards regression and chi-square tests. Significant differences were not identified between groups. Therefore, we elected to combine data from all enrollment phases for further analyses.

Cox proportional hazards regression analysis was used to investigate factors related to 2 outcomes (ST and FRT). Patients were censored if they had been lost to follow-up, died of conditions other than lymphoma, or were still alive at the end of the follow-up period on 5/30/2009. In order to assess the predictive ability of models, we used a split-sample approach to analysis so that reliability of the final multivariable models could be assessed using a separate subset of the data.[22] From all 160 patients, 2/3 of the dogs (n=106) were randomly selected to be the model building subset (modeling group) using Microsoft Excel program, and the remaining 1/3 (n=54) were reserved as the validation group for evaluation/validation of the final multivariable models.

Using the modeling group, Kaplan-Meier survival curves were evaluated for ST and FRT relative to all independent variables. Independent variables were also evaluated in univariable Cox proportional hazards models using both outcome variables and the proportionality assumption was evaluated. Variables with univariable P-values <0.25 were then passed into the multivariable model building. Backward selection was used to determine final multivariable models using a critical alpha for retention of 0.05. Variables that had been excluded based on their univariable results were introduced to the multivariable models to ensure that exclusion was appropriate. If these variables had Type III P-values <0.10 and had large hazard ratios (HR) suggesting there was a strong association with the outcome, they were retained in the multivariable models. Additionally, confounding was identified by >20% changes in parameter estimates when variables were individually removed from multivariable models; when identified, confounding variables were forced into multivariable models regardless of P-values. First-order interaction terms were then screened individually for all main effects that were included in the final models. HR and 95% confidence intervals (CI) were calculated from model results.

After multivariable models were finalized for ST and FRT, the validation group of dogs was used to evaluate their predictive reliability. Using the modeling group, the baseline hazard (i.e. the hazard probability when no covariates are present) of dying and of first cancer recurrence was estimated by the statistical software for a 180 day time period after initiating treatment. This baseline hazard probability was adjusted for the covariate pattern of each observation in the validation group using the regression parameter estimates which provided an adjusted probability of events (mortality and

cancer recurrence). For individuals with predictive probability estimates ≤0.50, it was interpreted that the model predicted that the event would *not* have occurred by 180 days (i.e. still alive or still in remission), whereas it was interpreted that the model had predicted the event (death or cancer recurrence) to have occurred if predictive probabilities were >0.50. These predicted outcomes were then compared to the actual patient outcomes and summarized by calculating the sensitivity and specificity and their associated 95% CI.

RESULTS

One hundred and sixty dogs were diagnosed with B-cell lymphoma and enrolled in the study. Sixty-six dogs met the inclusion criteria for enrollment phase 1, including one CD34+ dog and one dog with its CD34/MHC II status not determined. Enrollment phase 2 identified 45 CD34+ dogs and 49 CD34- dogs. Of the total 46 CD34+ patients, 7 had CD34 expression between 5% and 20% while the majority ranged between 20% and 95% CD34+ cells. Because no significant differences were identified between the enrollment phase populations, we elected to combine data from both phases, forming one population for further analysis.

For the first part of the study, 2/3 of patients were randomly selected for the modeling group, amounting to 106 dogs. The remaining 54 dogs were retained for the validation group. The test variables and patient distribution for both the modeling and validation groups are presented in **Table 1.2**. There were no statistical differences between the two groups, in so far as the proportion of dogs within each test variable category. For the modeling group, the median age was 8.5 years (range 3.1-15), with 38

females, 68 males, and 39 different dog breeds. There were 17 mix-breed dogs, 16 golden retrievers, 12 Labrador retrievers, 5 Rottweilers, and 3 or fewer of each of the remaining 35 breeds. The validation group had a median age of 8 years (range 3.8-16.8), with 26 females and 24 males. Twenty-five different dog breeds were represented and consisted of 12 mix-breed dogs, 9 Golden retrievers, 4 Labrador retrievers, 1 Rottweiler, and 3 or fewer of each of the remaining 21 breeds.

No statistical significance was found with gender or breed, although Golden retrievers and Labrador retrievers were over-represented in the overall study. Of the principal variables, the majority of dogs were greater than 7 years of age, had class II MHC high expression, medium cell size, negative CD34 expression, and underwent multi-agent chemotherapy.

For both ST and FRT univariable analyses, we identified three variables, class II MHC, cell size and treatment type, all of which had P-values <0.25, sufficient enough to advance to multivariable analysis (**Table 1.3)**. Gender had a P=0.22 for FRT analysis, but was not found to be significant during the multivariable analysis. We had also assigned cutoff values for MFI expression for CD5 and CD21 on neoplastic B-cells, but neither of these variables showed statistical significance and were eliminated early in the analysis. Expression of CD34 did not show any significant association with patient outcome. Patient age did attain statistical significance in the multivariable model building process even though it did not have a significant P-value <0.25 in univariable analysis.

Once the multivariable model building process was complete, four test variables showed significant correlation with patient outcome in the ST model, and two variables

were associated with outcome in the FRT model (**Table 1.4**). With all variables controlled for in the models, hazard ratios and their 95% CI were calculated, whereby a higher probability (HR >1) or lower probability (HR <1) of mortality or cancer recurrence were determined. The predictor variables for the ST model were as follows: class II MHC low expression had an HR=2.87 (CI 1.39-5.93, P=0.005); multi-agent treatment HR=0.24 (CI 0.09-0.59) and single-agent treatment HR=0.3 (CI 0.11-0.79); with an overall treatment category Type-III P-value=0.009; dog's age >7 years showed an HR=0.55 (CI 0.32-0.94, P=0.029); and B-lymphocyte large cell size had an HR=2.77 (CI 1.02-7.54, P=0.046).

The FRT analysis showed that the length of a patient's first remission was also correlated with class II MHC expression and treatment type but not cell size or patient age. Class II MHC low expression had an HR=3.49 (CI 1.51-8.08, P=0.004); multi-agent treatment HR was 0.18 (CI 0.07-0.5); and single-agent treatment HR=0.23 (CI 0.08-0.64) with a Type-III P-value=0.004.

The second part of the study allowed us to validate the associations between the significant test variables and patient outcome. This was accomplished by using the ST and FRT models to predict patient outcome of the remaining 54 dogs (the validation group), compare it to their observed clinical outcome, then calculate the sensitivity and specificity of the model predictions. The probability of mortality was generated for ST predictions, while the probability of first recurrence was produced for FRT predictions. For example, using just the multi-agent treated dogs from the validation group, **Table 1.5** illustrates how the model generated a probability of mortality for every possible

combination of significant variables that was associated with survival time (ST model predictions).

The sensitivity and specificity calculations for the ST and FRT models are outlined in **Figure 1.3** and stratified by treatment type. Only dogs with uncensored ST and FRT data were used in the accuracy calculations. In the ST model for instance, the overall sensitivity and specificity for all treatment types were 41% (CI 19-67%, n=33 uncensored dogs) and 88% (CI 60-98%, n=32 dogs), respectively. Similarly, when survival predictions for multi-agent treated dogs were evaluated separately, the ST model demonstrated a sensitivity of 22% (CI 4-60%, n=24 dogs) and a specificity of 93% (CI 64-100, n=23 dogs).

DISCUSSION

This study used FNA-FC to immunophenotype a population of canine multicentric B-cell lymphoma. Our results demonstrate the potential for a greater utility of flow cytometry beyond mere distinction of B versus T cell type status. Not only did our FNA-FC analysis confirm a cancer diagnosis and provide an immunophenotype, it identified new markers of prognosis in class II MHC expression and B-lymphocyte cell size. It also found no prognostic role for CD34 expression in lymphoma. Together, these findings highlight the heterogeneity of canine lymphoma and suggest that, like its human counterpart, can be subclassified to provide more prognostic information.

It is not surprising that dogs receiving multi-agent chemotherapy for lymphoma had lower hazard ratios (<1) compared to dogs only receiving prednisone treatment. Multi-agent therapies have been well-documented for their superiority in prolonging

patient remission and survival.[1] On the other hand, we were surprised to find that CD34 expression did not influence patient outcome, contrary to what we had hypothesized and different from our previous findings in blood from dogs with CD34+ acute lymphocytic leukemia (ALL).[14]

CD34 antigen is a cell surface sialomucin-like glycoprotein that is present on non-committed hematopoietic stem cells, small-vessel endothelial cells, and embryonic fibroblasts.[23] In health, it is expressed in about 1% of canine and human bone marrow cells and 0.01-0.1% in human peripheral blood.[23,24] The function of CD34 molecule on primitive stem cells is not fully defined but is thought to play a role in cell adhesion to the bone marrow stroma and maintenance of normal hematopoietic activity.[23]

There are few reports assessing the clinical significance of CD34 in diffuse B-cell lymphoma, and its evaluation is scarce within the veterinary literature. It is widely accepted that CD34 expression is a marker of progenitor cells, but in this study we found that the CD34+ dogs concurrently expressed the mature B-cell marker, CD21. It would have been interesting to see if there were a high number of double-positive CD34+CD21+ cells, but our flow cytometry panel does not combine these markers in the same test tube (Table 1.1). Thus, based on our observations and reports by others,[25-27] we find it reasonable to conclude that CD34 expression in (canine) B-cell lymphoma is an atypical phenotype and appears to have no prognostic role.

It is quite possible that the CD34+ lymphoma cases in this study represent a different disease/different subtype than our previously reported cases of CD34+ ALL.[14] Whereas all the CD34+ ALL cases had been class MHC negative (unpublished data), all of the B-cell lymphoma dogs in this study had positive class II MHC expression, albeit

some with lowered expression levels. In order to fully explain the differences between CD34 expression in lymphoma and ALL, it would have been ideal for this study to have performed flow cytometry on blood and bone marrow in addition to lymph node. Cytologically assessing the bone marrow and possibly the spleen may have also been helpful.

We consider the most significant finding of this study to be the association between low class II MHC expression and poor patient survival. This association parallels the findings seen in people with diffuse large B-cell lymphoma.[15-19] In this study, after controlling for other variables in the analysis, dogs with low class II MHC expression had a 2.87 times higher likelihood of mortality and a 3.49 times higher probability of cancer recurrence at any given time point compared to dogs with high class II MHC expression. A look into the cellular interactions of the cell-mediated immune response helps explain the correlation between reduced patient survival and low class II MHC expression.

Current evidence indicates that down-regulation of class II major histocompatibility complex is likely due to decreased expression of its principal transcription factor, CIITA, class II transactivator.[28,29] Although the precise mechanism is not delineated, lowered class II MHC expression appears to reduce the cell's ability to effectively interact with CD4+ T-lymphocytes, which consequently inhibits downstream immunosurveillance of the tumor.[15,30,31] A similar decrease in class I MHC expression, a related surface protein that presents "self-antigen" to cytotoxic T-lymphocytes, is well-established to be a mechanism for tumor immune escape in both solid tumors and hematopoietic malignancies.[32-36] It is now recognized that class II MHC molecules are

also important in presenting tumor-specific antigens and even their own idiotype (i.e. the targets of immune attack).[31,35,37] Thus, by down regulation of both classes of MHC receptors, the tumor seems to evade detection and promote cell survivability. This had led top researchers in the field of tumor immunology to conclude that the immune response to the tumor may be more prognostic than the nature of the tumor itself.[18,20] Certainly, further studies investigating canine B-cell lymphoma and its tumor immunology are warranted.

Cell size was found to be a significant predictor of ST but not for FRT. The reason for cell size not maintaining prognostic significance for FRT may be due to fewer dogs having reported first recurrence dates. In regard to survival time however, and with all other variables controlled for, dogs in the large cell size category showed a 2.77 times higher probability of mortality at any given time point compared to dogs in the other size category. It is also important to remember that all dogs regardless of their cell size designation, had larger sized neoplastic cells compared to normal lymphocytes. Thus, it is fair to conclude that cell size predicted a reduced survival time only when the neoplastic cells were very large. We speculate that these very large B-lymphocytes might correspond to a more immature cell type that has a more aggressive neoplastic behavior. The flow cytometric cutoff values used to distinguish the large neoplastic B-cells from the other size category should be further investigated and validated by using a prospective study design. It might also be helpful to compare cell size and immunophenotype, as determined by flow cytometry, with cell size and morphology as determined visually via FNA cytology or histopathology.

Patient age was an unexpected variable to show a significant association with ST during the multivariable analysis, even though it had no evidence for statistical significance in the univariable assessment. The most likely explanation is that patient age was a confounding variable that had some extraneous association with both the independent variables and the outcome variable, ST. Thus, the association between patient age and outcome may be a spurious relationship. We were able to control for this confounder (the effect of patient age on other test variables) by including it in the multivariable analysis. However, there may be unknown (residual) confounders that were not controlled for in our model, such as an owner's commitment to treat or financial considerations.

The final component of our study took our newly described prognostic variables and validated their association with patient outcome by testing the model on the 54 remaining dogs in our study population. The ST model predictions demonstrated an overall low sensitivity (41%, CI 19-67%) and high specificity (88%, CI 60-98%), with broad confidence intervals (Figure 1.3). When just the multi-agent treated dogs were evaluated, the ST model showed an even higher specificity (93%, CI 66-100%). The broad confidence intervals are likely a reflection of the small number of dogs in the validation group. Since the calculations did not include censored data, the number of dogs was further reduced to 33 or fewer. In regards to predicting mortality (death), the low sensitivity means a high number of false negative predictions, whereby the model erred on predicting patient survival when in fact, some dogs had actually died sooner than the 180 day mark. Likewise, the high specificity means a low number of false positive predictions of death, an essential criterion for any good confirmatory diagnostic test,

especially when dire consequences (euthanasia) might result from its findings. Thus, it appears that the model can identify which patients have the poorest prognosis with a high level of specificity. This may help clinicians guide their client's decision of whether or not to treat their dog.

This study, like many others in canine lymphoma, was retrospective in nature and has its limitations and biases One unforeseen bias, which only affected the validation component results, occurred during determination of the cutoff values used to distinguish one test category from another (i.e. high versus low). Early in the study, we determined the cutoff values using all 160 dogs before they were randomly distributed to either the modeling or validation groups. Thus, even after randomization, the validation group was already biased, forcing a limited conclusion about their results.

In summary, we have shown that flow cytometry of lymph node aspirates is useful in the diagnosis and prognosis of canine lymphoma. We identified new markers of prognosis in class II MHC expression and B-lymphocyte cell size. We found that CD34 expression, while atypical in lymphoma, does not impact patient survival. Most exciting was the investigation into the role of cancer immunosurveillance. The concept that the immune response to cancer may be more prognostically important than the cancer type itself has far reaching implications in cancer research and drug development. Future prospective studies of canine lymphoma are warranted. This will allow us to better understand the complex nature and diversity of lymphoma, with its subclassifications and the many interdependent variables that influence its development, progression, treatment and outcome.

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TABLES AND FIGURES

Table 1.1 Flow cytometry antibody panel for fine-needle aspirate lymph node evaluation

Test tube	FITC-labeled antibody	Clone	PE-labeled antibody	Clone
1	no antibody		no antibody	
2	MIgG1 (non-canine antigen, neg. control)	Cat# MCA928A	CD45 (common leukocyte antigen)	YKIX16.13
3	CD18 (myeloid origin, some lymphocytes)	YFC118.3	MIgG1	Cat# MCA928A
4	CD8 (cytotoxic T-cells)	YCATE55.9	CD4 (T helper cells, canine neutrophils)	YKIX302.9
5	CD5 (T-cells)	YKIX322.3	CD21 (mature B-cells)	CA2.1D6
6	CD3 (T-cells)	CA17.2A12	CD45	YKIX16.13
7	CD4 (T helper cells, canine neutrophils)	YKIX302.9	CD14 (myeloid antigen)	UCHM1
8	Class II MHC (monocytes, lymphocytes)	YKIX334.2	CD34 (hematopoietic stem cells)	1H6

Table 1.2 Test variables and patient distribution

Variable[a]	Category	n Dogs in Modeling Group[c]	Percent w/in category %	n Dogs in Validation Group[c]	Percent w/in category %
Age[b]	≤7 years	36	*35*	21	*39*
	>7 years	67	*65*	33	*61*
Gender	Female	38	*36*	26	*48*
	Male	68	*64*	28	*52*
Treatment	Pred-only	7	*7*	5	*9*
	Single-agent	27	*25*	8	*15*
	Multi-agent	72	*68*	41	*76*
Class II MHC	High	91	*86*	47	*89*
	Low	15	*14*	6	*11*
Cell Size	Medium	99	*93*	50	*93*
	Large	7	*7*	4	*7*
CD34	Negative	78	*74*	35	*66*
	Positive	28	*26*	18	*34*
Total		**106** [c]		**54**[c]	
		160 [c]			

[a] Dog breed, enrollment phase, CD5, and CD21 were additional variables analyzed.
[b] Median age of dogs in modeling group was 8.5 years (range 3.1 to 15.6) and of the validation group was 8 years (range 3.8 to 16.8).
[c] Not all category information was available for some patients.

Table 1.3 Univariable analysis of the modeling group (n=106 dogs)[a]

Variable	Category	n Dogs[a]	Survival Time - ST (probability of mortality)			First Remission Time - FRT (probability of recurrence)		
			Hazard Ratio	95% CI	Type-III P-value	Hazard Ratio	95% CI	Type-III P-value
Age	≤7 years	34	Reference[b]		0.32	Reference[b]		0.57
	>7 years	69	0.78	0.48-1.27		0.86	0.51-1.44	
Gender	Female	38	Reference[b]		0.77	Reference[b]		0.22
	Male	68	0.93	0.56-1.53		0.724	0.43-1.22	
Breed	Other[c]	60	Reference[b]		0.84	Reference[b]		0.97
	gold ret.	16	0.83	0.41-1.68		1.01	0.5-2.05	
	Lab ret.	12	0.76	0.35-1.66		1.11	0.49-2.53	
	Mix	17	1.09	0.56-2.12		1.17	0.6-2.28	
Treatment	Pred-only	7	Reference[b]		0.055	Reference[b]		0.0063
	Single-agent	27	0.343	0.13-0.9		0.228	0.08-0.65	
	Multi-agent	72	0.352	0.15-0.84		0.204	0.08-0.54	
Class II MHC	High	91	Reference[b]		0.011	Reference[b]		0.0064
	Low	15	2.296	1.22-4.34		3.129	1.38-7.1	
Cell Size	Medium	99	Reference[b]		0.097	Reference[b]		0.23
	Large	7	2.194	0.87-5.55		1.75	0.7-4.39	
CD34	Negative	78	Reference[b]		0.91	Reference[b]		0.63
	Positive	28	0.971	0.57-1.65		0.86	0.47-1.59	
CD21	Low	18	Reference[b]		0.69	Reference[b]		0.97
	High	88	1.147	0.59-2.25		0.99	0.52-1.87	
CD5	Low	92	Reference[b]		0.67	Reference[b]		0.35
	High	14	0.857	0.42-1.73		0.67	0.29-1.55	

[a] Not all category information was available for some patients.
[b] Reference category has a hazard ratio of 1. Hazard ratio >1 indicates a higher risk for dying.
[c] "Other" refers to all dogs identified as purebred other than golden and labrador retrievers; "Mix" refers to all dogs identified as mixed breed.

Table 1.4 Results of multivariable analysis for the modeling group (n=106 dogs)

Variable	Category	Hazard Ratio	95% CI	Type 3 P-value
Survival Time – ST Model				
Class II MHC	High	Reference[a]		0.005
	Low	2.87	1.39-5.93	
Treatment	Pred-only	Reference[a]		0.009
	Single-agent	0.30	0.11-0.79	
	Multi-agent	0.24	0.09-0.59	
Age	≤7 years	Reference[a]		0.029
	>7 years	0.55	0.32-0.94	
Cell Size	Medium	Reference[a]		0.046
	Large	2.77	1.02-7.54	
First Remission Time – FRT Model				
Class II MHC	High	Reference[a]		0.004
	Low	3.49	1.51-8.08	
Treatment	Pred-only	Reference[a]		0.004
	Single-agent	0.23	0.08-0.64	
	Multi-agent	0.18	0.07-0.50	

[a] Reference category has a hazard ratio of 1. Hazard ratio >1 indicates a higher risk for dying.

Table 1.5 Predictions using the survival time model: Probability of mortality at 180 days post-onset of treatment for dogs in the validation group that were treated with multi-agent chemotherapy (n=41)[a]

Treatment	Class II MHC	Cell Size	Age	n Dogs[a]	Probability of mortality at 180 days[b]	95% CI
Multi-agent	High	Medium	≤7	15	0.30	0.16-0.41
			>7	21	0.17	0.08-0.26
		Large	≤7	0	0.62	0-0.87
			>7	0	0.41	0.01-0.65
	Low	Medium	≤7	1	0.63	0.16-0.84
			>7	1	0.42	0.16-0.61
		Large	≤7	0	0.94	0-1
			>7	2	0.78	0.07-0.95

[a] Class II MHC status not determined in one multi-agent treated dog.
[b] If probability ≤0.5, then dog more likely to still be alive at 180 days post-onset of treatment.
If probability >0.5, then dog more likely to have died before 180 days.

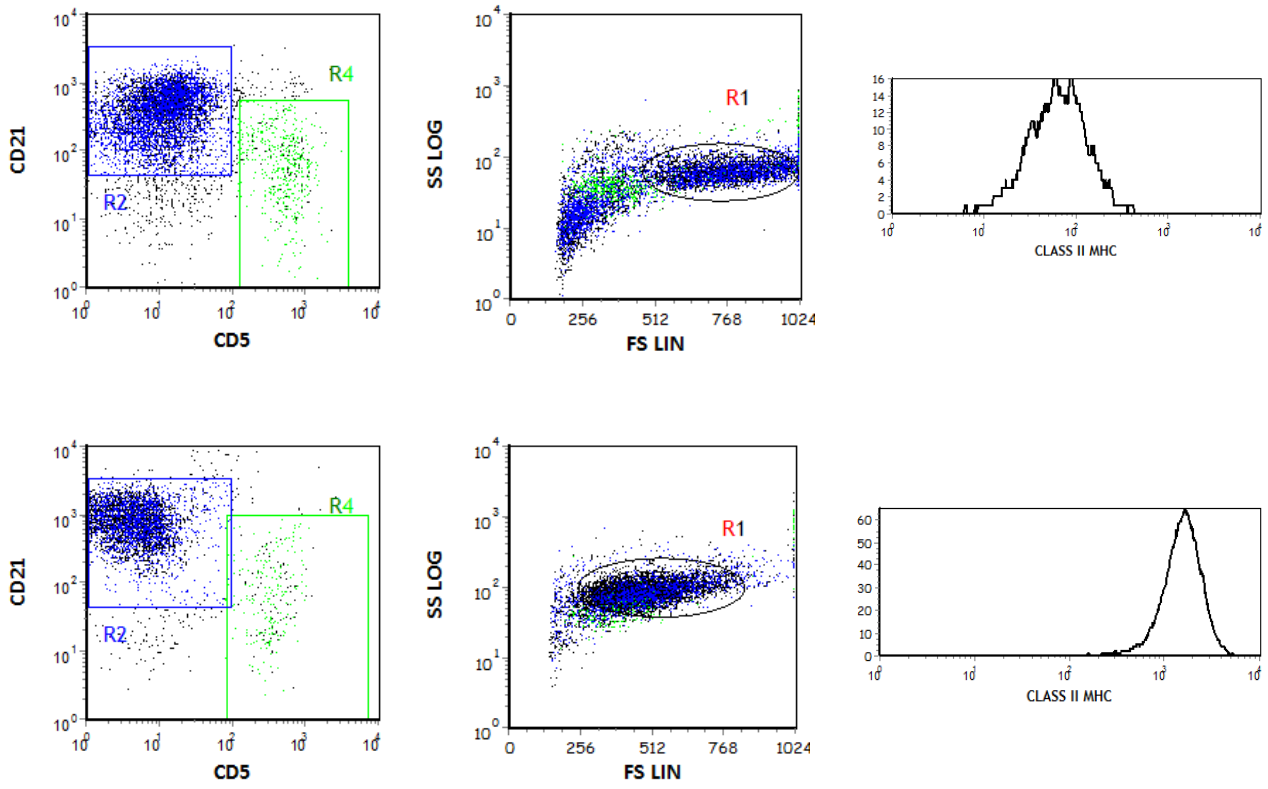

Figure 1.1 Flow cytometry determination of class II MHC expression levels on neoplastic cells. The large neoplastic cells are identified and gated (R1) in the middle boxes. A color gate (R2) is drawn around the CD21+ B-lymphocytes so that the tumor cells can be color-identified within the R1 gate of the scatterplots. The R1 gate can then be readjusted so that mostly only colored neoplastic cells are within it. Class II MHC expression was measured only on the cells within the final R1 gate. The top row boxes depict a B-cell lymphoma patient with class II MHC low-expression, while the bottom row boxes depict a dog with class II MHC high-expression.

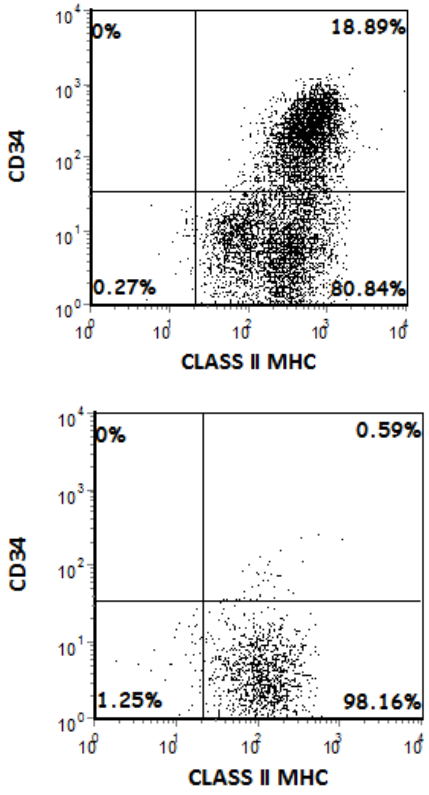

Figure 1.2 Sample flow cytometry scattergrams of test tube number 8 depicting a CD34+ lymphoma patient (top box, where ~19% of gated cells are CD34+) versus a CD34- lymphoma patient (bottom box). Quadrants are set on isotype controls.

Accuracy of mortality predictions for ALL treatment types

	Observed dead	Observed alive	
Predicted dead	7	2	9
Predicted alive	10	14	24
	17	16	Total 33 dogs*

Sensitivity = 7/17 = 41% (CI 19-67%)
Specificity = 14/16 = 88% (CI 60-98%)

Accuracy of first recurrence predictions for ALL treatment types

	Observed YES recur.	Observed NO recur.	
Predicted YES recur.	4	1	5
Predicted NO recur.	12	15	27
	16	16	Total 32 dogs*

Sensitivity = 4/16 = 25% (CI 8-53%)
Specificity = 15/16 = 94% (CI 68-100%)

Accuracy of mortality predictions for MULTI-AGENT treatment only

	Observed dead	Observed alive	
Predicted dead	2	1	3
Predicted alive	7	14	21
	9	15	Total 24 dogs*

Sensitivity = 2/9 = 22% (CI 4-60%)
Specificity = 14/15 = 93% (CI 66-100%)

Accuracy of first recurrence predictions for MULTI-AGENT treatment only

	Observed YES recur.	Observed NO recur.	
Predicted YES recur.	1	1	2
Predicted NO recur.	8	13	21
	9	14	Total 23 dogs*

Sensitivity = 1/9 = 11% (CI 1-49%)
Specificity = 13/14 = 93% (CI 64-100%)

Accuracy of mortality predictions for SINGLE-AGENT and PRED-ONLY treatment

	Observed dead	Observed alive	
Predicted dead	5	1	6
Predicted alive	3	0	3
	8	1	Total 9 dogs*

Sensitivity = 5/8 = 63% (CI 26-90%)
Specificity = 0% (CI 0-95%)

Accuracy of first recurrence predictions for SINGLE-AGENT and PRED-ONLY treatment

	Observed YES recur.	Observed NO recur.	
Predicted YES recur.	3	0	3
Predicted NO recur.	4	2	6
	7	2	Total 9 dogs*

Sensitivity = 3/7 = 43% (CI 12-80%)
Specificity = 2/2 = 100% (CI 20-100%)

* Only dogs with uncensored ST and FRT were used in sensitivity and specificity calculations.

Figure 1.3 Sensitivity and specificity calculations for patient outcome predictions of the validation group. Predictions using the ST model (probability of mortality) and FRT model (probability first recurrence) were compared to observed patient outcomes at 180 days post-onset of treatment. Only dogs with uncensored ST and FRT were used in the calculations. Top row boxes represent calculations for overall model predictions (all treatment types combined); middle row boxes for the multi-agent treatment cases; and bottom row boxes for single-agent and prednisone-only treated cases together.

CHAPTER 2

AN IMMUNOPHENOTYPIC AND MORPHOLOGIC ASSESSMENT OF CANINE CD34+ ACUTE LEUKEMIA

ABSTRACT

Background: CD34 antigen is a hematopoietic stem cell marker for acute leukemia, although not all acute leukemias express it.

Hypothesis: Some canine CD34+ leukemias will not be categorized as an acute leukemia solely based on traditional morphologic criteria of their neoplastic cells in peripheral blood. CD34+ acute leukemias will be qualitatively different from CD34-negative acute leukemias in terms of cellular morphology, patient signalment, and more severe in their degree of presenting cytopenias and survival.

Methods: Using flow cytometry and CBC data of peripheral blood, 11 dogs with CD34+ acute leukemia were retrospectively compared to 11 CD34- acute leukemia dogs.

Results: The CD34+ dogs were classified as follows: 8 had acute undifferentiated leukemia (AUL), 2 had undefined acute lymphoblastic leukemia (CD3-CD5+ undefined ALL) and 1 had acute myeloid leukemia (AML). CD34- dogs were classified as follows: 1 AUL, 1 CD3-CD5+ undefined ALL, 5 T-cell ALL, and 4 B-cell ALL. Most dogs were affected with single or bicytopenia, with both groups having a similar frequency of anemia and thrombocytopenia. CD34+ dogs had significantly more severe anemia and were significantly younger at time of diagnosis. The groups did not differ in total white blood count, blast cell count, flow cytometry cell size, or survival time. CD34+ and CD34- leukemic cells subjectively did not differ greatly in their morphologic appearances.

Conclusions and Clinical Importance: Flow cytometry of peripheral blood enhances the traditional morphologic-based classification of acute leukemias, particularly when CD34 antigen is expressed. Some acute leukemias may not be accurately classified as such when only a morphologic evaluation of peripheral blood is performed.

INTRODUCTION

Leukemia is a progressive, malignant proliferation of hematopoietic cells, usually originating in the bone marrow and extending into peripheral blood circulation. Less often, it can arise in the spleen and infiltrate other organs in advanced disease. Broadly speaking, leukemia is classified by its cell of origin, being either myeloid or lymphoid, and further by its degree of cellular differentiation, termed acute or chronic. The acute versus chronic terminology correlates with the neoplasm's pathobiologic behavior.[1-3] Acute leukemia represents a malignancy of immature cells, yielding an aggressive and rapid disease progression that is often accompanied by single or multiple cytopenias, namely anemia, neutropenia, and thrombocytopenia.[3] The immature "blast" cells typically have a morphology characterized by large nuclei containing fine to coarse chromatin and visible nucleoli, indicating active cellular replication. In contrast, chronic leukemia is a proliferation of well-differentiated, mature cells and typically has an indolent nature. These cells can often be indistinguishable from their normal, non-neoplastic counterparts.

The traditional diagnosis and classification of leukemia has been based on a morphologic account of the neoplastic cells in the blood and bone marrow, whereby acute leukemia has a required visualization of >20% blast cells (or >30% historically) in the bone marrow and/or >20% blast cells in circulation.[4-6] Occasionally, some cases of acute leukemia can have such poorly-differentiated cells, either with or without readily apparent nucleoli or completely lacking lineage-specific characteristics, that a pathologist cannot accurately classify their "acute" status or distinguish their myeloid versus lymphoid origin. Even with past use of cytochemical stains, the morphologic-based

classification scheme for leukemia has been challenged by aberrant cellular morphology and a relative lack of cytochemical stain specificity, such that misclassifications have occurred.[7-9] Fortunately, the advent of immunophenotyping via flow cytometry has helped resolve many of the classification challenges and has fast become the gold standard technique for leukemia diagnosis, in part because of its ease of application and high specificity and accuracy.[10,11] For example, the immunologic cell-surface markers CD3, CD21, and CD14, to name only three, are used in standard hematologic flow cytometry panels to accurately identify T-lymphocyte, B-lymphocyte, and myeloid (monocytic) lineages, respectively. Similarly, CD34 antigen is a hematopoietic stem cell marker that is used to support a diagnosis of acute leukemia, although not all acute leukemias may express it.[6,12,13] Flow cytometry and positive CD34 expression can be particularly helpful if a given case of either acute myeloid leukemia (AML) or acute lymphoblastic leukemia (ALL) does not exhibit typical morphologic cellular criteria via light microscopy. In our Clinical Pathology and Clinical Immunology Services at Colorado State University (CSU), we have observed several such cases where morphologic criteria have been insufficient, thus requiring immunophenotyping to more accurately classify an acute leukemia.

In this pilot study, we have focused our attention on canine acute leukemia of both myeloid and lymphoid origin, and we try to bridge the gap between flow cytometric immunophenotyping and the traditional morphologic classification of the leukemic cells present in blood. Because a canine-specific monoclonal antibody for CD34 is only available for a flow cytometric application, it is difficult to definitively evaluate the morphology of CD34+ neoplastic cells. To the authors' knowledge, there are no

published direct accounts that describe the morphology of canine CD34+ leukemic cells. Hence, one goal of this study is to do so, by using the combined efforts of flow cytometry data and blood film evaluation. Also, we have examined a population of client-owned dogs that have been diagnosed with CD34+ acute leukemia via flow cytometry and have compared them to CD34-negative patients that had their acute leukemia classified via more traditional criteria, specifically by cell morphology and >20% blasts in blood circulation. In this comparison, our goal is to show that flow cytometry enhances the traditional classification of acute leukemias and further identifies CD34+ leukemias as a distinct subset, not just in terms of CD34 expression but in their overall cell morphology, clinical presentation, and patient outcome. We suspect that leukemias with CD34+ expression have the most aggressive clinical behavior and poor outcome of all the documented acute leukemic proliferations. Our hypotheses are that (1) some CD34+ leukemias will not be categorized as acute leukemias solely based on traditional morphologic-based criteria; and that (2) CD34+ acute leukemias will be qualitatively different from CD34-negative acute leukemias in terms of cellular morphology, patient signalment, and more severe in their degree of presenting cytopenias and survival.

METHODS AND MATERIALS

Study Subjects

In order to be eligible for this retrospective study, dogs must have been examined at CSU Veterinary Teaching Hospital during 2004-2009 and diagnosed with acute leukemia, as determined by results of their initial CBC and flow cytometric analysis, which were performed on whole blood in EDTA (ethylenediamine tetraacetic acid). A

search through the Clinical Immunology Service's flow cytometry database identified suitable subjects. Dogs were enrolled in Group 1, the CD34+ acute leukemias, if they had >1% of neoplastic cells expressing CD34 on flow cytometry and visible blast cells on blood film evaluation. If dogs had negative flow cytometric staining for CD34 and >20% blast cells on blood film evaluation, then they were placed in Group 2, the CD34- acute leukemias. Blast cells were defined as medium to large cells whose diameter was equal to/or larger than a neutrophil diameter and contained either visible nucleoli or displayed a clear morphologic similarity with blast cells in every way except lacking visible nucleoli. Collectively, this blast cell population was called the "immature cells" to distinguish it from the subset of nucleoli-containing blast cells. Medical records were used to determine a patient's treatment and survival time, calculated in days from time of diagnosis (initial flow cytometry analysis) to the time of death or euthanasia. Dogs lost to follow-up were censored in survival analysis.

Hematologic Parameters and Morphology

Blood samples were processed using an ADVIA 120 hematology analyzer[a] to obtain the total white blood cell count (WBC), preliminary leukocyte differential, hematocrit (HCT), and platelet count. A manual packed cell volume (PCV) verified the analyzer HCT findings. Blood films were stained with Wright-Giemsa and a manual differential performed. For purposes of this study, blood film slides were retrieved so that a single clinical pathologist (MDW) could perform a 200-cell leukocyte differential (including percentages for the immature cells and nucleoli-containing subset of blast cells) on all cases, as available. Platelet number and/or presence of platelet clumps were also assessed. Bands were recorded when present, but not used in analysis for this study,

as they did not alter any patient's severity grade for neutropenia. The presence of cytopenias was based on reference limits established at CSU and categorized according to severity using a grading system for PCV, neutrophilia, neutropenia, and thrombocytopenia (**Table 2.1**).[5,14-16] Dogs with significant platelet clumping but a measured count above 100,000/µl were considered to have adequate platelet numbers that were likely within the reference interval. Dogs with significant platelet clumping but a measured count below 100,000/µl were excluded from platelet interpretation.

For each case available, the morphologic assessment of the immature cell population and all cell types were performed by two clinical pathologists (MDW and PRA). The morphologic criteria used to describe the immature cells included: cell size (cell diameter compared against a neutrophil cell diameter - an internal-control); nuclear shape; the density of chromatin; the number, size and appearance of nucleoli; the volume and color or cytoplasm; and the presence of cytoplasmic granules and/or vacuoles.

Flow Cytometry and Leukemia Classification

Leukemia immunophenotype and classification were based upon the patients' flow cytometry analysis, using a panel that consisted of antibodies against: anti-CD3 (clone CA17.2A12)[b], CD4 (clone YKIX302.9)[b], CD5 (YKIX322.3)[b], CD8 (YCATE55.9)[b], CD14 (UCHM1)[b], CD18 (clone YFC118.3)[b], CD21 (clone CA2.1D6)[b], CD34 (clone 1H6)[c], CD45 (YKIX716.13)[b] and class II MHC (YKIX334.2)[b]. The methodology used was identical to that previously described by Williams et al.[17] Briefly stated, 400µl whole blood was placed in a vial where red blood cells were lysed in two rounds, leukocytes resuspended in 200µl phosphate-buffered saline (PBS)-2% fetal bovine serum (FBS), then divided amongst 8 wells of a 96-well plate. The cell aliquots

were incubated 15 minutes with the conjugated antibodies, according to protocol previously described[17], then washed twice, resuspended and analyzed immediately. All samples were analyzed within 48 hours of collection. Cytometric analysis was performed using a Coulter XL flow cytometer[d] and data analysis carried out with FCS Express.[e] As a guideline to determine the percentage of cells stained with each antibody, gates were set based on the isotype controls (supplied by the same manufacturer) such that <2% of cells were positive (CD34+). Relative cell size of the gated neoplastic cell populations was determined by measuring geometric mean linear forward scatter (FS) and in part, by comparing it with an internal control, the forward scatter of the CD4+CD14+ neutrophils and our laboratory's established normal blood lymphocyte geometric mean cell size.

The acute leukemias were classified as myeloid in origin if the neoplastic cell population had negative staining for lymphoid antigens CD3, CD5, CD8, CD21, and positive staining for CD14, CD18 and +/- CD4 (only if no reaction with other lymphoid markers). Acute lymphoblastic leukemias had to be negative for CD14, and were classified as B-cell in origin if positive for CD21 +/- CD5 and T-cell in origin if positive for any combination of CD3, CD4, CD5, and CD8. Distinction between ALL and Stage V (leukemic phase) lymphoma could not definitively be made unless clinical history revealed that lymphadenopathy and/or hepatosplenomegaly had preceded observable blast cells in blood circulation. In cases where flow cytometry analysis could not accurately determine cell origin, a diagnosis of AUL, acute undifferentiated leukemia or undefined CD3-CD5+ ALL was made. All diagnoses were reviewed and confirmed by the authors (MDW and ACA).

Data Analysis

Statistical analysis was performed using GraphPad Prism 5.0.[f] Mann-Whitney tests were used to compare the two groups in their patient age, gender, neoplastic cell size (geometric mean linear forward scatter), PCV, WBC, immature cell percentage, nucleoli-blast cell percentage, neutrophil count, and platelet count. Archived blood films were required to calculate the percentages of immature and nucleoli-blast cells. A log-rank test and Kaplan-Meier survival curves compared patient survival between groups. Statistical significance was set at $P<0.05$ for all tests.

RESULTS

Twenty-two dogs met the inclusion criteria for the study. Eleven dogs were diagnosed with CD34+ acute leukemia (Group 1) based on their flow cytometry results, while an equal number of dogs were CD34- (Group 2) on flow cytometry and confirmed as having acute leukemia based on the presence of >20% blast cells in circulation. Using the combination of flow cytometry and cell morphology, all dogs were classified by immunophenotype into one of six categories (**Table 2.2**). Within Group 1, one dog was diagnosed with AML, subtype M4, based on CD4-CD14+ expression and morphology. Two dogs had undefined CD3-CD5+ ALL, while the remaining eight dogs were classified as AUL, showing only CD34+ expression. Within Group 2, only one dog was diagnosed with AUL, while the other ten had ALL as follows: four dogs with B-cell ALL, five dogs with T-cell ALL, and one dog with undefined CD3-CD5+ ALL.

Total white blood cell counts were not significantly different between Groups 1 and 2, and their WBC ranges were very wide. The WBC range for Group 1 was 5.1-376

x 10^3/µl (median 62.7 x 10^3/µl, interquartile range 16-192.5 x 10^3/µl), and the CD34+ neoplastic cells accounted for 1.4-92.7% (median 33.7%) of the WBC. Group 2 had a WBC range of 15-274.1 x 10^3/µl (median 75.6 x 10^3/µl, interquartile range 22.8-181.4 x 10^3/µl). On flow cytometry, the median geometric mean cell size of the neoplastic cell populations did not significantly differ between groups.

There were 6/11 CD34+ cases and 10/11 CD34- cases with archived blood films available for review, and with them, the percentage of immature cells and nucleoli-blast cells were assessed. For these sixteen cases, the morphology of the neoplastic cell populations is described later in the results section. The six CD34+ cases in Group 1 had an immature cell range of 32-77% (median 51.5%) and a nucleoli-blast cell range of 7-14% (median 11.5%). The ten CD34- cases in Group 2 had an immature cell range of 22-73% (median 43.5%) and a nucleoli-blast cell range of 7-45% (median 24.5%). There was no statistically significant difference between the groups' immature and nucleoli-blast cell percentages (P=0.55, **Figure 2.1**; P=0.07, **Figure 2.2**), nor were there any significant associations between the blast cell percentages and anemia, neutropenia, or thrombocytopenia.

The frequency and severity of all cytopenias were compared between Groups 1 and 2 (**Table 2.3** and **Table 2.4**) and graded according to the defined scale listed in Table 2.1. Seventeen dogs (77%) in the study were anemic: seven had grade 1 anemia (PCV 30-39%), nine had grade 2 anemia (PCV 20-29%), and one had grade 3 anemia (PCV <20%). While no statistical difference existed in the frequency of anemia, Group 1 had statistically more severe anemia than Group 2 (P=0.02, **Figure 2.3**). The CD34+ group

had a median PCV of 24% (interquartile range 23-28%) compared to the CD34- group median PCV of 36% (interquartile range 32-41%).

The mature neutrophil count was compared between groups, and neutrophilia was present in seven dogs; three in Group 1 and four in Group 2. Neutropenia only existed in three dogs, which were all in Group 1. The one AML dog of Group 1 was excluded from neutrophil count comparison. The neutrophil count range for Group 1 was 0.3-27 x 10^3/µl (median 4.2 x 10^3/µl), and Group 2 had a range of 3.3-29 x 10^3/µl (median 8.7 x 10^3/µl). There was no statistically significant difference between the frequency or severity of the neutrophilia/neutropenia seen in Groups 1 and 2, although a trend of neutropenia in the CD34+ group might be emerging (P=0.07, **Figure 2.4**).

Differences in thrombocytopenia frequency and severity were not statistically significant either (P=0.23, **Figure 2.5**). Overall, thrombocytopenia was present in 16/21 (76%) dogs, with Group 1 median of 84,000 plts/µl (interquartile range 42-111 x10^3plts/ µl) and group 2 median of 98,000 plts/µl (interquartile range 81-155 x10^3plts/ µl). One dog from Group 2 was excluded because it had significant platelet clumping with a platelet count <100,000/ µl. No dogs had platelet counts above the reference interval.

The clinical characteristics of the two groups were compared in regard to patient signalment, biochemistry data (as available), treatment and survival. No breed or gender differences existed; each group contained 7 males and 4 females, Group 1 had three Golden retrievers while Group 2 had one. Patient age at diagnosis, however, showed a statistically significant difference (P=0.04, **Figure 2.6**) with Group 1 CD34+ dogs diagnosed at a younger median age of 8.3 years (range 2.2-11.4 yrs; interquartile range 3.6-10.6 yrs) compared to 10.5 years (range 6.6-16.2 yrs; interquartile range 8.8-12.4 yrs) for Group 2.

Review of patient medical records showed no significant differences in biochemistry data, and no dog had cancer-associated hypercalcemia. Treatment regimes were variable and short-lived. Most acute leukemia patients had short survival times. There were 9/11 dogs in the CD34+ group and 10/11 dogs in the CD34- group for which date and cause of death/euthanasia were known. Group 1 survival time ranged 1-147 days (median 24 days) while Group 2 survival time spanned 0-303 days (median 57 days). No significant difference in patient survival was observed (P=0.42, **Figure 2.7**).

The morphologic assessment of both CD34+ and CD34- acute leukemias was performed using the available blood films from 6/11 cases and 10/11 cases, respectively. The immature cell count of the six CD34+ cases ranged 32-77% of the 200-cell differential, and it is presumed that the CD34+ cells were within that proportion. A ratio of the CD34+ cell count (on flow cytometry) to immature cell count (on light microscopy) was calculated to help provide a relative likelihood that a viewed immature cell on blood film evaluation would correspond to a CD34+ neoplastic cell on flow cytometry (**Table 2.5).** The immature cell populations observed in Group 2 blood films accounted for 22-73% of the 200-cell differential.

The morphology of Group 1's immature cells is summarized below and depicted in **Figures 2.8-2.10**:

- **Cell size**: medium to large
  - 4 cases had majority immature cells that were larger than a neutrophil; 2 cases equal to/or larger than a neutrophil.
- **Nuclear features**: round nuclei with fine to coarse chromatin density
  - Majority of immature cells had round nuclei; rare cells had some membrane clefts or cerebriform shapes.
  - The chromatin density was fine in 5 cases and coarse in 1 case.
- **Nucleoli**: 1-2 small, faint nucleoli in 13-32% of immature cells

- **Cytoplasmic features**: minimal to moderate quantity, variable color, rare contents
    - 4 cases had dark basophilic cytoplasm; 2 cases had lightly basophilic cytoplasm.
    - 5 cases had minority of immature cells containing punctate clear vacuoles; 1 case contained many vacuoles.
    - Within the immature cell populations, azurophilic granules were absent in 2 cases, few in 2 cases, and rare in 2 cases.

The morphology of Group 2's immature cells was similar to that of Group 1 but more variable in their nuclear features and in their proportion of normal-appearing small lymphocytes (**Figures 2.11-2.13**):

- **Cell size**: variable
    - 4 cases had majority immature cells that were larger than a neutrophil; 3 cases equal to/or larger than a neutrophil; 1 case with an equal mix of small, normal-appearing lymphocytes and immature cells larger than a neutrophil; and 2 cases with a majority of small, normal-appearing lymphocytes but still >20% immature cells larger than a neutrophil.
- **Nuclear features**: round nuclei with variable membrane clefts and chromatin densities
    - 9 cases had majority immature cells with round nuclei and few to many cells with membrane clefts; 1 case (CD8+ T-cell leukemia) had predominantly cerebriform shaped nuclei.
    - Half of the cases had fine chromatin density, while the other half had predominantly coarse chromatin.
- **Nucleoli**: variable presence and number, but mostly small and faint. Higher proportion of immature cells with visible nucleoli compared to Group 1.
- **Cytoplasmic features**: usually moderate quantity, light to dark basophilic and contain variable contents
    - 7 cases had moderate cytoplasm, 1 case minimal, and 2 cases (CD8+ ALL) having moderate cytoplasm with pseudopodia or projections.
    - 7 cases had light colored and 3 cases had dark colored basophilic cytoplasm.
    - 7 cases had majority immature cells with no cytoplasmic contents and only some cells containing clear punctate vacuoles and/or azurophilic granules; 3 cases had azurophilic granules in the majority of their immature cells (2 T-cell ALL and 1 AUL).

DISCUSSION

In this study, we used flow cytometry to identify CD34+ acute leukemias and used the data to help describe and compare their morphologic and clinical characteristics. The majority of Group 1 acute leukemias did not express CD molecules beyond CD34 and had poorly differentiated neoplastic cells that were classified either as AUL (73%), undefined CD3-CD5+ ALL (18%), or a single case of AML (9%). In contrast, 82% of Group 2's CD34-negative cases showed differentiation along a lymphoid lineage, with near equal representation of B and T-cell origin, all of which required a morphologic account of >20% immature blast cells in circulation to be deemed an acute leukemia. It is interesting that we did not observe many cases of AML in this small study, either CD34 positive or negative, given that the reported prevalence within canine acute leukemia is around 50%.[5,8] Some possible contributing factors include differences in geographic disease distribution, but also that our flow cytometry antibody panel at CSU does not include many myeloid-specific markers. There is a paucity of readily available myeloid markers for use in the dog, and while many myeloid leukemias are positive for CD14, CD18 +/- CD4, immature myeloblast cells may not always express those antigens. Thus, it is possible that some of the CD34+ acute undifferentiated leukemias were myeloid in origin but we were unable to identify it.

The purpose of morphologically evaluating CD34+ leukemias was not only to identify any potentially distinct features in their cell appearance but to see if "acute" leukemia classification would have been achieved without the use of flow cytometry. If traditional criteria were used alone, would the neoplastic CD34+ cell population contain sufficient (>20%) blast cell features? Using our working definition of blast cell

morphology, the immature cells of Group 1, many of which were attributed to a CD34+ phenotype (Table 2.5), accounted for 32-77% (median 51.5%) of the 200-cell differential, enough to qualify all six cases for acute leukemia classification. Their actual morphology, however, was quite variable. The immature cell populations consisted of medium to large sized cells with fine to coarse nuclear chromatin, variably present nucleoli and a moderate amount of basophilic cytoplasm. In contrast, when the same cells were evaluated under a stricter nucleoli-containing blast cell requirement, none of the six CD34+ cases made the >20% blast cell cutoff to be morphologically designated as an acute leukemia. The nucleoli-blast count of the six CD34+ cases ranged 7-14% (median 11.5%) of the 200-cell differential. Similarly, half of the ten evaluated CD34- cases would not have been designated an acute leukemia with the more stringent nucleoli-containing blast requirement (Figure 2.2).

Using the microscope, we categorized the large immature cells into a single population of blast cells, even while some contained a nucleolus and others did not. Must a nucleolus be discernable to be considered a blast cell? We found it logical to define the blast cell population as being immature cells along a continuum rather than a fixed morphological point within the active proliferating pool. Currently, there is no consensus on the definition for a blast cell. While it is well-accepted that a nucleolus-containing cell represents an immature proliferating cell, there is no clear morphologic cutoff for when an immature cell is no longer part of the proliferating population. Hence, we considered the blast cell population to represent the active proliferative pool, with and without conspicuous nucleoli. It is on this basis that we defined and justified our blast cell definition for use in this study.

There is even no wide consensus for the optimal cutoff percentage needed to classify a blast cell population as being positive for CD34 expression on flow cytometry. Some researchers/diagnosticians have used a 5, 10, 20 or even 30% cutoff point for bone marrow evaluation.[5,18-20] The CD34 expression level in normal, healthy canines and humans is around 1% of bone marrow cells and 0.01-0.1% of (human) blood.[9,18] We used a cutoff of >1% of circulating neoplastic cells having CD34 expression to be deemed a CD34+ leukemia (i.e. >10x the expected normal for CD34+ cells in blood). The bottom line is that CD34 expression, when present, is helpful in confirming an acute leukemia.

Most dogs in this study were affected by single or multiple cytopenias (20/22 dogs, 91%) with almost half having bicytopenia. Cytopenias often accompany acute leukemia, and the incidence found here was similar to previous reports.[5,21] While there was no statistically significant difference in the frequency of cytopenias between groups, we noticed that only the CD34+ category showed evidence of neutropenia (Table 2.3). Also, CD34+ cases tended to have more bi- and tricytopenias (9/11) compared to the more common single cytopenias of CD34- cases, where only 4/11 had bicytopenia and none had pancytopenia (Table 2.4). It will be interesting to observe whether or not this difference trend in cytopenia frequency persists as we add future cases to our study.

For cytopenias, only the severity of anemia showed a significant difference between groups, with CD34+ cases having more severe anemia at a median PCV of 24% compared to 36% (Figure 2.3). It is difficult to identify the cause of anemia or any other cytopenia, as the etiology is likely multifactorial. The pathogenesis may include myelophthisis, secondary immune-mediated hemolysis, anemia of chronic disease, and

hemorrhage that is secondary to platelet deficiency, dysfunction, or disseminated intravascular coagulation.[22] The production of inhibitory factors is also likely to contribute to a cancer-associated cytopenias.[3,22]

Patient age at diagnosis was the second variable to show a significant difference between groups. CD34+ dogs were diagnosed with leukemia at an earlier age (median 8.3 years) compared to the CD34- dogs (median 10.5 years), but like anemia, the findings are preliminary and warrant further investigation.

There were some limitations to our pilot study with the weakest element being the small number of patients. We compared eleven CD34+ dogs to eleven CD34- dogs, and only sixteen in which blood films were available for review. It would have been ideal for every dog to not only have had available blood films but bone marrow aspirates performed as well. Bone marrow evaluation might have helped assess disease manifestation and response to cytopenias, however, its absence highlights the fact that bone marrow aspirates are not always necessary to confirm a leukemia diagnosis. All of the cases in our study had their acute leukemia diagnosed and confirmed simply with peripheral blood, using flow cytometric immunophenotyping and traditional morphologic criteria. It is important to recognize, however, that some acute leukemias may fail to be accurately classified as such when only a morphologic evaluation of peripheral blood is used. As veterinary medicine becomes more sophisticated in its diagnostic workup of animals, even more advanced tests, such as gene expression profiling and microarray analysis, will stand by the side of old favorites like the CBC and light microscope.

FOOTNOTES

[a] Siemens Healthcare Diagnostics, Tarrytown, NY
[b] Serotec Inc, Raleigh, NC
[c] B-D Biosciences, San Jose, CA
[d] Beckman Coulter Inc, Fullerton, CA
[e] De Novo Software, Ontario, Canada
[f] GraphPad, La Jolla, CA

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TABLES AND FIGURES

Table 2.1 Grading scale for cytopenias

Blood Cell Abnormality	Grade 1 (mild)	Grade 2 (moderate)	Grade 3 (marked)	Reference Interval
Anemia PCV (%)	30-39	20-29	<20	40-55
Neutrophilia neutrophil count ($x10^3/\mu l$)	11.1-19.0	20.0-49.9	≥50.0	2.6-11.0
Neutropenia neutrophil count ($x10^3/\mu l$)	2.0-2.6	1.0-1.9	<1.0	2.6-11.0
Thrombocytopenia platelet count ($x10^3/\mu l$)	101-199	30-100	<30	200-500

Table 2.2 Immunophenotype of leukemias

Leukemia Classification	Number of Dogs	
	Group 1, CD34+	Group 2, CD34-
AML (likely M4)	1	
ALL, CD21+ B-cell		4
ALL, CD4+ T-cell		1
ALL, CD8+ T-cell		3
ALL, CD3+CD5+ T-cell		1
ALL, CD3-CD5+ undefined	2	1
AUL, acute undifferentiated leukemia	8	1
Total, n=22	11	11

Table 2.3 Summary of cytopenias

Type of Cytopenia	Within Ref. Interval		Grade 1 (mild)		Grade 2 (moderate)		Grade 3 (marked)		TOTAL # Dogs with Cytopenias	
	CD34+	CD34-	CD34+	CD34-	CD34+	CD34-	CD34+	CD34-	Group 1 CD34+	Group 2 CD34-
Anemia	2	3	0	7	8	1	1	0	9/11	8/11
Neutrophilia	4	7	1	2	2	2	1*	0	3/10*	4/11
Neutropenia			1	0	1	0	1	0	3/10*	0
Thrombo-cytopenia	1	4	3	0	5	6	2	0	10/11	6/10

* = Single case of AML excluded from neutrophil count analysis

Table 2.4 Frequency of single and multiple cytopenias

Number of Cytopenias	Group 1, CD34+	Group 2, CD34-
0	1	1
1	1	6
2	6	4
3	3	0
Total, n=22	10/11	10/11

Table 2.5 Group 1, CD34+ cases for morphologic assessment via blood film evaluation.

Patient Immunophenotype	WBC	% CD34+ Cells	% Immature Cells	Ratio
	(cells/µl)	(on flow cytometry)	(on light microscopy)	(CD34+ cells to immature cells)
ALL CD3-CD5+ Undefined	376,000	92.7	77	1.2
AUL	206,300	33.7	62	0.54
ALL CD3-CD5+ Undefined	144,300	39.1	66	0.59
AUL	16,000	35.0	40	0.88
AUL	13,500	31.7	41	0.77
AUL	5,100	20.4	32	0.64

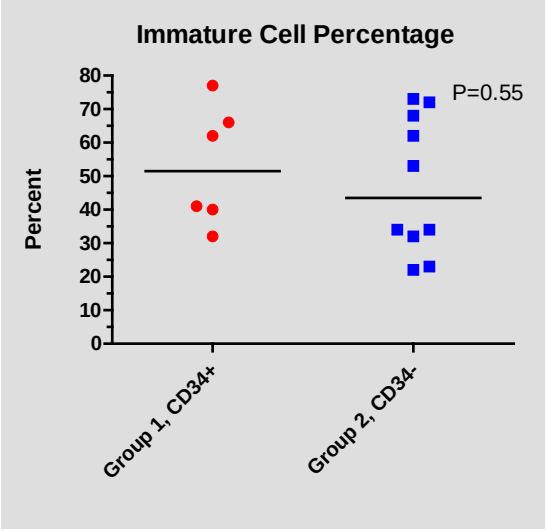

Figure 2.1 Comparison of immature cell percentage between groups.

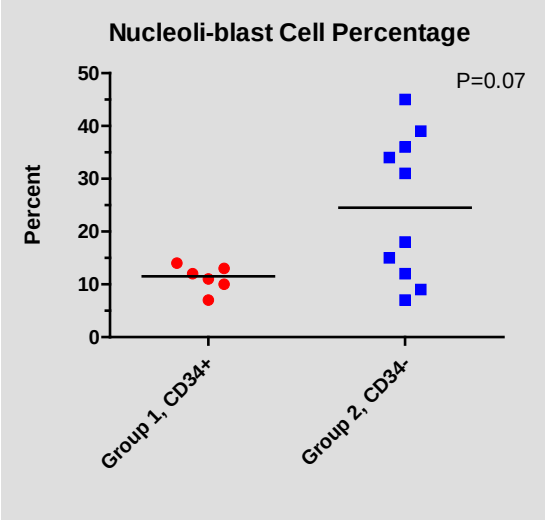

Figure 2.2 Comparison of nucleoli-blast cell percentage between groups.

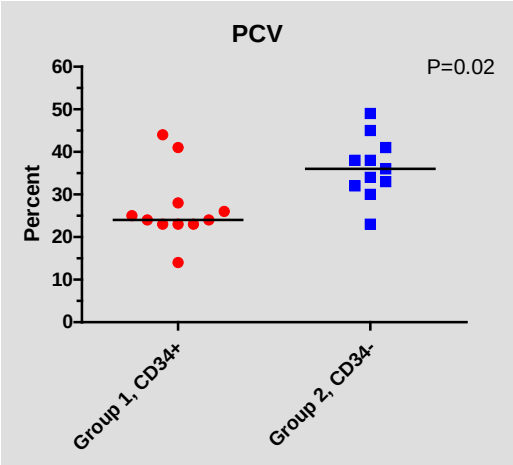

Figure 2.3 Comparison of anemia between groups.

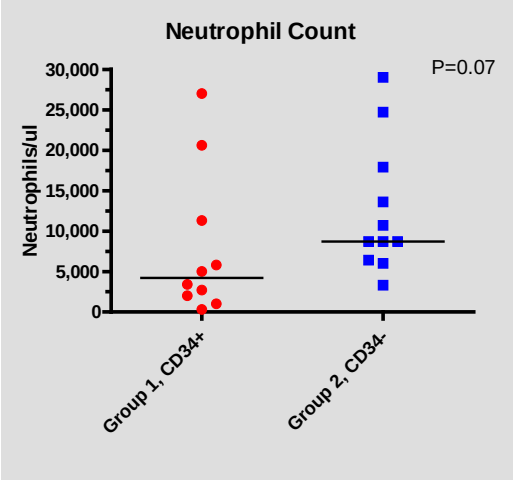

Figure 2.4 Comparison of neutrophil count between groups.

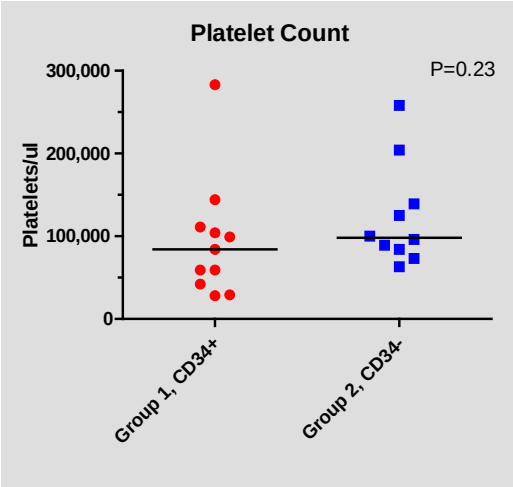

Figure 2.5 Comparison of platelet count between groups.

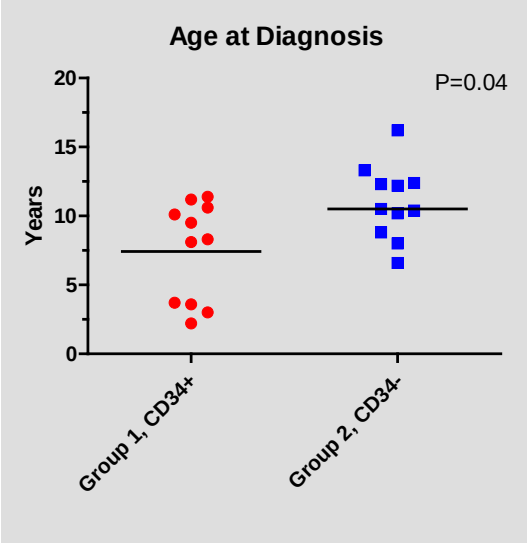

Figure 2.6 Comparison of age between groups.

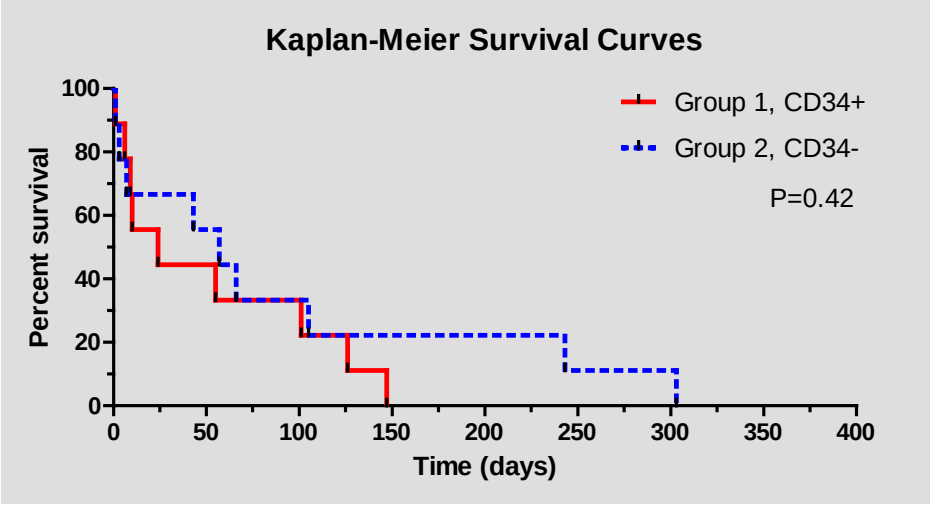

Figure 2.7 Survival analysis compared between groups.

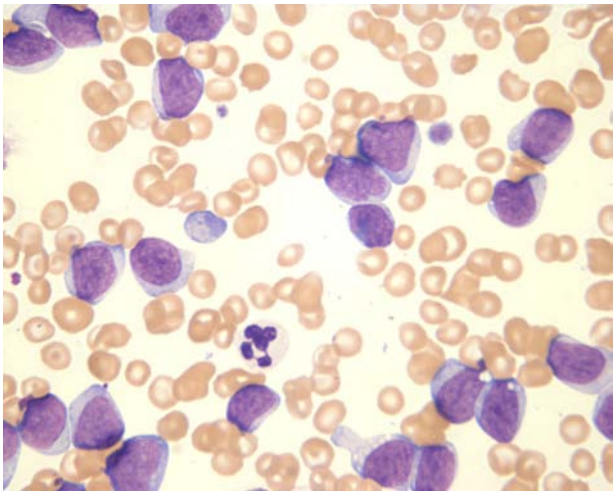

Figure 2.8 CD34+ ALL, CD3-CD5+ undefined

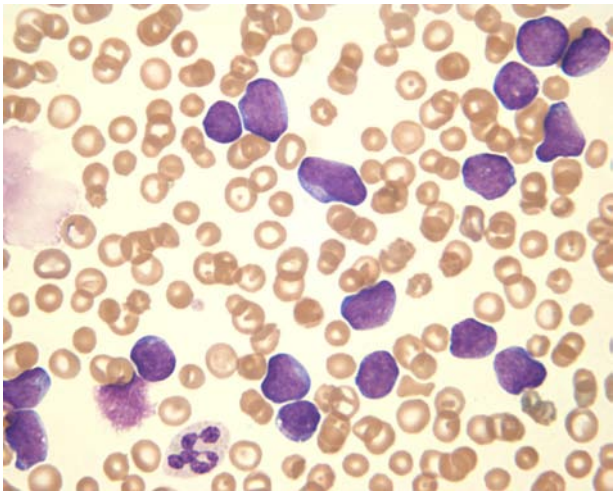

Figure 2.9 CD34+ ALL, CD3-CD5+ undefined

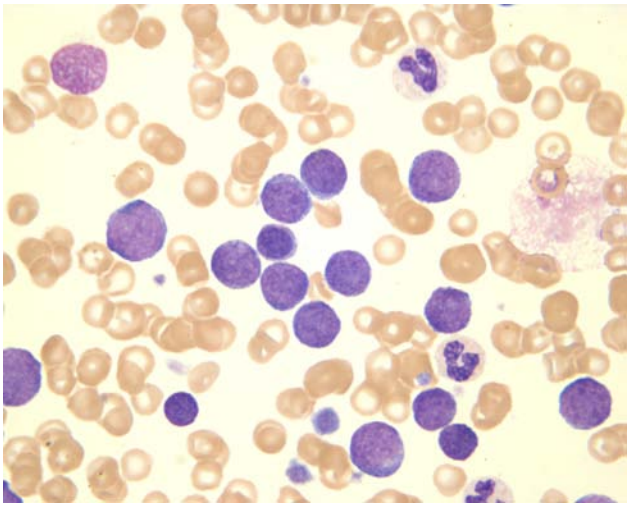

Figure 2.10 CD34+ AUL

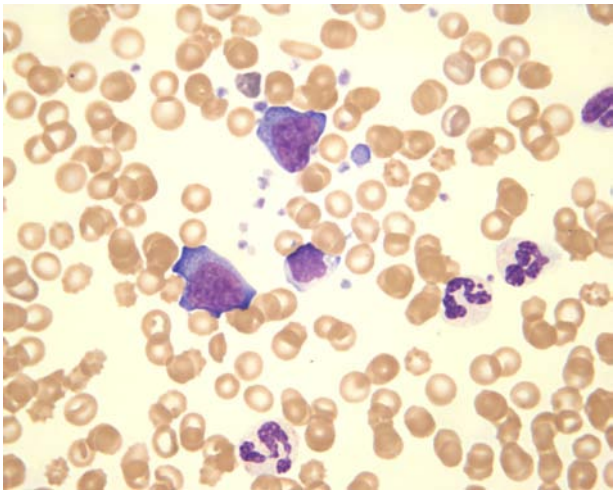

Figure 2.11 CD34- ALL, B-cell

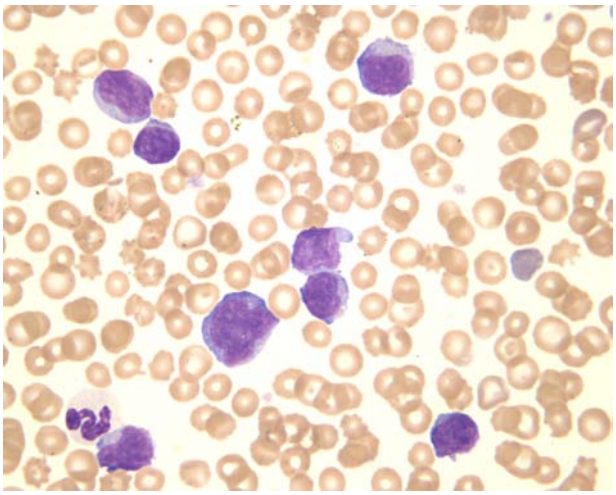

Figure 2.12 CD34- ALL, CD8+ T-cell

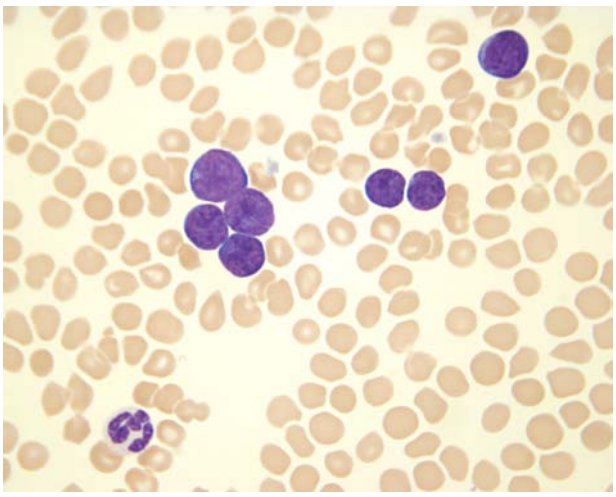

Figure 2.13 CD34- ALL, CD3-CD5+ undefined

CONCLUSION

The studies described in this thesis have shown the benefits and versatility of flow cytometry in the diagnosis, classification and prognosis of canine multicentric lymphoma and acute leukemia. Whether applying flow cytometry to lymph node fine-needle aspirates or to peripheral blood, the assay is minimally invasive, highly specific and offers a wealth of potential clinical information that can help identify the presence of cancer, describe its immunological characteristics, and provide insight as the how the body may respond to the cancer. In chapter one, new prognostic markers were identified in class II major histocompatibility complex and cell size of the neoplastic B-lymphocytes. CD34 expression was shown not to have any impact on lymphoma patient outcome, although the verdict is still out as for its prognostic role in canine acute leukemia. Chapter two's study characterized the morphology of CD34 leukemic cells and laid the ground work to determine if CD34 expression in canine acute leukemia warrants subclassification.

The work herein offers three points to help guide the future directions for canine lymphoma and leukemia research. The first point is more of a challenge to the commonplace retrospective study designs seen in canine lymphoma. We highlighted a relatively straightforward and interesting study design using a multivariable statistical model to analyze, predict and validate the significant associations in question. Although our study had biases upon which to improve, chapter one's multivariable analysis tested the strength and clinical utility of the found associations with patient outcome. We look forward to re-validating our lymphoma outcome prediction model with a prospective and independent set of patients.

The rapidly expanding field of tumor immunology is another point worthy of mention, as it is making the scientific community reevaluate the roles of the innate and adaptive immune responses. Whether it is exploring lowered class II MHC expression on tumor cells or features of tumor-infiltrating immune cells, the tumor microenvironment is ripe for investigation and novel drug development. Gaining a better understanding of how cancer cells interact with the immune system will most certainly lead to major breakthroughs in lymphoma/leukemia treatment in the near future.

Lastly, our work emphasizes the need to combine the use of morphology and immunophenotyping to more accurately classify lymphomas and leukemias. Even as newer technologies take hold, such as gene expression profiling and microarray analysis, it will be important to unite these assays and their classification schemes to yield more cohesive prognostic information.