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Applications of Flow Cytometry in Veterinary Research and Small Animal Clinical Practice

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Abstract

Flow cytometry is a rapid, versatile and powerful technique that allows for qualitative and quantitative assessment of multiple parameters of individuals cells and particles. With the flexibility of flow cytometry, and the reliability of results, this technology has become an essential veterinary research instrument with important clinical diagnostic capabilities, particularly in the fields of immunology and hematology. This methodology has played a key role in the discovery of immunological cells and multiple aspects of canine immune function. Flow cytometric techniques have been instrumental in the identification of regulatory T lymphocytes and roles they play in autoimmune diseases, as well as characterizing and measuring cellular products, such as cytokines and other proteins. Flow cytometry has also been used in the pharmacodynamic monitoring of immunosuppressive therapies in dogs. Besides immunological cells, flow cytometry allows for the evaluation of erythrocytes, specifically the quantitation of reticulocytes, detection of anti-erythrocyte antibodies, and diagnose of erythrocyte parasites. Similar techniques can also be applied to canine platelets to diagnose immature reticulated platelets and anti-platelet antibodies. Flow cytometry can also be used to assess various aspects of platelet function. Flow cytometry is most often applied in veterinary oncology to diagnose and stage lymphoid neoplasia and leukemia, and can also be used to assess neoplastic and non-neoplastic marrow conditions. This article will cover some of the most common and popular uses of flow cytometry in small animals, and discuss the influences that this technology has on veterinary research and small animal clinical medicine.

ABBREVIATIONS

FSC: Forward-angle scatter; **SSC:** Side-angle scatter; **FITC:** Fluorescein isothiocyanate; **PE:** Phycoerythrin; **APC:** Allophycocyanin; **CD**: Cluster of Differentiation; **NK**: Natural Killer; **IL**: Interleukin; **Tregs**: Regulatory T cells; **FOXP3**: Forkhead box P3; **DCs**: Dendritic cells; **IFN-γ**: Interferon gamma; **PBMCs**: Peripheral blood mononucleated cells; **TLRs**: Toll-like receptors; **ROS**; Reactive oxygen species; **CFSE**; Carboxyfluorescein succinimidyl ester; **IMTP**: Immune-mediated thrombocytopenia; **GP**: Glycoproteins; **IMHA**: Immune-mediated hemolytic anemia; **COX**: Cyclooxygenase; **IMPA**: Immune-mediated polyarthritis

INTRODUCTION

Flow cytometry, a laser-based biophysical technology employed in cell counting, cell sorting and cellular marker detection, is routinely used in veterinary medicine. There have been significant advancements in flow cytometric technology over the past 30 years. Flow cytometers are highly complex and expensive instruments that have traditionally required a dedicated, highly trained professional to operate but, with technological advancements, flow cytometric analysis is becoming simpler, and can be applied to an ever widening variety of applications. Initially, flow cytometry was used as a cell counter and research tool; however, with changes and improvements to instrument design, flow cytometry has not only become an extremely effective and popular research methodology, but has also become an integral component to clinical veterinary medicine. In fact, flow cytometric techniques that were once believed to solely be used for research assessment, are currently being used by veterinarians to diagnose and monitor therapy in clinic patients on an individual basis. In many veterinary clinical pathology laboratories, the incorporation of flow cytometry into hematology analyzers has replaced manual counting of hematologic cells, allowed for assessment of cellular maturity, and provided additional diagnostic modalities. User-friendly versions of these laser-based flow cytometry hematology

analyzers have also recently attained common usage in general veterinary practice. Today, flow cytometers remain essential research instruments with important clinical diagnostic capabilities but, with the flexibly of these units, and the reliability of results, the use of flow cytometry in veterinary medicine will continue to grow and be applied to a vast number of both research and clinical applications. Due to the seemingly endless number on possible uses of flow cytometry, a discussion on all uses of this methodology is beyond the scope of this article, therefore, in this article, the authors have selected some of the most common and popular uses of flow cytometry in small animals, particularly dogs, and will discuss the influences of flow cytometry on veterinary research and clinical medicine. Laser-based flow cytometry hematology analyzers will not be discussed.

FLOW CYTOMETRY PRINCIPLES

Flow cytometers are complex instruments that consist of optics, lasers, electronic networks, advanced software, and most often a fluidics system. The detailed operations of a flow cytometer are beyond the scope of this article. However, the basic concept of flow cytometry is that cells or particles flow through a laser to identify or measure the characteristics of that particular cell or particle. The sample of interest is suspended in fluid, and a pressurized line directs the cells to flow into a central core that forces the cells to form in a single file line. As the sample continues to flow, the aligned cells will pass through the laser, thereby analyzing one cell or particle at a time. [1-3]

As individual cells pass through the laser beam, two types of light scatter occur: forward-angle scatter (FSC) and side-angle scatter (SSC). The light that passes through each individual cell in a forward direction is the forward-angle scatter and represents the refractive index of the cell, which roughly correlates to size. The light that scatters in a 90-degree angle is the side-angle scatter, and represents the internal complexity of the cell. The unique light scatter properties of a particular cell type can be used to distinguish and identify subpopulations of cells, making this technique an integral part in the laser-based clinical hematology analyzers. [2,3]

To enhance the capabilities of these instruments, fluorochromes can be conjugated to antibodies that target specific antigens on a cell. These fluorescently labeled antibodies are added to the sample and incubated, and then weakly bound non-specific and unbound antibodies are washed off before the labeled sample is injected into the flow cytometer. As the fluorescently labeled cell passes through the laser, the fluorochromes will emit a light of a specific wavelength which will be collected by a detector, converted to a digital output and transferred to a computer for analysis. By incorporating multiple fluorochrome dyes that emit light at different wavelengths, such as fluorescein isothiocyanate (FITC), phycoerythrin (PE), and allophycocyanin (APC), multiple antigens can be identified on the same sample at the same time. The intensity of the emitted light is directly proportional to the quantity of bound antibody. Unfortunately, when compared to humans, there is limited availability of antibodies specific to dogs. However, depending on the antigen of interest, there may be appropriate cross-reactivity with antibodies from other species to allow flow cytometric analysis. An additional benefit of some flow cytometers is the ability to sort subpopulations of fluorescent-labeled cells that can be collected and further analyzed [2,3].

EVALUATION OF THE CANINE IMMUNE SYSTEM BY FLOW CYTOMETRY

Single, two and three parameter flow cytometry

Flow cytometry has played a key role in discovery of immunological cells, and has helped facilitate evaluation of canine immune function. Canine peripheral blood leukocyte subsets, in particular $CD4^+$ and $CD8^+$ T cells, $CD21^+$ B cells, $CD5^{lo}$ natural killer (NK) cells and $CD14^+$ monocytes/macrophages have been stained, identified with species-specific antibodies, and sorted by flow cytometry. [4-13] Additionally, using three color flow cytometry analysis, novel canine cell populations, in particular $CD3^+CD4^+CD8^+$ double positive T cells with features of activated T cells, have been phenotypically identified in the peripheral blood of dogs. [14]

The discovery of a population of suppressive $CD4^+$ T cells characterized by high constitutive expression of the IL-2Rα chain (CD25) led to remarkable progress in elucidating the phenomenon of cell-mediated suppression and its importance in self-tolerance and regulation of adaptive immune responses. [15-17] In classical suppression assays, adult mice that expressed $CD4^+CD25^+$ T cells were significantly less likely to develop organ-specific autoimmune disease than mice that expressed $CD4^+CD25^-$ T cells. [18] The identification of these cell populations lead to the discovery of a key mechanism of peripheral tolerance and a critical component of the host immune system, regulatory T cells (Tregs), which are a small population of T lymphocytes that possess immunomodulatory properties and aid in the suppression of autoimmune responses. Using flow cytometry, $CD4^+$ T cells with the highest CD25 expression were shown to be enriched for FOXP3, a transcription factor that is highly expressed in Tregs in humans. [19,20] Recently, using similar flow cytometric techniques, $CD4^+CD25^+FOXP3^+$ regulatory T cells were described in dogs (Weaver et al., unpublished observation, 2013). [19-25] With analytical three-color flow cytometry and fluorescence-activated cell sorting, canine Tregs have been characterized phenotypically similar to humans, providing direct evidence of Treg regulatory function in dogs, which can also serve as a model for human diseases. [26-28]

Dendritic cells (DCs), the most potent antigen-presenting cells, represent a heterogeneous group of bone-marrow derived cells, and serve as sentinels of the immune system, capable of capturing and processing antigens. Canine DCs have been identified, via flow cytometry, in the peripheral blood [10], gastrointestinal tract [29] and skin [30], with panels of canine-specific and cross-reactive antihuman monoclonal antibodies. Current protocols for isolation and generation of canine monocyte- and bone marrow derived DCs involve extensive flow cytometric analysis of multiple phenotypical and functional changes in DCs upon maturation. [6,7,31-36]

The use of flow cytometry to identify the phenotype and function of immune cells is not limited to peripheral blood. Microglial cells are the main immune effector cell of the brain, and respond to diseases within the central nervous system. Once activated, microglial cells are capable of several immunological

functions, including phagocytosis, modulation of T-cell response, and production of cytokines. The extensive immune phenotyping of the surface markers expression in canine microglial cells have been identified by single color flow cytometry analysis. [37,38]

Along with the ability to label and categorize individual cells via fluorescent antibodies, flow cytometry is also capable of identifying and measuring cellular products such as cytokines, proteins, and other factors. Cytokines are important mediators of immune responses, and are produced by a variety of activated lymphocytes. The advent of intracellular cytokine assessment by flow cytometry through staining of permeabilized cells with fluorochrome-conjugated anti-cytokine antibodies allows cytokine levels in a large numbers of cells to be rapidly examined. Several studies have described flow cytometric assays for the measurement of cytokines involved in T cell activation and polarization. Requiring only a small volume of blood, a rapid whole-blood flow cytometric assay has been developed to measure interferon-γ (IFN-γ) and interleukin-4 (IL-4) produced from canine $CD4^+$ and $CD8^+$ lymphocytes. [39]. In our laboratory, we have evaluated the effects of *in vitro* and *in vivo* cyclosporine exposure on a panel of cytokines, including IL-2, IL-4 and IFN-γ, produced by activated T cells to ascertain their potential as markers of immunosuppression in dogs. [12,13] Interferon γ, predominantly produced by NK and Natural Killer T cells, is a fundamental component of the innate immune response, and is vital to the immunological response to viral and intracellular bacterial infections and tumor control. In canine peripheral blood mononucleated cells (PBMC) assessed by one or two color surface expression and intracellular staining flow cytometry approaches, Huang and others recently discovered $CD5^{lo}$ cells that closely resembled human NK cells by expressing NK-specific cell markers and producing high levels of IFN-γ. [8] Finally, in addition to cytokine detection, flow cytometry is capable of determining the purity of samples, for example via the detection of intracellular staining of CD79α in canine B-cells after being magnetically separated. [11]

Multiparameter and polychromatic flow cytometry

Multiparameter flow cytometry is a powerful analytical tool that enables the rapid measurement of multiple physical and chemical characteristics of individual cells. Recently, a multiparametric 4-color flow cytometry assay was used to evaluate the changes related to the maturation of lymphocyte subsets as in young dogs. [40] Multiparameter flow cytomery approaches have also successfully been applied for the separation and phenotypic characterization of several canine DC populations. Purified and sorted by a 4-color flow cytometer, canine $CD11c^+$/$HLA\text{-}DR^+$/$CD14^-$/$DM5^-$ cell populations were determined to have the same functional and morphological properties as human myeloid DCs. [10]

The development of polychromatic cytometry (greater than 4 colors) has contributed to significant progress in the field of human immunology. Recently, 12 color panels were developed to simultaneously investigate the direct activation of multiple DC subsets, monocytes, and NKs, and their relationships within the PBMC environment after stimulation with a live-influenza virus or toll-like receptor (TLR) agonists. [41] Currently, the complex polychromatic cytometry approach has not been widely utilized in clinical veterinary medicine. However, a recent report using 7-color multiparameter flow cytometry provided a comprehensive approach to characterize canine lymphocyte phenotypes expressing multiple markers in healthy research dogs. [42]

Functional assays in flow cytometry

The function of immune cells, in particular neutrophils, macrophages, monocytes and DCs, can be evaluated by multiple functional flow cytometry methods. Phagocytosis is an important early step in triggering adaptive immune responses, which require processing of bacterial pathogens and presentation of their antigens to $CD4^+$ and $CD8^+$ T cells. [43] Several reports characterized phagocytosis in canine neutrophils, PBMC and peripheral blood-derived monocytes/macrophages by using FITC-latex beads [9,44] and *Salmonella enterica* serotype Kentucky labeled with GFP, a plasmid with green fluorescence (Harris et al., unpublished observation, 2010). Additionally, flow cytometry has been employed to evaluate the active uptake of FITC-labelled dextran by canine monocyte-derived DCs. [32,33] Finally, the phagocytic properties of canine microglial cells were measured using flow cytometry by using heat-killed and lyophilized FITC-labelled *Staphylococcus aureus* as an antigen. [37,38]

Flow cytometry is uniquely suited for the measurement of the oxidative burst and superoxide production in neutrophils and monocyte/macrophages. Compared to more conventional techniques, the major advantages of flow cytometry for the assessment of these cells are that only a very small volume of blood is required for analysis and that the results are objective and quantifiable. Neutrophils produce reactive oxygen species (ROS) and generate superoxide anion essential to their bactericidal function. Apoptosis is the process of programmed cell death in response to a stress that leads to characteristic cell changes and death. The mitochondria play a vital role in one of the major apoptosis signaling pathways, and mitochondrial dysfunction is part of the pathogenesis of multiple viral, bacterial and parasite infections. Recently, using flow cytometry, it was determined that the oxidative stress and acceleration of apoptosis associated with chronic renal disease in dogs decreases neutrophil production of superoxide. [45]. An additional study compared the status of apoptosis of peripheral blood leukocytes in dogs with demodicosis and healthy dogs, and found that in dogs with demodecosis a significantly greater percentage of cells exhibited externalization of phosphatidylserine, a hallmark sign of depolarized mitochondrial membrane potentials and early apoptosis. [46]. Extrinsic apoptosis can be evaluated by the assessment of the cellular surface expression of CD95 (Fas) and CD178 (Fas ligand). Recently, CD95 and CD178 molecules were shown to induce apoptosis in $CD4^+$ and $CD8^+$ T cells isolated from peripheral blood and spleen of dogs naturally infected with *Leishmania* [5]. Analysis of the functional ability of canine microglial cells to produce ROS has been performed by using dihydrorhodamine and flow cytometric analysis. [37]

Cell proliferation analyses are crucial for evaluating cell growth and differentiation, and are typically based on average DNA content or on cellular metabolism parameters. Carboxyfluorescein succinimidyl ester (CFSE) is a novel cell

tracing fluorescent dye used to examine the proliferative activity of cells by labeling a parent generation and determining the inheritance by the daughter generation. A recent report used flow cytometry to assess the effects of synthetic agonists on TLR 9 and the regulation of canine lymphocyte proliferation by CFSE division. [47] Also, canine $CD4^+CD8^+$ double positive T cells express high proliferation potential based on the CFSE division technique [14]. Proliferative response of T cells in dogs have been also assessed with monoclonal antibodies to IL-2R (CD25) to detect the percentage of $CD4^+CD25^{low}$ activated T cells. [48-50]

Flow Cytometric Analysis of Erythrocytes

Some of the major applications of flow cytometry for erythrocytes include the quantitation of reticulocytes, detection of anti-erythrocyte antibodies, and the detection and diagnose of erythrocyte parasites.

Reticulocytes are immature red blood cells (RBCs) that contain a reticular (mesh-like) network of residual ribosomal RNA, and quantifying reticulocytes serves as a reliable measure of marrow red cell production. Although several types of fluorochromes are capable of labeling reticulocytes, thiazole orange is the most common label used to detect canine reticulocytes via flow cytometry. Thiazole orange will passively enter the reticulocyte, bind to residual RNA, and fluoresce when exposed to laser light of the appropriate wavelength. When compared to traditional manual reticulocyte counts, the thiazole orange technique correlates well as long as reticulocyte percentages are greater than 2% of all RBCs. However, as the percentage of reticulocytes decreases, so does the accuracy of the test, suggesting that flow cytometric assessment of immature erythrocytes is best performed in patients with a normal or elevated reticulocyte count. [51]

Immune-mediated hemolytic anemia (IMHA) in dogs is mediated by the presence of antibody bound to the surface of the RBC. While the traditional means of detecting these antibodies is the direct antiglobulin (Coombs') test, flow cytometry has often been used as a simple and rapid alternative means of detecting anti-RBC antibodies, of quantitating complement involvement, and of determining the precise immunoglobulin sub-type (IgG or IgM) involved. [52-54]

Flow cytometric techniques have also been developed for detecting and quantifying RBC parasites in dogs. Parasitemia with low numbers of organisms such as *Babesia* can be difficult to detect by the standard method of examination of stained blood smears and, although very sensitive polymerase chain reaction (PCR) based methods have now been developed for organism detection, such methods are so sensitive that even a few parasitized RBCs can lead to a strong positive result. Flow cytometry offers a practical 'middle ground', in that even a small number of parasitized cells can be detected, but the precise number of infected cells can be accurately and rapidly counted. Stains that bind to protozoal nucleotides, particularly hydroethidine, have been successfully used to measure RBC parasitemia in dogs affected with *Babesia gibsoni* and *Babesia canis.* [55,56] One potential drawback with using a non-specific nucleotide label rather than an organism-specific antibody label for detecting the presence of RBC organisms is that a positive flow cytometry result may not necessarily be detecting the target organism, but may instead be detecting other sources of nucleotide, such as the residual RNA within reticulocytes [57] or other RBC organisms such as the hemotropic mycoplasma [58].

Morpological and Functional Evaluation of Canine Platelets

Flow cytometry is capable of evaluating several aspects of platelets, including quantitation of immature (reticulated) platelets, detection of anti-platelet antibodies, and the detection of activated platelets. Flow cytometric assessment of platelets can be performed on multiple sample types, including washed platelets, gel-filtered platelets, platelet-rich plasma, and whole blood samples; however, whole blood has become the preferred sample type due to minimal sample processing and reduced risk of artifactual *in vitro* platelet activation. The use of whole blood not only allows for the assessment of platelet function in near physiologic conditions, where platelets can interact with other blood cells, but can also allow for the assessment of platelet function with the use of a small sample volume. Furthermore, unlike many other tests of platelet function, flow cytometry is capable of evaluating platelet function in thrombocytopenic and anemic patients.

Newly formed platelets that have recently been released into circulation are known as reticulated platelets, and still contain some residual mRNA. [59]. The percentage of circulating reticulated platelets can reflect megakaryocyte proliferation, and an elevated percentage of these new platelets usually indicate accelerated platelet production, while a lower percentage suggests poor megakaryocyte function. [59] Reticulated platelet numbers can help determine whether thrombocytopenia is caused by decreased platelet production or platelet consumption/destruction. As with reticulocytes, thiazole orange can be used to detect the residual mRNA in reticulated platelets. However, at least in humans, this fluorochrome is not specific for residual platelet RNA, and approximately half of the thiazole orange staining is associated with uptake in dense granules. [60]

Immune-mediated thrombocytopenia (IMTP) is associated with binding of anti-platelet antibodies to the platelet surface membrane, leading to destruction of the platelet by phagocytic cells. Blood samples from dogs with suspected IMTP can be combined with a fluorescently labelled species-specific anti-IgG and/or anti-IgM antibody and analyzed via flow cytometry in combination with platelet-specific labels. [61] According to Kristensen and others, approximately 67% of dogs with suspected IMTP were positive anti-platelet antibodies, however, it may be difficult to distinguish between primary and secondary immune mediated thrombocytopenia. [62,63] Ideally, flow cytometry should be performed within 24 hours of sample collection, and the assay also has limited sensitivity and specificity, reducing the clinical effectiveness of this diagnostic test.

Flow cytometry can measure the activation state and reactivity of circulating platelets, diagnose inherited and acquired platelet disorders, evaluate leukocyte-platelet aggregation and procoagulant changes to the platelet surface, and facilitate the assessment and monitoring of antiplatelet therapy. Unlike platelet aggregometry, where platelet activation is necessary,

flow cytometry can evaluate surface protein expression both before and after platelet activation. Samples are typically labeled with two fluorescently conjugated monoclonal antibodies. The first antibody is usually specific for glycoproteins Ib, IIb, and IIIa (ie. anti-CD41 or anti-CD61), molecules common to all platelets, and serves to identify the platelet. Some samples can be left unlabeled, however, and in these instances identification of the platelet is based on characteristic forward and side scatter. The second fluorescently conjugated antibody is specific to the epitope or receptor of interest. In an attempt to stabilize the platelet membrane, the sample can be fixed before or after the addition of antibodies, although this process may adversely affect the binding of some antibodies. Flow cytometry techniques have been developed to identify activated platelets as well as microparticles released from activated platelets. [64-67]

There are several markers that indicate the presence of an activated platelet, including glycoprotein IIb/IIIa (GP IIb/IIIa), P-selectin, and leukocyte-platelet aggregates. The primary mediator of platelet aggregation involves a conformational change of the surface GPIIb/IIIa complex, enabling the binding of fibrinogen to stabilize the platelet plug [68]. With inactivated platelets, this receptor is in a resting conformation and, upon activation, there is a conformation change, allowing this receptor to bind fibrinogen. The monoclonal antibody PAC-1 only binds to the GP IIb/IIIa receptor in an activated conformation: therefore, a PAC-1 negative platelet is considered to be inactivated or resting, while a PAC-1 positive platelet is considered to be activated [69]. One of the molecules released from platelet α-granules during activation is P-selectin. Platelet P-selectin, CD62, is a cell adhesion molecule that mediates platelet and leukocyte aggregation and generates procoagulant microparticles that contain active tissue factor and enhance fibrin deposition. Anti-CD62 monoclonal antibody binds to P-selectin expressed on the platelet surface only after activation and granule release, and a P-selectin negative platelet should therefore be considered to be inactivated or resting. [63,66,67] Platelet P-selectin has been shown to be expressed in higher concentrations in dogs with primary immune-mediated hemolytic anemia (IMHA) [64], and provides evidence that platelet activation could contribute to the high rate of thromboembolic complications seen in IMHA patients. The ability of P-selectin to mediate platelet and leukocyte aggregation can also be detected with flow cytometry, and leukocyte platelet aggregates are a sensitive marker of *in vivo* platelet activation. [67]

In resting platelets, the aminophospholipid phosphatidylserine is located in the inner leaflet of the platelet plasma membrane but, upon activation, platelet phosphatidylserine is exposed on platelet surface membranes and binds to prothrombin, accelerating the conversion of prothrombin to thrombin, and expediting the formation of a blood clot [70]. Flow cytometric detection of annexin V, a protein that preferentially binds to phosphatidylserine on the surface of platelets, can also detect the presence of activated platelets. The inherited inability of platelets to express phosphatidylserine is known as Scott Syndrome, and has been described in humans and dogs [71]. Flow cytometry can also be used to diagnose several other heritable platelet disorders, such as Glanzmann thrombasthenia (deficiencies in the GPIIb/IIIa complex) and storage pool defects. [72,73]

Since mature platelets do not contain a nucleus and are therefore incapable of mRNA synthesis, and because COX-1 is usually constitutively expressed by cells whereas COX-2 expression is more typically induced, it was long believed that COX-1 was the only COX isoform expressed in circulating platelets, and that COX induction could happen only at the level of the bone marrow. However, a flow cytometry technique utilizing permeabilization methods has been described that has enabled identification and quantification of the expression of both COX-1 and COX-2 in circulating human and canine platelets. [74,75]

Despite the significant benefits of flow cytometry for evaluating platelets, there are several disadvantages and limitations to this methodology. Ideally, to avoid *ex vivo* platelet activation, samples should be processed within hours of collection, reducing the clinical practicability of this methodology. Additionally, while flow cytometry is excellent at determining the expression of proteins and receptors on the surface of activated platelets, the technique does not evaluate the platelet's actual ability to form a platelet plug. Finally, activation of platelets prior to collection leads to binding to the vascular endothelium or loss of surface receptors/proteins, preventing these platelets from being appropriately assessed by flow cytometry.

Immunophenotyping in Canine Neoplasia

In human medicine, flow cytometry has become an essential component of the assessment, diagnose, and staging of cancer. Flow cytometry is primarily used for immunophenotyping, which is the use of a panel of antibodies to identify cell lineage, for example B or T cell lymphoma. Antibodies are directed toward molecules expressed by hematopoietic lineages and lymphoid subsets. The range of immunophenotyping panels used to assess canine neoplasia is still constrained because of associated expense and a limited availability of species-specific antibodies. In veterinary oncology, although flow cytometry is most often applied to hemolymphic neoplasia [76-78], particularly lymphoma, this methodology has also been used for the assessment of other neoplastic conditions such as osteosarcoma and transitional cell carcinoma. Immunophenotyping can also play a pivotal role in the assessment of canine leukemia by defining the cell lineage, stage of maturation, and prognostic information. [76,79]

In dogs with lymphoma, flow cytometry is not intended to replace conventional diagnostic techniques such as cytology or histopathology, but instead is used to assist in the classification and staging of specific tumor types. Based on flow cytometric studies in dogs, there is predominately one type of B-cell lymphoma, while T-cell lymphomas will typically have a variety of phenotypes. Large cell lymphoma, which is similar to an immunoblastic subtype characterized by the World Health Organization, is the most common form lymphoma in dogs, and originates from a B-cell lineage. On the cell surface, most B-cell lymphomas will express CD1, CD21, CD45RA, CD79a, CD90, and MHC class II, while most T-cell lymphomas are characterized by CD3, CD4, CD8, and αβ-TCR antigens. The presence of hypercalcemia in T-cell lymphoma is most frequently associated with CD4 and αβ-TCR antigens. [76,80]

One advantage of flow cytometry is that the technique can rapidly evaluate a large number of different cell types within a

sample, and use multiple simultaneous labels to identify specific cells within a heterogeneous cell population. This is particularly invaluable when applied to bone marrow samples in patients with lymphoreticular neoplasia or 'pre-leukemic' leukemia, where a particular malignant cell type within the marrow can be identified and characterized from within a soup of multiple different cells. [78, 81-83]. In patients without neoplasia, similar methodology can be used to quantify the distribution of particular cell types within the marrow, including cells that are typically very difficult to count, such as stem cells [84-86], and cell sorting techniques can be used to isolate purified populations of specific cells [87].

Pharmacodynamic Monitoring in Dogs

One exciting new clinical application of flow cytometry in dogs is the pharmacodynamic monitoring of immunosuppressive drugs such as cyclosporine. Cyclosporine is a powerful immunosuppressive agent which inhibits T cell production of cytokines such as IL-2 and IFN-γ, and is being used in an increased frequency in veterinary medicine. In dogs, a microemulsion formulation of cyclosporine has FDA approval for the treatment of canine atopic dermatitis, and this formulation is also used off label as an immunosuppressive agent for the treatment of other conditions such as inflammatory bowel disease, anal furunculosis, IMHA, IMTP, and immune-mediated polyarthritis [88]. When treating life-threatening conditions such as IMHA or IMTP, getting the treatment right as soon as possible can make a huge difference in patient survival. Unfortunately, the best way to use cyclosporine in these conditions is unknown. Patients are usually started at high cyclosporine doses, with therapy adjusted based on pharmacokinetic monitoring of cyclosporine blood concentrations. In our experience, however, dogs can have variable cyclosporine blood concentrations at the same oral drug dose and, even at comparable drug blood concentrations, some dogs will respond positively to medical management while others will not. Pharmacokinetic monitoring alone, therefore, does not necessarily lead to acceptable treatment outcomes.

In human medicine, pharmacodynamic monitoring is now used to supplement pharmacokinetic monitoring, with the goal of improving treatment outcomes in clinical patients receiving cyclosporine. [89,90] Pharmacodynamic monitoring is the use of an assay to measure the biological effects of a drug on a specific target cell or tissue. Cyclosporine is a mainstay drug in the field of human transplantation medicine [91,92] and, because effective immunosuppression is essential in transplant patients, a range of pharmacodynamic assays have been developed to evaluate the effects of cyclosporine on T cell function. Such assays have now also been adapted for use in dogs.

Our research group has over the past few years worked to develop pharmacodynamic assays for the monitoring of cyclosporine therapy in dogs. Initially, we performed an *in vitro* study using flow cytometry to measure the effects of incubation with cyclosporine on activated T cell expression of both surface markers (CD25 and CD95) and intracellular cytokines (IL-2, IL-4, and IFN-γ) in order to identify candidate markers for subsequent pharmacodymamic studies [12]. Dogs were then given oral cyclosporine at various doses, and flow cytometry was used to evaluate T cell expression of IL-2, IL-4, and IFN-γ. Lower cyclosporine doses were found to suppress IFN-γ expression, and higher drug doses suppressed both IL-2 and IFN-γ [13]. While our study demonstrated that flow cytometry could be utilized to explore new markers for use in pharmacodynamic assays of cyclosporine therapy in dogs, we also encountered some of the limitations common seen with flow cytometry as a routine diagnostic tool, in that the technique was too labor intense and time sensitive for routine clinical use. However, we were able to use the information gained from our flow cytometric assay to then develop and refine a more robust quantitative reverse transcription polymerase chain reaction assay to measure activated T cell expression of IL-2 and IFN-γ for routine use in clinical patients.

DISCUSSION AND CONCLUSION

Flow cytometry is a laser-based technology that uses light scatter and fluorescence properties to identify, count and assess function in selected subpopulations of cells. Some of the most common applications in small animal medicine include in-practice hematology analyzers, precise assessment of cells within the immune system, detection of antibodies on RBCs and platelets, identification of erythrocyte parasites, assessment of aspects of platelet function, characterization of bone marrow cell types, immunophenotyping of neoplastic cells, and pharmacodynamic monitoring. As technology continues to grow and become more user-friendly, the potential benefits and uses of flow cytometry in small animal veterinary medicine are practically endless.

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