NOVEMBER 2018

Reference point.

Flow cytometry

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Flow Cytometry at Marshfield Labs

Marshfield Labs is celebrating our first year of flow cytometry service. Veterinary flow cytometry was developed with the combined efforts of three veterinary clinical pathologists with both interest and experience in flow cytometry and the extensive experience of technologists with more than 40 years experience in human flow cytometry. Having this service available in Marshfield has improved our ability to accurately diagnose and immunophenotype hematopoietic malignancies and has markedly reduced the turn around time.

What is Flow and How Does It Work?

Flow cytometry involves the analysis of cells in a fluid suspension as they flow single file in a linear stream across an illuminated light path. Cells are incubated with fluorescently labeled antibodies prior to analysis. Exposure to several different lasers (see Fig. 1 below) provides information on cell size, internal and nuclear complexity, and fluorescent intensity of specific antibody labeling.

This example illustrates the general principles of flow cytometry. Cells (either blood or a tissue sample in a liquid medium) enter into the flow cytometer and pass single file past a series of lasers. A variety of data is obtained via the varied interactions of these cells and the laser light. The manner in which cells scatter the light as the cell passes the laser provides information on both cell size (forward scatter – FSC) and internal/nuclear complexity (side scatter – SSC). Cells can also be incubated with various antibody combinations, each labeled with a distinct fluorescent marker. This example illustrates a T-helper cell (Th), which is binding antibodies directed against both CD3 and CD4. The CD3 antibody is labeled with a

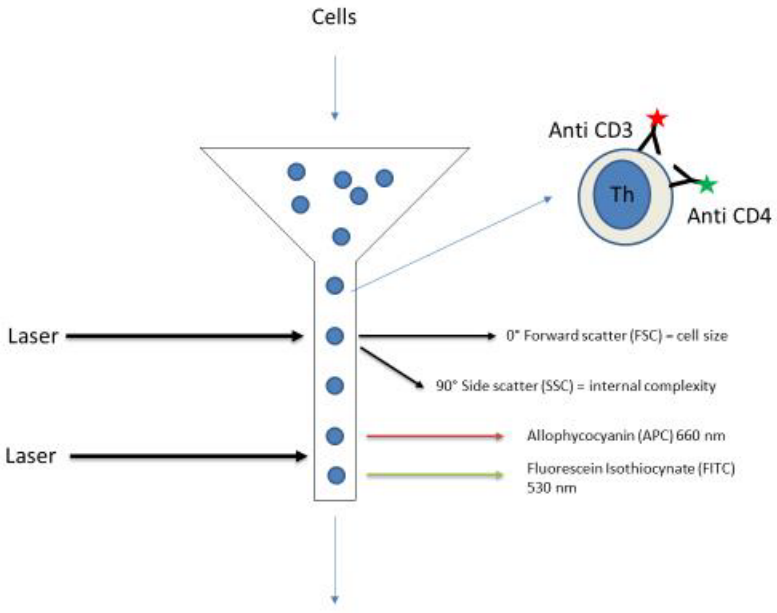

Fig. 1 – Example of how flow cytometry works.

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fluorescent molecule, allophycocyanin (APC), which when excited by a laser, will emit red light. The CD4 antibody is labeled with a green fluorescent molecule, fluorescein isothiocyanate (FITC). The flow cytometer detects the light scatter properties and any bound antibodies on each cell, and allows for the powerful analysis of cell populations via the aid of a computer.

In a single analysis, a flow cytometer will analyze the light scatter and fluorescent emittance properties (indicating the level of antibody binding and thus antigen expression of each cell) from thousands of individual cells. It sends this data to a computer, which enables the pathologist and technicians to interpret and analyze this data in a more organized manner. This data is commonly viewed in cytograms to make interpretation easier. Cytograms are a two dimensional graphical representation of individual cell properties. Two categories of data are viewable in each cytogram, presented on an X and Y axis. Each axis can represent any of the data types gathered and noted above. Figure 2 below illustrates a graph of cell size (forward scatter – FSC) on the X axis and internal/nuclear complexity (side scatter – SSC) on the vertical Y axis. This is related to how a laser based hematology analyzer works, and shows the overall distribution of cells in a sample by size and internal complexity.

This analysis allows us to more specifically characterize cell populations in such samples as peripheral blood, cavitary fluids, and aspirates from solid tissues to help determine whether a benign, mixed cell distribution is present or an atypical neoplastic cell population is present. It also helps to determine the phenotype of confirmed hematologic malignancies. While this is primarily used to diagnose and phenotype lymphoproliferative disorders, it's also useful if a possible myelomonocytic origin is suspected. Flow cytometry is also a very useful tool to aid in prognostication of diagnosed lymphoid malignancies.

Figure 2 is a cytogram showing the distribution of normal canine leukocytes in a blood sample, organized by forward (FSC – indicating cell size) and side scatter (SSC – representing internal/nuclear complexity). Neutrophils comprise the majority of cells present. They are intermediate in size, but consistent with their multi-lobulated nuclear structure, have the greatest amount of nuclear complexity, or side scatter. Small mature lymphocytes are the next numerous cell type in this sample. Their small size and simple, round nucleus is represented by their position near the bottom left of this plot (small amounts of forward and side light scatter). Finally, monocytes (seen in green) are larger than both neutrophils and small lymphocytes. Their nuclear structure is intermediate in complexity (often indented/lobulated, but not as complex as that of a neutrophil). Figure 3 (next page) illustrates how antibody binding is represented and interpreted on

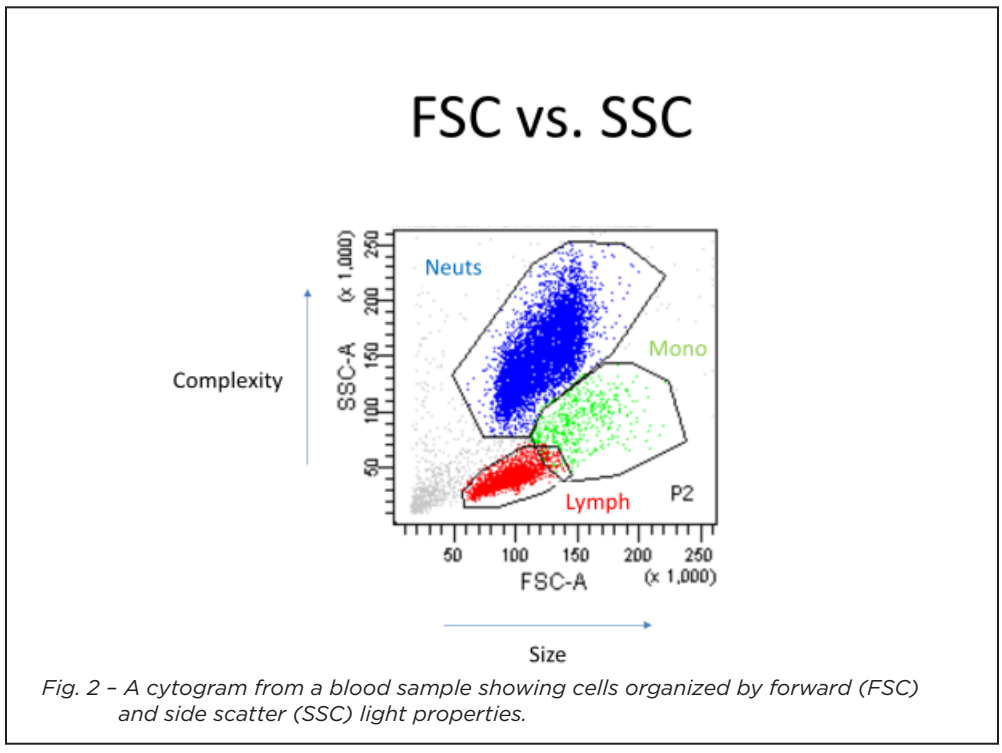

Fig. 2 – A cytogram from a blood sample showing cells organized by forward (FSC) and side scatter (SSC) light properties.

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a cytogram. Expression of antibody binding is read in a positive and negative manner. Cells appearing at the top and to the right of the midlines of the Y and X axes respectively represent positive antibody binding for a specific antibody. The opposite holds true for negative expression, appearing below and to the left of midline for each axis.

In this instance, instead of light scatter properties, cells are graphed on the X and Y axes according to whether they bound antibodies against CD4 (Th) and CD8 (Tc). Antibody binding by each cell is displayed on the X or Y axis by whether they bind an antibody or not. Cytograms commonly display this difference by referencing the middle of the axis in question. Below or to the left of midline equals no antibody binding for that axis, while cells that are above or to the right of midline are positively binding for an antibody. In Figure 3, cells in the upper left quadrant would be CD4+, CD8-, while those in the lower right quadrant would be CD4-, CD8+. Those in the upper right and lower left quadrants would be positive and negative for both respectively.

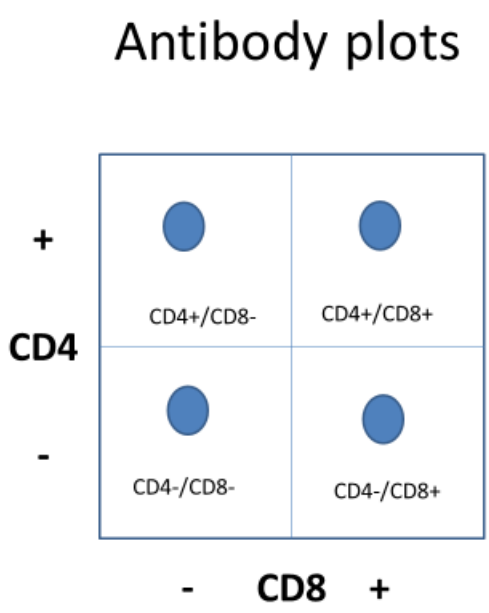

Fig. 3 – An example of how antibody binding can be shown on a cytogram to distinguish different cell populations.

When Should I Recommend It?

To enable the optimal utility of the sample and the data obtained, Marshfield Labs recommends using flow cytometry in the following instances:

- Solid tissues/cavitary fluids
  - Confirming and immunophenotyping large cell lymphomas
  - Distinguishing small and intermediate size lymphomas from atypical reactive states
- Peripheral blood
  - Immunophenotyping diagnosed leukemia
  - Confirming and immunophenotyping suspected leukemia
  - Distinguishing a persistent lymphocytosis from a reactive state

With all malignancies, flow cytometry may also provide prognostic information.

Limitations of Flow Cytometry

- Flow cytometry can't always distinguish inflammation/infection from neoplasia. An important example of this phenomenon is the expansion of CD8+ T-cells that can occur with *E. canis* infection.

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- Because flow cytometry involves the analysis of individual cells, samples with low cell concentrations of interest are more difficult to analyze. As a general rule, flow cytometry is recommended for samples in which the target cell population is greater than 5,000/uL.

- Samples for flow cytometry can be compromised by cell degradation as a result of sample aging and cell fragility.

- Samples that have 50% or less cell viability upon receipt in nearly all cases prevent accurate evaluation.

- Cell viability should be preserved if recommended guidelines are followed. Sample viability assays are performed before full analysis. If sample is nonviable, only a viability charge will be incurred.

Flow Cytometry Panels

Antibodies used in veterinary flow cytometry at Marshfield Labs:	
Dogs:	
T-cell	CD3, CD4, CD5, CD8
B-cell	CD21
Myelomonocytic	CD14, CD18
Others:	CD34, CD45, MHCII
Cats:	
T-cell	CD4, CD5, CD8
B-cell	CD21

Our pathologists keep up to date on the latest veterinary flow cytometry literature and research trends, and will update our panels with new diagnostic antibodies as they emerge.

Flow Cytometry and Prognosis

Flow cytometry can provide prognostic information in many patients.

Examples include:

- A more favorable prognosis with small to intermediate sized T-cells lacking the pan-leukocyte antigen CD45 in cases of T-zone lymphoma/leukemia (Seelig, Avery et. al., JVIM 2014: 28:878-886).

- Expression of high levels of MHCII has also been shown to be associated with a better prognosis in cases of B-cell lymphoma (Rao, Lana et. al., JVIM 2011: 25:1097-1105).

- In canine leukemias, a worse prognosis in CD8+ T-cell chronic lymphocytic leukemias has been observed with higher cell counts. In B-cell leukemias in the dog, a larger cell size has been associated with a worse prognosis (Williams, Avery et. al., JVIM 2008: 22:596-601).

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Other studies also exist describing the prognosis associated with varied antigen expression in cases of hematologic malignancies. In addition to a diagnosis, this information gives you and your clients more power and confidence in deciding a plan of treatment for their loved, valued pets.

Two Case Examples

1. Chronic lymphocytic leukemia (CLL) in a dog.

This case is from a 9 year old M/N mixed breed dog named "Max". He presented to his veterinarian for routine blood work for a dental exam and cleaning. A CBC revealed a severe leukocytosis characterized by a marked elevation in often somewhat atypical appearing small to intermediate sized lymphocytes (see Table 1 and Figure 4 below). He was clinically healthy, with no lymphadenopathy or organomegaly noted. No prior history of disease was noted and serologic testing for Heartworm/*Anaplasma/Ehrlichia canis*/Lyme was negative.

There is a severe leukocytosis with a population of atypical appearing, small to intermediate sized lymphocytes (classified as others in the differential count) having round to indented nuclei (see Fig. 4). Small lymphocytes were also increased in concentration as were monocytes and eosinophils. Platelet number was decreased, but they were aggregated in the smear and the estimated platelet count appeared normal.

Analyte	Value	RI
RBC	6.04 M/uL	5.39-8.70
Hgb	14.1 g/dL	13.4-20.7
HCT	45.8%	38.3-56.5
PLT	**137 K/uL (clumps noted)**	143-448
WBC	**212.7 K/uL ↑**	4.9-17.6
Neut	10,635 cells/uL	2,940-12,670
Lymphs	**6,381 cells/uL ↑**	1,060-4,950
Monos	**2,127 cells/uL ↑**	130-1,150
Eos	**2,127 cells/uL ↑**	70-1,490
Basos	0	0-100
Others/Unclassified	**191,430 cells/uL ↑**	

Table 1 - Table of blood data from "Max".

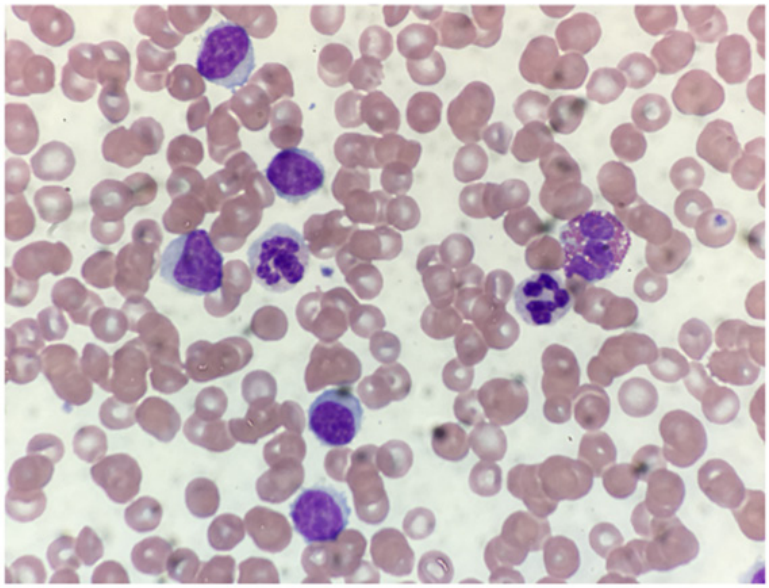

Fig. 4 – Blood smear from "Max".

This representative image from the smear shows five small to intermediate sized lymphocytes, along with two neutrophils and an eosinophil.

Flow cytometry was submitted on a subsequent blood sample from Max. Flow analysis (see Fig. 5 below) revealed a predominating population of small to intermediate

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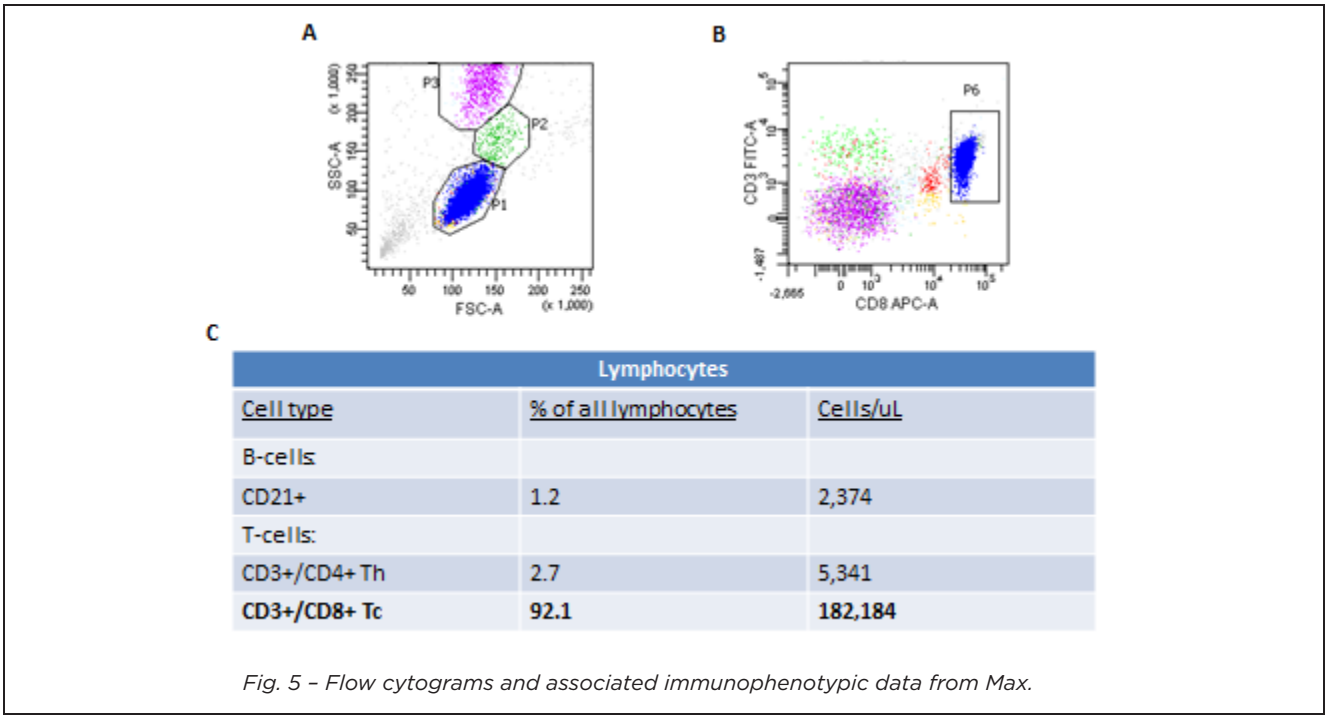
sized lymphocytes, consistent with that found on the blood smear and CBC. Nearly all were CD3/5/8+, consistent with a severe increase in CD8+ T-cells. Cells were CD45+ and CD34-. These findings were consistent with a case of T-cell lymphoproliferative disease. Given the lack of any noted lymphadenopathy/organomegaly and the negative *E. canis* serology, these findings were consistent with a case of CD8+ T-cell chronic lymphocytic leukemia. Based on prior studies, the median survival in this case was likely less favorable given the very high cell count on presentation (A study by Williams, Avery et. al., JVIM 2008: 22:596-601 demonstrated that CD8+ T-cell leukemias with greater than 30,000 lymphocytes at presentation had a median survival time of 130 days (max 350 days) and those with less than 30,000 lymphocytes had a median survival of 1,000 days (max 1,400)).



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Lymphocytes		
Cell type	% of all lymphocytes	Cells/uL
B-cells:		
CD21+	1.2	2,374
T-cells:		
CD3+/CD4+ Th	2.7	5,341
CD3+/CD8+ Tc	**92.1**	**182,184**

Fig. 5 – Flow cytograms and associated immunophenotypic data from Max.

(A) This cytogram displays nuclear complexity on the Y axis (SSC – side scatter) and cell size on the X axis (FSC – forward scatter). The most abundant cell type in this blood sample is a population of small to intermediate sized lymphocytes, labeled as box P1 (colored blue). Neutrophils and monocytes are found in boxes P3 (magenta) and P2 (green) respectively. (B) This cytogram shows the intensity of cells staining for CD3 expression on the Y axis, and CD8 on the X axis. As seen, nearly all the lymphocytes (box P1, colored blue) in (A) are CD3+/CD8+. (C) This table denotes the specific proportions and concentrations of specific lymphocyte subsets.

2. Nodal large B-cell lymphoma.

This case is from a 10 year old F/S Corgi named "Sweetie". She presented with a 1 week history of peripheral lymphadenopathy. Weakness and vomiting were noted. CBC revealed a left shift with a mild anemia. A reticulocyte count was not ordered, but the anemia was presumed to be either non-regenerative or very mildly regenerative in nature. Circulating atypical lymphocytes were not noted. Hypoproteinemia was found. No identifiable organomegaly was found. FNA of a lymph node revealed a homogenous expansion of large immature lymphocytes, consistent with lymphoma (see Fig. 6 next page).

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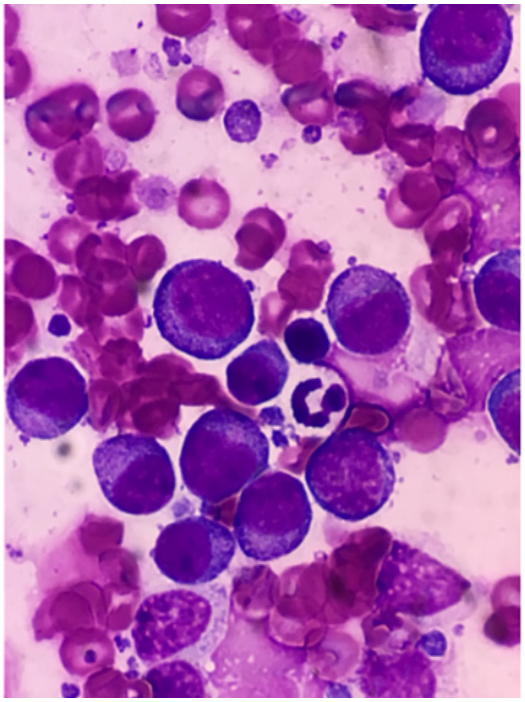
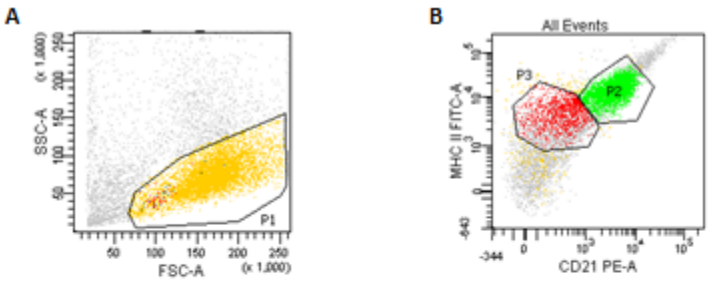


Fig. 6 – Lymph Node FNA from an 11 year old F/S dog, "Sweetie".

Figure 6 shows a Wright Giemsa stain, 1000x magnification. Slides submitted were of excellent quality and high cellularity. While small mature lymphocytes were found, nearly all of the nucleated cells present consisted of a population of large immature lymphocytes. They possessed large round nuclei, immature open chromatin patterns, and had multiple prominent nucleoli. Mitotic activity was increased, and scattered tingible body macrophages were seen. Plasma cells were not noted, and the blood background contained small numbers of ruptured cells and scattered lymphoglandular bodies. The diagnosis was consistent with lymphoma.

A lymph node aspirate was submitted for subsequent flow cytometry analysis to determine immunophenotype. Flow results are illustrated in Figure 7 below. Flow cytometry indicated a homogenous expansion of large CD21+ lymphocytes, consistent with B-cell lymphoma. These lymphocytes expressed high levels of MHCII, which in studies has been shown to have a more favorable prognosis, with a median survival time to approximately 300 days with treatment (Rao, Lana et. al., JVIM 2011: 25:1097-1105).



C

Lymphocytes	
Cell type	% of all lymphocytes
B-cells:	
CD21+	**73.7**
MHCII+	**100**
T-cells:	
CD3+/CD4+ Th	0.4
CD3+/CD8+ Tc	0.2

Fig. 7 – Flow cytograms and associated immunophenotypic data from "Sweetie".

(A) This cytogram displays nuclear complexity on the Y axis (SSC – side scatter) and cell size on the X axis (FSC – forward scatter). Consistent with the cytology, this node contains a homogenous population of large sized lymphocytes, labeled as box P1 (colored yellow). The dots colored grey represent ruptured cell material and other objects such as platelets. (B) This cytogram shows the intensity of cells staining for MHCII expression on the Y axis,

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and CD21 on the X axis. As seen, nearly all the lymphocytes (box P2, colored green) are CD21+, and express high levels of MHCII. Very few T-lymphocytes (cytograms not shown) were present. (C) This table denotes the specific proportions of lymphocytes in this node.

References

- Rao, Lana et. al., JVIM 2011: 25:1097-1105 (MHCII/B-cell lymphoma).
- Williams, Avery et. al., JVIM 2008: 22:596-601 (Canine leukemia prognosis).
- Seelig, Avery et. al., JVIM 2014: 28:878-886 (T-zone paper).
- Deravi, Berke et. al., Vet Imm and Immunopath 2017: 191:5-13 (T-cell lymphoma prognosis).

Submission Guidelines

Submission guidelines for flow cytometry samples can be found in our on-line test menu, test code "VFLOWC". Consultation is encouraged. Pathologists are happy to discuss case specifics and how flow cytometry may help in patient evaluation. When submitting a specimen for flow, it is important to remember to sample the site of involvement. For example, to assess peripheral blood lymphocytosis, submit peripheral blood. To assess nodal lymphoma, an aspirate of an affected node should be submitted.

Validation studies have been performed for peripheral blood in both cats and dogs and for solid tissues and cavitary fluids from dogs.

- For flow cytometry on blood samples:
    - Recommended sample required is one EDTA tube containing 2 mL whole blood.
    - Results of recent CBC or an additional EDTA tube to run a CBC concurrently. A CBC run within 48 hours of collection of the flow sample (with blood smears) is strongly recommended, as it is extremely important in helping our pathologists provide the most accurate and best diagnosis possible. While you can provide copies of in house blood work or a CBC from another outside lab, if possible a CBC run at Marshfield is preferred (and can be run from the extra EDTA tube submitted).

- For cavitary fluid specimens (e.g., thoracic and abdominal fluid – from dogs only at this time):
    - A minimum of 500 uL of fluid in an EDTA tube is required (no CBC is needed).

- For solid tissue submissions (liver/spleen/lymph nodes – again, dogs only at this time):
    - Aspirate the site of interest several times into 1 mL of saline and 0.1 mL of serum from the same patient in a red top tube (RTT). The needle can be rinsed in this fluid after each aspiration, repeating 2-4 times.
    - Instructions for this procedure are found in our online test menu, and if you have any questions, please feel free to contact one of our pathologists. They'd be more than happy to help in any way they can!

Sample Submission Form

Please see a copy of our flow cytometry submission form on the following page. We request that this form be filled out as completely as possible when submitting your sample, as it helps our pathologists interpret the flow cytometry data and helps provide you and your clients with the best possible report.

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FLOW CYTOMETRY SUBMISSION FORM

Owner Name:	**Account Name:** R _ _ _ R
Animal Name/ID:	**Address:**
Species:	**Phone:**
Breed:	**Fax:**
Sex: F, M, F/S, M/N **Age/DOB:**	**Email:**
Collection Date/Time:	**Veterinarian:**
Apply patient label here: (Please also label all samples that accompany this form)	

PLEASE NOTE: Submission of a blood sample within 48 hours of collection is requested for optimum cell viability. Sample viability can be affected by a variety of factors, of which include age of specimen, proper refrigeration, necrosis, etc. If you have any questions, please call, a pathologist would be happy to discuss sample collection prior to submission with you.

Patient History: (history of infectious disease [e.g Ehrlichiosis], autoimmune disease, neoplasia, other abnormalities)

_____

_____

Clinically healthy?: Yes☐ No☐ **Patient on steroids?** Yes ☐ No☐

Please provide details: _____

SAMPLE SITE, TYPE

☐**Peripheral blood** (EDTA tube- minimum volume 1cc)

☐ **CBC to be performed at Marshfield Labs** (*submit a fresh blood smear and a second EDTA tube*)

OR

☐ **CBC results provided** (*include results or Marshfield accession number. **NOTE**: Results must be from a specimen drawn within 48 hrs of the flow cytometric specimen to be accepted*)

☐**Non Blood Tissue**

☐ **Non Blood Tissue** (Liver, Spleen, Lymph node, Thymus) (See Collection /Volume Requirements in our online Test Reference Manual)

☐ **Please specify tissue** _____

☐**Cavitary Fluids**

☐ **Cavitary Fluid** (See Collection /Volume Requirements in our online Test Reference Manual)

☐ **Please specify fluid source** _____

Lymphoid neoplasia confirmed by cytology or histology? Yes☐ No☐

(please include a copy of the pathology report or CYT number if performed at Marshfield)