Canine Peripheral Blood Lymphocyte Phenotyping by 7-Color Multiparameter Flow Cytometry

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OBJECTIVE: To characterize baseline canine lymphocyte phenotypes including lymphocytes coexpressing multiple markers by novel 7-color multiparameter flow cytometry.
STUDY DESIGN: Fresh canine peripheral blood lymphocytes of 79 healthy 26-week-old Beagle or Beagle-mix dogs were stained and analyzed.
RESULTS: The high number of samples and acquired flow data (averaging 1.9×10^5 cells/sample) allowed the detection of minor lymphocyte subsets coexpressing multiple lymphocyte markers. The averaged percentages of major lymphocyte subsets of CD3+, CD4+, CD8+, CD21+ and γδ TCR+ cells from this study were 74.0, 43.6, 14.3, 9.6, and 0.2, respectively, which were comparable but uniquely different from other reports as they were simultaneously detected in the same sample. We demonstrated that the commonly used CD21 and CD3 monoclonal antibody (mAb) clones, previously recommended not to be used in the same staining, could and should be used together with the proper steps of lymphocyte gating. We found a high percentage (10.3%) of unidentified CD21−CD3+CD4−CD8−γδTCR− lymphocyte subset that has never been reported. The intensive gating strategy and the mean percentages of each lymphocyte subset to their parent subsets and to the total lymphocyte population are presented and discussed.
CONCLUSION: The canine lymphocyte phenotypes were fully characterized. This novel multiparameter flow cytometry method is a powerful approach to in-crease the accuracy of lymphocyte phenotyping in dogs. (Anal Quant Cytopathol Histopathol 2013;35:197–204)

Keywords: Beagle dog, canine diseases, canine lymphocytes, flow cytometry, lymphocyte phenotyping.

The characterization of lymphocyte subset distribution in clinically normal dogs provides the basis

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to study any changes in dogs affected by infectious agents, neoplasia, or other pathologic conditions. The phenotyping of canine lymphocytes by flow cytometer has been done in various breeds,[1,2] age groups,[1-7] and clinical conditions.[2,8,9] Most of these studies were performed using single-color,[1,2,4,6] dual-color,[2,7,10] or triple-color[7,10] flow cytometry. The limited availability of monoclonal antibodies (mAbs) specific to canine lymphocyte markers restricts the subsets, which may be analyzed. When primary mAbs of identical isotype are used, it requires direct conjugation with compatible fluorochromes to use them concurrently. Cross-reaction of specific mAbs among different species helps expand the pools of compatible mAbs. Faldyna et al[11] characterized a panel of commercially available mAbs specific to human lymphocytes. They confirmed 3 clones of anti-human CD21 that stained canine CD21 the same way as an anti-canine CD21 clone and could be used as alternatives, but all of them were the same mouse IgG1 isotype.

This study utilized commercially available directly conjugated mAbs as well as unconjugated mAbs with their corresponding secondary conjugates to develop a compatible 7-color multiparameter flow cytometry protocol to identify the phenotypes of canine peripheral blood lymphocytes (PBLs), the coexpression of multiple lymphocyte markers, and their distribution in relation to each other. The intensive gating strategy and the mean percentages of each lymphocyte subset to their parent subsets and to the total lymphocyte population are presented and discussed.

Materials and Methods

Animals

The same 79 healthy Beagle or Beagle-mix breed dogs at 26 weeks of age used in a study to evaluate the T cell responses induced by various canine vaccines were used in this study. All dogs were born within a 1-week period, vaccinated with various routine canine vaccines, and handled under the Code of Federal Regulations (9 CFR 3.6.C) and Institutional Animal Care and Use Committee approved protocol (#RD11-090, 9/16/11). The data presented here were on fresh peripheral blood mononuclear cells (PBMCs) from all dogs regardless of the vaccines they received.

Blood Samples

Blood samples (7 mL) were collected in Vacutainer cell preparation tubes with sodium citrate as anticoagulant and Ficoll as density gradient separation solution (BD Diagnostics, Franklin Lakes, New Jersey, U.S.A.). The blood tubes were centrifuged at 1,500× g for 25 minutes at room temperature (RT) within 1 hour after blood collection to separate PBMCs from whole blood.

Multiparameter Flow Cytometry

PBMC Isolation. The PBMCs used in this study were from the same PBMC population isolated to evaluate the T cell responses induced by various canine vaccines in another study. The PBMCs were collected from the cell preparation tubes, and red blood cells were lysed by adding 6 mL of buffered water (0.15 M Na_2HPO_4 and 0.15 M KH_2PO_4) for 1 minute followed by 3 mL of 3× PBS. The pellets were washed once with 10 mL PBS then resuspended in RPMI++ (RPMI 1640 with L-glutamine and 25 mM HEPES, supplemented with 15% fetal bovine serum (Atlanta Biologicals, Norcross, Georgia, U.S.A.), penicillin (150 IU/mL)/streptomycin (150 µg/mL)/amphotericin B (0.38 µg/mL) solution (Mediatech, Inc., Herndon, Virginia, U.S.A.), and 1.5 µg/mL gentamicin (Invitrogen, Grand Island, New York, U.S.A.). The cells in PBMC suspensions were counted and adjusted to 5×10^6 cells/mL with RPMI++.

Antibody Panels. The details of specific primary antibodies and secondary antibody conjugates used in this study are listed in Table I.

PBL Staining. A total of 200 µL of each PBMC suspension with 10^6 cells were added in each well of a 96-well, flat-bottomed tissue culture microtitration plate. After 2 hours' incubation at 37°C most monocytes attached to the bottom of the plate. The unattached PBLs were transferred to a 96-well, round-bottomed tissue culture microtitration plate. The plate was centrifuged at 800× g for 1 minute and the supernatant was removed by rapid flicking of the plate in all washing steps. The PBLs were washed once with PBS++ (PBS with 0.5% bovine serum albumin and 0.1% sodium azide). The primary antibody mix was added to all wells (50 µL), the cells resuspended, and the plate was incubated at RT for 30 minutes. After incubation the plate was washed twice with PBS++. All wells then received 50 µL of the secondary antibody mix, the cells were resuspended, and the plate was incubated at RT for another 30 minutes and washed 3 times with PBS++. The 1% solution of ultrapure formaldehyde (Poly-

Table I *List of Primary Antibodies and Secondary Conjugates Used in this Study*

Source	Catalog #/clone	Primary antibodies, dye	Isotype	Secondary antibodies, dye	Source	Catalog #
AbD Serotec	MCA1774F CA17.2A12	Mouse anti-canine CD3, FITC[a]	Mouse IgG1			
VMRD	DH29A	Mouse anti-canine CD4	Mouse IgM	Goat anti-mouse IgM, Phycoerythrin-Cy7	Southern BioTech	1020-17
AbD Serotec	MCA1039A700 YCATE55.9	Rat anti-canine CD8α, Alexa Fluor 700[a]	Rat IgG1			
AbD Serotec	MCA1781PE CA2.1D6	Mouse anti-canine CD21, Phycoerythrin[a]	Mouse IgG1			
US Biological	I1903-17B	Goat anti-canine IgG (Fc), Biotin	Goat PAb[b]	Streptavidin-DyeMer 488/615	Invitrogen	S-32386
UC Davis	CA20.8H1	Mouse anti-canine γδ TCR	Mouse IgG2a	Goat anti-mouse IgG2a, Phycoerythrin-Cy5.5	Invitrogen	M32218
eBioscience	50-0250-42 P4A10	Mouse anti-canine CD25, eFluor 660[a]	Mouse IgG1			

[a]Commercially direct-conjugated.
[b]Polyclonal antibody.
Source list: AbD Serotec, Raleigh, North Carolina; eBioscience, San Diego, California; Invitrogen: Life Technologies Corporation, Carlsbad, California; SouthernBiotech: SouthernBiotechnology Associates Inc., Birmingham, Alabama; UC Davis: Dr. Peter Moore's laboratory, University of California, Davis; US Biological: United State Biological, Salem, Massachusetts; and VMRD: Veterinary Medical Research and Development Inc., Pullman, Washington.

science, Warrington, Pennsylvania, U.S.A.) in PBS was added to all wells (250 µL) before transferred to flow tubes (BD Biosciences, San Jose, California, U.S.A.). The tubes were stored at 4°C until analyzed within 1 day.

Flow Cytometry Analysis. The personnel of the Flow Cytometry Facility of the Office of Biotechnology, Iowa State University, acquired the data from the stained PBLs by a cytometer (FACSCanto, BD Biosciences). The 2 lasers 488 nm Argon and 633 nm HeNe were used. The 488 nm Argon laser excited 5 fluorochromes: FITC, PE, DyeMer 488/615, PE-Cy5.5 and PE-Cy7 using filters 530/30, 575/26, 610/20, 695/40 and 780/60, respectively. The 633 nm HeNe laser excited 2 fluorochromes: efluor 660 and AlexaFluor 700 using filters 660/20 and 730/45, respectively. The averaged total acquisition was 1.9×10^5 cells/sample.

Flow Data Analysis. The cell analysis software FlowJo version 9.3.2 (Tree Star Inc., Ashland, Oregon, U.S.A.) was used to analyze the flow cytometry data. All plots mentioned here are from Figure 1. From all PBLs the single cells were gated on forward scatter–height and forward scatter–area plot (plot A1) to eliminate doublet cells. The lymphocytes[1,4,12] were then gated on side scatter–area and forward scatter–area plot (plot A2). On CD3/CD21 plot of lymphocytes (plot A3), the 3 well-separated populations CD21+, CD21–CD3–, and CD21–CD3+ were gated. The surface immunoglobulin G (sIgG+) gates were placed on these 3 populations (plots B1, B2, B3) as well as the 4 quadrant gates on CD4/CD8 plots (plots C1, C2, C3). The cells in each of the 3 major CD4/CD8 subsets of CD21–CD3+ cells (plot C3) were gated for γδ TCR+ (plots D1, D2, D3) and CD25+ (plots E1, E2, E3) cells.

Statistical Analysis. The JMP statistical software (JMP 10, SAS Institute Inc., Cary, North Carolina, U.S.A.) was used to analyze normal data distribution of each lymphocyte subset percentages. The outliers were identified and eliminated before the mean and standard deviation (SD) analysis.

Results

The gating strategy of canine lymphocyte immunophenotyping is demonstrated graphically in Figure 1. The gating of each plot was adjusted in individual samples. All samples contained high numbers of cells with high granularity (plot A2). The phenotypes of individual lymphocyte populations and their distributions, relative to their parent subsets and the total lymphocytes, are summarized in Table II. Minor lymphocyte subsets representing <1% of their parent subsets were not presented.

The averaged relative percentages of CD3+, CD4+, CD8+, CD21+ and γδ TCR+ from total lymphocytes were 74.0, 43.6, 14.3, 9.6, and 0.2, respectively. The multiparameter flow cytometry analysis showed a well-separated CD21+ population with

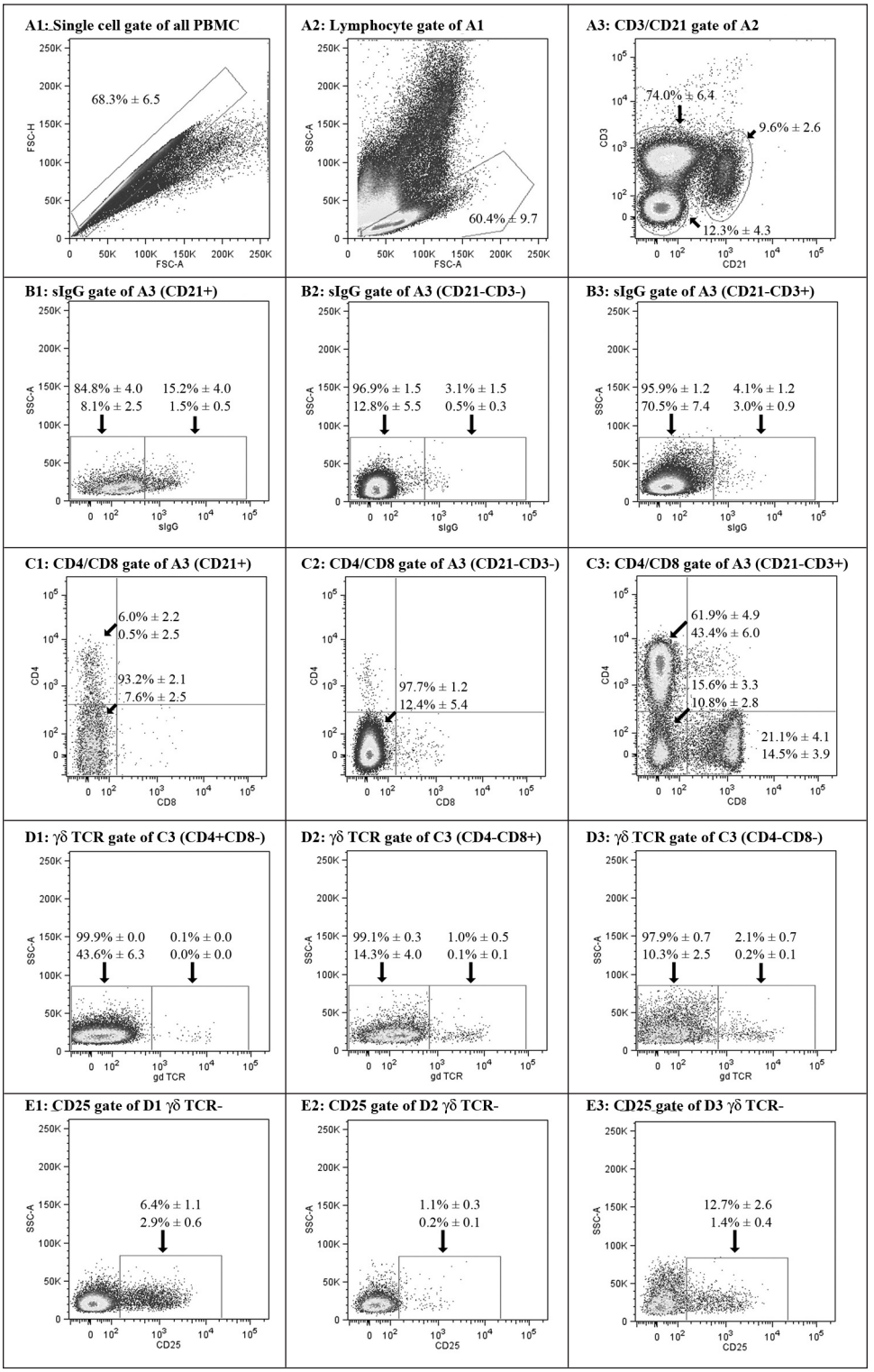

Figure 1 Graphic gating strategy of a representative sample. Percentages of gated population of the parent population (first row of numbers) and of the total lymphocyte (A2) (second row of numbers in B, C, D, and E) are shown.

the majority of these cells also expressing CD3 fluorescence (plot A3). Another 10.3% of the PBLs were unidentified (CD21 – CD3 + CD4 – CD8 – γδ TCR–). The 2.9% of the CD21 – CD3 + CD4 + CD8 – γδ TCR–

Table II *Mean and Standard Deviation of Canine Lymphocyte Subset*

Plot	CD21	CD3	CD4	CD8	Other markers	% of parent population		% of total lymphocytes		Proposed phenotype included
						Mean	SD	Mean	SD	
A1						68.3	6.5			Single cells
A2						60.4	9.7			Lymphocytes
A3	CD21–	CD3+				74.0	6.4			All T cells
A3	CD21–	CD3–				12.3	4.3			
A3	CD21+					9.6	2.6			All B cells
B1	CD21+				sIgG+	15.2	4.0	1.5	0.5	IgG memory B cells
B1	CD21+				sIgG–	84.8	4.0	8.1	2.5	Virgin and non-IgG memory B cells
B2	CD21–	CD3–			sIgG+	3.1	1.5	0.5	0.3	
B2	CD21–	CD3–			sIgG–	96.9	1.5	12.8	5.5	
B3	CD21–	CD3+			sIgG+	4.1	1.2	3.0	0.9	
B3	CD21–	CD3+			sIgG–	95.9	1.2	70.5	7.4	NK cells
C1	CD21+		CD4+	CD8–		6.0	2.2	0.5	0.2	
C1	CD21+		CD4–	CD8–		93.2	2.1	7.6	2.5	
C2	CD21–	CD3–	CD4–	CD8–		97.7	1.2	12.4	5.4	Non-adherent monocytes
C3	CD21–	CD3+	CD4+	CD8–		61.9	4.9	43.4	6.0	
C3	CD21–	CD3+	CD4–	CD8+		21.1	4.1	14.5	3.9	
C3	CD21–	CD3+	CD4–	CD8–		15.6	3.3	10.8	2.8	
D1	CD21–	CD3+	CD4+	CD8–	γδ TCR–	99.9	0.0	43.6	6.3	T helper cells
D2	CD21–	CD3+	CD4–	CD8+	γδ TCR–	99.1	0.3	14.3	4.0	Cytotoxic T cells
D3	CD21–	CD3+	CD4–	CD8–	γδ TCR+	2.1	0.7	0.2	0.1	γδ TCR+ T cells
D3	CD21–	CD3+	CD4–	CD8–	γδ TCR–	97.9	0.7	10.3	2.5	Unidentified T cells
E1	CD21–	CD3+	CD4+	CD8–	CD25+	6.4	1.1	2.9	0.6	Treg
E2	CD21–	CD3+	CD4–	CD8+	CD25+	1.1	0.3	0.2	0.1	
E3	CD21–	CD3+	CD4–	CD8–	CD25+	12.7	2.6	1.4	0.4	

SD = standard deviation.

subset that expressed CD25 may represent regulatory T cells (Treg).

Discussion

The averaged percentages of CD3+, CD4+, CD8+, CD21+ and γδ TCR+ cells from the 26-week old Beagles were 74.0, 43.6, 14.3, 9.6, and 0.2, which were comparable to other reports. Faldyna et al[4] studied fresh PBMCs of 12 adult Beagles using single color flow cytometry and reported their subset percentages at 88.1, 46.1, 21.8, 12.1, and 2.1, respectively. Byrne et al[1] studied lymphocyte subset distribution also in Beagles using single color flow cytometry. They reported percentages of CD3+, CD4+, CD8+, and CD21+ at 79.5, 45.1, 17.0, and 22.7 in 2-year-old dogs and 79.5, 30.9, 18.9, and 13.8 in 9-year-old dogs. Faldyna et al[4] reported changes in lymphocyte subset distribution in different age groups using single color flow cytometry. The study in 38 Beagles of 6 age groups (newborn to > 5 years) found that the CD21+ percentage decreased while the CD8+ percentage increased with advancing age. The changes were more apparent in the first year of age. There were no age-related changes in CD4+ and γδ TCR+ percentages, so the CD4+/CD8+ ratio also decreased in older dogs. Villaescusa et al[10] performed a longitudinal study of PBL subsets of the same 13 Beagle dogs from 7–15 months of age using dual and triple color flow cytometry and reported a decrease of lymphocyte, CD3+, CD3+CD4+ and CD21+ subsets but an increase of CD3+CD8+ lymphocytes with increasing age. The findings were in agreement with the earlier studies. The authors concluded that the changes related to the maturation of lymphocyte subsets occurred during early life in Beagle dogs.

The CD21 molecule or the complement receptor (CR2) has been used to identify B cells, and the CD3 molecule has been used to identify T cells.[1,4,7,10] The anti-canine CD21 and anti-canine CD3 mAbs used in this study were from the anti-canine CD21 clone CA2.1D6 and anti-canine CD3 clone CA17.2A12 from Dr. Peter Moore's laboratory (University of California, Davis, U.S.A.). The companies that market these antibodies (AbD Serotec, Raleigh,

North Carolina, U.S.A. and United States Biological, Salem, Massachusetts) recommended that these 2 clones should not be used together in dual color flow cytometry due to nonspecific interactions between the 2 reagents. Both companies confirmed their recommendation when contacted but did not disclose the nature of the interference. There were other reports that studied the nonspecific interaction between these same CD21/CD3 mAbs in canine PBMC by flow cytometry. Yagihara et al[2] did not observe any nonspecific interaction in their preliminary study. Villaescusa et al[7] stained the PBMCs from clinically normal German shepherd (n = 11) and Labrador retriever (n = 12) dogs using mAbs from the same CD3 and CD21 mAb clones. The authors did not mention any nonspecific interaction of the 2 mAbs or the coexpression of the 2 markers but later[10] stained Beagle dog PBMCs with the same direct conjugated CD3-FITC and CD21-PE mAb clones in 2 steps with an additional step of 1% bovine serum albumin in PBS in between. The multiparameter flow cytometry analysis in this study showed a well-separated CD21+ population with the majority of these cells also expressing CD3 fluorescence and could be interpreted as a nonspecific interaction between the 2 antibodies (plot A3). When the CD21+ population was further analyzed, there were only a few cells that coexpressed the CD4 marker (6.0% of CD21+ cells/0.5% of total lymphocytes) but no substantial CD8+ or γδ TCR coexpression with CD21 (plot C1). The coexpression of CD21 and CD3 was reported in 29% of the dogs of many different breeds (no Beagles) with lymphoproliferative disorders characterized by small-cell lymphocytosis.[2] Wilkerson et al[8] found the same coexpression of these B and T cell markers on canine lymphoma cells from 29 different dog breeds. They concluded that the aberrant CD molecule expression is not uncommon in canine lymphomas and can be used as diagnostic marker for malignancy. They utilized the dual staining of these 2 mAb clones to diagnose canine B cell, T cell, or mixed cell lymphoma from lymph node–derived lymphocytes. We suggest that these 2 commonly used CD21/CD3 mAb clones can and should be used together with the proper steps of lymphocyte identification gating during the flow data analysis. If this FITC-conjugated CD3 mAb clone is used without the CD21 mAb, the CD3+ cell gating may include a substantial number of CD21+ cells. This could contribute to the much higher averaged CD3+ subset percentages in Beagle dogs reported by Byrne et al[1] and Faldyna et al.[4] Both reports stained PBMC with the same CD3 mAb clone as used in this study and had FITC as the secondary conjugate in a single color flow cytometry analysis. They identified 79.5% and 88.1% CD3+ subset, which was 5.5% and 14.1% higher than our results from multicolor staining and analyzing by gating CD3 together with CD21 marker.

This study and those of Byrne et al[1] and Faldyna et al[4] used FITC as direct or secondary conjugate for the CD3 marker. FITC has a similar range of fluorescent spectrum as normal tissue endogenous autofluorescence, which originates mainly from mitochondria and lysosomes.[13] Vremec et al[14] enriched mouse dendritic cells and found up to 30% of autofluorescent cells, which were brightest in PE and FITC channels of flow cytometer. Mitchell et al[15] found multiple sources of autofluorescence present in most myeloid cells. Even though autofluorescence has not been mentioned as a significant problem in lymphoid cell research, it is possible that FITC fluorescence detected in the canine CD21+ population may be due to endogenous autofluorescence rather than the nonspecific binding of the CD3 mAb to CD21+ cells. In our unstained canine PBMC control, 2.17% and 0.45% of the total lymphocytes expressed fluorescence in FITC and PE channels, while almost no cell had any expression in other channels (data not shown). Other choices of fluorochrome for CD3 and other canine CD3-specific mAb clones should be screened to observe whether they also detect CD3 fluorochrome on a high percentage of CD21+ cells.

Our study detected only 0.2% of total lymphocytes that were γδ TCR+ cells. Faldyna et al[4] used the same mAb to canine γδ TCR which stained 2.1% of the lymphocytes. However, the CD21−CD3+ CD4−CD8− γδ TCR− subset represented 10.3% of all lymphocytes (plot D3), and 12.7% of those expressed CD25 (plot E3). These CD21−CD3+CD4− CD8− γδ TCR− cells remained unidentified. These cells may be speculated to be γδ TCR cells that did not express the surface γδ TCR antigen detectable by the γδ TCR mAb used in this study.

The polyclonal antibody (pAb) to canine IgG stained 15.2% of CD21+ cells in this study. Coffman and Cohn[16] identified the B cells with IgG1 on their surface as memory B cells, the precursors of IgG1 antibody producing cells, while the virgin B cells had IgM and IgD on their surface. This memory B cells marker helps differentiate IgG memory B cells from virgin B cells and showed the ability of B cells

to be primed either by vaccination or natural exposure.

The CD21–CD3– (non-B, non-T) lymphocyte subset possibly includes contaminating nonadherent monocytes. This group of Beagle dogs had 12.3% of this lymphocyte subset similar to 12% reported in Labrador retriever and 17% in German shepherd dogs.[10] In the Beagle dogs study by Villaescusa et al[7] the PBMC without CD3 and CD21 cell markers varied from approximately 16% at 7 months of age and 21.5% 34 weeks later. The same cell population percentages in their control adult Beagle dogs >4 years of age in the same period of the study were 20.3% and 27.9%, respectively. The decrease in B cell percentage in the blood by age may contribute to the relative increase in this lymphocyte subset percentage over time. Huang et al[17] described canine killer NK cells as $CD5^{lo}$ with CD3+ and varying levels of CD8+. We did not include NK cell specific marker in this study and thus cannot accurately identify the NK cell population.

The CD4+CD25+ population was identified as a part of the Treg phenotype in humans[18,19] and other animal species[20-22] when coexpressed with the transcription factor forkhead box P3 (FoxP3). There was 2.9% of the CD21–CD3+CD4+CD8– subset that expressed CD25 in the circulation of the dogs in this study (plot E1). We did not stain for FoxP3 antigen and thus cannot confirm whether they are part of the natural Treg. Daneshvar et al[23] studied the alteration of canine mononuclear cell subpopulations after immunization with gentamicin-attenuated *Leishmania infantum*. They identified CD4+CD25+ T cells in lymph node as Treg, without using FoxP3 staining. Felix De Lima et al[24] reported that the reduction of the same phenotype of CD4+CD25+ Treg cells in lymph nodes correlated with protection against leishmaniasis.

This study did not include staining to exclude dead cells due to the utilization of fresh cells, stained within 6 hours of blood collection. The dead cell staining would occupy a useful fluorochrome channel of the flow cytometer and reduced the possible choices of important lymphocyte markers.

Conclusions

The novel multiparameter flow cytometry presented here is a powerful tool to increase the accuracy of lymphocyte phenotyping by simultaneously staining lymphocytes with specific antibodies to 6 B and T cell markers, which made it possible to identify B and T cell phenotypes and their distribution directly related to each other. Careful selection of compatible antibody/conjugate panel and instrument compensation are necessary to obtain the optimum signals for all fluorochromes used in the system. Different antibody panels can be designed to define more specific lymphocyte subsets and their functions. The high number of dogs (n = 79) and number of acquired cells from each sample (averaged 1.9×10^5 cells) in this study allowed the detection of minor lymphocyte subsets in this group of dogs. These results presented baseline normal canine lymphocyte phenotypes. This report does not represent dogs of different breed, age group, or physiological condition and should be interpreted according to the materials and methods described.

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References

1. Byrne KM, Kim HW, Chew BP, Reinhart GA, Hayek MG: A standardized gating technique for the generation of flow cytometry data for normal canine and normal feline blood lymphocytes. Vet Immunol Immunopathol 2000;73:167-182
2. Yagihara H, Uematsu Y, Koike A, Tamura K, Isotani M, Yamaguchi T, Ono K, Washizu T, Bonkobara M: Immunophenotyping and gene rearrangement analysis in dogs with lymphoproliferative disorders characterized by small-cell lymphocytosis. J Vet Diagn Invest 2009;21:197-202
3. Moreno J, Nieto J, Chamizo C, Gonzalez F, Blanco F, Barker DC, Alva J: The immune response and PBMC subsets in canine visceral leishmaniasis before, and after, chemotherapy. Vet Immunol Immunopathol 1999;71:181-195
4. Faldyna M, Leva L, Knotigova P, Toman M: Lymphocyte subsets in peripheral blood of dogs: A flow cytometric study. Vet Immunol Immunopathol 2001;82:23-37
5. Blount DG, Pritchard DI, Heaton PR: Age-related alterations to immune parameters in Labrador retriever dogs. Vet Immunol Immunopathol 2005;108:399-407
6. Reis AB, Carneiro CM, Carvalho MG, Teixeira-Carvalho A, Giunchetti RC, Mayrink W, Genaro O, Correa-Oliveira R, Martins-Filho OA: Establishment of a microplate assay for flow cytometric assessment and its use for the evaluation of age-related phenotypic changes in canine whole blood leukocytes. Vet Immunol Immunopathol 2005;103:173-185
7. Villaescusa A, García-Sancho M, Rodríguez-Franco F, Sainz A: Early-life longitudinal survey of peripheral blood lymphocyte subsets in Beagle dogs. Vet Immunol Immunopathol 2012;149:126-131
8. Wilkerson MJ, Dolce K, Koopman T, Shuman W, Chun R, Garrett L, Barber L, Avery A: Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules. Vet Immunol Immunopathol 2005;106:179-196

9. Itoh H, Horiuchi Y, Nagasaki T, Sakonju I, Kakuta T, Fukushima U, Uchide T, Yamashita M, Kuwabara M, Yusa S, Takase K: Evaluation of immunological status in tumor-bearing dogs. Vet Immunol Immunopathol 2009;132:85-90
10. Villaescusa A, García-Sancho M, Delgado AM, Tesouro MÁ, Rodríguez-Franco F, Sainz A: Immunophenotypic evaluation of working Labrador Retrievers and German Shepherd dogs living in the same environment. Vet J 2012;193:602-605
11. Faldyna M, Samankova P, Leva L, Cerny J, Oujezdska J, Rehakova Z, Sinkora J: Cross-reactive anti-human monoclonal antibodies as a tool for B-cell identification in dogs and pigs. Vet Immunol Immunopathol 2007;119:56-62
12. Loken MR, Brosnan JM, Bach BA, Ault KA: Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. Cytometry 1990;11:453-459
13. Monici M: Cell and tissue autofluorescence research and diagnostic applications. Biotech Ann Rev 2005;11:227-256
14. Vremec D, Pooley J, Hochrein H, Wu L, Shortman K: CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. J Immunol 2000;164:2978-2986
15. Mitchell AJ, Pradel LC, Chasson L, Van RN, Grau GE, Hunt NH, Chimini G: Technical advance: Autofluorescence as a tool for myeloid cell analysis. J Leukocyte Biol 2010;88:597-603
16. Coffman RL, Cohn M: The class of surface immunoglobulin on virgin and memory B lymphocytes. J Immunol 1977;118: 1806-1815
17. Huang YC, Hung SW, Jan TR, Liao KW, Cheng CH, Wang YS, Chu RM: CD5-low expression lymphocytes in canine peripheral blood show characteristics of natural killer cells. J Leukoc Biol 2008;84:1501-1510
18. Sun L, Yi S, O'Connell PJ: IL-10 is required for human CD4(+)CD25(+) regulatory T cell-mediated suppression of xenogeneic proliferation. Immunol Cell Biol 2010;88:477-485
19. Fukumura Y, Takase M, Mitani K, Suda K, Imamhasan A, Nobukawa B, Ueda A, Abe H, Yao T: Amount of CD4+ CD25+ regulatory T cells in autoimmune pancreatitis and pilonidal sinus. Pancreas 2012;41:910-915
20. Zhang B, Zhang A, Qu Y, Liu J, Niu Z, Zhao Y: Development of mouse CD4(+)CD25(+)Foxp3(+) regulatory T cells in xenogeneic pig thymic grafts. Transpl Immunol 2009;20:180-185
21. Gerner W, Stadler M, Hammer SE, Klein D, Saalmuller A: Sensitive detection of Foxp3 expression in bovine lymphocytes by flow cytometry. Vet Immunol Immunopathol 2010; 138:154-158
22. Wongyanin P, Buranapraditkun S, Chokeshai-Usaha K, Thanawonguwech R, Suradhat: Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). Vet Immunol Immunopathol 2010;133:170-182
23. Daneshvar H, Sedghy F, Dabiri S, Kamiabi H, Molaei MM, Phillips S, Burchmore R: Alteration in mononuclear cell subpopulations in dogs immunized with gentamicin-attenuated Leishmania infantum. Parasitology 2012;21:1-8
24. Felix De Lima VM, Ikeda FA, Rossi CN, Feitosa MM, Vasconcelos RO, Nunes CM, Goto H: Diminished CD4+/CD25+ T cell and increased IFN-gamma levels occur in dogs vaccinated with Leishmune in an endemic area for visceral leishmaniasis. Vet Immunol Immunopathol 2010;135:296-302