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Peripheral blood lymphocyte subtypes in dogs with different stages of myxomatous mitral valve disease

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Abstract

Background: Data on alterations in peripheral blood lymphocyte (PBL) subtypes in dogs with myxomatous mitral valve disease (MMVD) is lacking.

Objectives: To investigate PBL subtypes and their correlation with parameters of inflammation and MMVD progression markers in dogs with different stages of MMVD.

Animals: Seventy-eight client-owned dogs: 65 with MMVD (American College of Veterinary Internal Medicine [ACVIM] classification stages B2, C, and D) and 13 healthy controls.

Methods: Prospective cross-sectional study. Complete cardiac assessment, flow cytometry (T lymphocytes [CD3+], their subtypes [CD3+CD4+, CD3+CD8+, CD3+CD4+CD8+, CD3+CD4−CD8−], and B lymphocytes [CD45+CD21+]) and measurement of N-terminal pro B-type natriuretic peptide, cardiac troponin I, and C-reactive protein concentrations were performed.

Results: The percentage of CD3+CD4+ lymphocytes was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .003$), the percentage of CD3+CD8+ lymphocytes was significantly higher in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$), CD3+CD8+ lymphocyte concentration was significantly higher in unstable ACVIM C and D patients ($P = .05$), and the CD3+CD4+/CD3+CD8+ ratio was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$) compared with healthy controls.

Conclusions and Clinical Importance: The percentages of CD3+CD4+ and CD3+CD8+ PBL and CD4+/CD8+ ratio were altered in MMVD dogs with congestive heart failure (ACVIM C, D), but not in ACVIM B2, suggesting involvement of these PBL subtypes in the pathogenesis of congestive heart failure in dogs with MMVD.

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; CHF, congestive heart failure; CRP, C-reactive protein; cTnI, cardiac troponin I; DCM, dilated cardiomyopathy; DN T, double negative T (lymphocytes); DP T, double positive T (lymphocytes); MMVD, myxomatous mitral valve disease; NT-proBNP, N-terminal pro B-type natriuretic peptide; PBL, peripheral blood lymphocyte; WBC, white blood cell.

KEYWORDS
canine, CD21 B lymphocytes, CD3 T lymphocytes, CD4 T helper lymphocytes, CD45, CD8 cytotoxic T lymphocytes, congestive heart failure, degenerative mitral valve disease, flow cytometry

1 | INTRODUCTION

Myxomatous mitral valve disease (MMVD) is common in older dogs,[1] and can progress over time to congestive heart failure (CHF). Several pathophysiological mechanisms are involved in the development and progression of CHF, including neurohumoral activation, immune activation, and inflammation, the latter being under intensive investigation in humans with heart failure.[2-6] Studies have shown that inflammation is also present in dogs with CHF.[7-11] One of the components of the inflammatory response is adaptive immunity, which consists of T lymphocytes (CD3+) including their subtypes, T helper (CD4+) and cytotoxic T lymphocytes (CD8+) and B lymphocytes (CD21+), which are the major subtypes of peripheral blood lymphocytes (PBLs). Lymphocytes are known to orchestrate inflammation by controlling other inflammatory cells and their responses. Their role in the context of cardiovascular disease has been studied extensively in mice and humans, and they are known to promote inflammation of the heart, apoptosis, fibrosis, and remodeling.[12-15] Possibly reflecting this involvement, lymphocytes and their subtype concentrations and percentages have been found to be altered in peripheral blood in people with cardiovascular diseases.[16-19]

In contrast to studies in mice and humans, a single study has investigated changes in PBL subtypes in dogs with cardiac disease.[20] It was found that the percentages of both CD4+ peripheral blood mononuclear cells and CD8+ lymphocytes were decreased in dogs with heart failure caused by dilated cardiomyopathy (DCM) and MMVD compared to healthy dogs.[20] Another study,[21] presented only as an abstract, found a decrease in the percentage of CD4+ and an increase in the percentage of CD8+ lymphocyte subtypes in German shepherds with primary mitral insufficiency.

Our hypothesis was that PBL subtypes in dogs with MMVD (American College of Veterinary Internal Medicine [ACVIM] stages B2, C, D) change with the progression of the disease and differ from those of healthy dogs and that these changes are more pronounced in advanced disease. Therefore, our aim was to investigate changes in the concentrations and percentages of PBL subtypes by multicolor flow cytometry and to correlate PBL subtypes with a biomarker of heart disease severity (N-terminal pro-B type natriuretic peptide, [NT-proBNP]), a biomarker of myocardial damage (cardiac troponin I [cTnI]), the acute phase protein, C-reactive protein (CRP), and with neutrophils and monocytes as cells representing innate immunity, and selected echocardiographic and clinical variables.

2 | MATERIALS AND METHODS

2.1 | Study population

Seventy-eight client-owned dogs were included in this prospective cross-sectional study. Of these, 65 were dogs with various stages of MMVD with or without CHF and 13 were healthy control dogs. Dogs were presented to the cardiology service of the small animal clinic for evaluation (MMVD patients) or health assessment (control dogs) between August 2018 and November 2019.

We included dogs with MMVD of ACVIM classes B2, C, and D.[1] We excluded dogs with concomitant systemic disease (including metabolic, neoplastic, and inflammatory diseases), as well as those with local inflammatory conditions, and those that had received systemic or local immunosuppressive treatment or antibiotics in the past month. We excluded patients as necessary based on history, clinical examination findings, results of hematologic and biochemical analyses, echocardiography, and, if indicated, thoracic radiographs, ECG, abdominal ultrasound findings, and urinalysis. Dogs with asymptomatic MMVD without signs of cardiac remodeling (ACVIM stage B1) were not included. Diagnosis of MMVD and heart failure was made by an experienced veterinarian based on history, clinical examination findings, thoracic radiographs, ECG, and echocardiography using 2-dimensional, M-mode, color, and spectral Doppler modes (GE Vivid E9, General Electric Healthcare). Routine hematologic and biochemical analyses were performed in each dog.

We included healthy small- and medium-sized breed dogs of both sexes older than 5 years of age. They were considered healthy on the basis of clinical examination, echocardiography, and routine hematological and biochemical analyses.

Written informed consent was signed by the owners before enrollment in the study.

2.2 | Groups of MMVD patients in the study

The ACVIM classification was used to divide MMVD dogs into 3 groups. The ACVIM B2 group included asymptomatic treated or untreated dogs with cardiac remodeling and no signs of CHF. The second group included treated dogs in ACVIM stage C, but with stable (compensated) CHF only. The third group included treated dogs in ACVIM stages C and D with advanced decompensated (unstable) CHF, according to the presence of clinical signs such as tachypnea, tachycardia, radiographic signs of pulmonary edema, and echocardiographic signs of increased left atrial pressure.

2.3 | Blood sampling

Blood obtained by jugular or cephalic venipuncture was collected in tubes containing ethylenediaminetetraacetic acid (for CBC with white blood cell [WBC] differential count, flow cytometry, and NT-proBNP) and serum separator tubes (for routine biochemical, cTnI and CRP

analyses). Samples for routine blood analyses (CBC and WBC differential count, biochemistry) were processed by the in-house laboratory within 2 hours. Samples for flow cytometry were stored in the dark at room temperature (approximately 21°C) and analyzed within 24 hours. Samples for NT-proBNP, cTnI, and CRP measurements were centrifuged immediately at 1500*g* at 4°C for 15 minutes (for NT-proBNP) or after complete coagulation at 1300*g* at room temperature for 10 minutes (for cTnI and CRP). Obtained plasma (for NT-proBNP) and serum (for cTnI and CRP) samples were separated and stored at −80°C until analysis.

2.4 | Routine hematological and biochemistry analyses

Hematological analyses were performed using an automated laser-based hematology analyzer (ADVIA 120, Siemens, Munich, Germany). Blood biochemistry was performed using an automated biochemistry analyzer (RX Daytona, Randox, Crumlin, United Kingdom; glucose, urea, creatinine, alanine aminotransferase, alkaline phosphatase, total protein, albumin) and electrolyte analyzer (ILyte, Instrumentation Laboratory, Lexington, Massachusetts; sodium, potassium, chloride).

2.5 | Flow cytometry

Multicolor flow cytometry was used to analyze fresh whole blood samples and determine the percentages of T lymphocytes (CD3+), T helper lymphocytes (CD3+CD4+), cytotoxic T lymphocytes (CD3+CD8+), double positive T (DP T) lymphocytes (CD3+CD4+CD8+), double negative T (DN T) lymphocytes (CD3+CD4−CD8−), and B lymphocytes (CD45+CD21+). Cells were labeled with monoclonal rat and mouse anti-canine antibodies against CD3 (clone CA17.2A12), CD4 (clone YKIX302.9), CD8 (clone YCATE55.9) in a mix (CD3:FITC/CD4:RPE/CD8:Alexa Fluor 647, ref: TC014), CD45 (clone YKIX716.13; Alexa Fluor 488, ref: MCA1042A488), and CD21 (clone CA2.1D6; Alexa Fluor 647, ref: MCA1781A647). All monoclonal antibodies used were manufactured by Bio-Rad Laboratories Inc (Hercules, California). The whole blood lysis method was used according to the manufacturer's protocol.[22] Blood samples were incubated for 30 minutes at 2°C to 8°C with a commercially available triple color cocktail of monoclonal antibodies against CD3, CD4, CD8 in 1 tube and a combination of anti-CD45 and anti-CD21 monoclonal antibodies in another tube. Erythrocytes were lysed using a commercially available red blood cell lysing solution (BD FACS Lysing Solution; BD Biosciences, San Jose, California; ref: 349202) according to the manufacturer's instructions. The samples were centrifuged and the supernatant discarded. Then, 0.1% bovine serum albumin (ref: A9418; Sigma-Aldrich, St. Louis, Missouri)/phosphate-buffered saline (ref: P4417; Sigma-Aldrich) was added and, after centrifugation, the cells were resuspended in phosphate-buffered saline solution and analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, California) with FACSDiva software, version 8.0.1 (BD Biosciences). The flow cytometer was calibrated using BD FACSDiva CS&T Research Beads (BD Biosciences). Compensation controls were performed to correct for fluorescence spillover.

Forward and side scatters were used to gate PBLs based on their size and granularity. For each sample, data for 100 000 events in the lymphocyte gate were acquired for the first tube (CD3, CD4, CD8), and data for 50 000 events were acquired for another tube (CD45, CD21). Forward scatter, side scatter, and fluorescence were used to calculate percentages of lymphocyte subtypes. The absolute concentrations of PBL subtypes were calculated based on CBC and WBC differential count and flow cytometry results.

2.6 | Circulating biomarkers

Plasma NT-proBNP concentrations (pmol/L) were measured using an IDEXX ELISA (IDEXX Laboratories, Leipzig, Germany). Serum cTnI concentrations (μg/L) were measured using a high-sensitivity immunoassay (ADVIA Centaur TnI-Ultra; Siemens). Serum CRP concentrations (ng/mL) were measured using an ELISA (Canine CRP ELISA; Alpco, Salem, New Hampshire).

2.7 | Statistical analysis

Data were analyzed using commercially available software (IBM SPSS 24.0, Chicago, Illinois). The Shapiro-Wilk test was used to determine data distribution. On the basis of the findings, parametric tests or nonparametric tests were used to compare data among groups of dogs. Accordingly, 1-way analysis of variance with post hoc Tukey honestly significant difference (for normally distributed data) or Kruskal-Wallis test followed by pairwise comparisons and Bonferroni adjustments (for non-normally distributed data) were performed to compare variables between groups of patients and healthy dogs. Sex differences were evaluated using Chi-squared analyses. Correlations between variables were performed using the Spearman test (nonparametric data) or Pearson test (parametric data). Results for non-normally distributed data are reported as median and interquartile range and for normally distributed data as mean and SD. For the purpose of correlation analyses, MMVD dogs were divided into 2 groups: ACVIM B2 group and CHF group (all dogs with ACVIM C and D combined). Values of $P < .05$ were considered significant.

3 | RESULTS

One-hundred seventy-three MMVD patients were screened for eligibility, 108 of which were not included in the study because they did not meet the inclusion criteria (n = 99) or the owners did not want to participate in the study (n = 9). At the time of enrollment, 28 healthy dogs were recruited and tested for suitability as control dogs, of which 15 were excluded because they did not meet the inclusion criteria (because of subclinical heart disease [n = 13] or other

pathology [n = 2]). A total of 65 MMVD dogs and 13 healthy control dogs were included in the study. Baseline characteristics of the included dogs are shown in Table 1.

There was a significant age difference between each group of MMVD patients and healthy control dogs, with the healthy dogs being significantly younger than ACVIM B2 ($P = .02$), stable ACVIM C ($P < .001$), and unstable ACVIM C and D dogs ($P = .001$), but no significant age difference was found among the 3 groups of MMVD patients ($P > .05$). It was difficult to include old healthy dogs and many were rejected because of various health problems (mostly asymptomatic MMVD). No significant differences in sex ($P = .12$) and weight ($P > .05$) were found between the cardiac patient and healthy dog groups.

Lymphocyte and their subtype concentrations and percentages are presented in Table 2 and Figures 1 and 2. The percentage of CD3+CD4+ lymphocytes was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .003$) compared with healthy dogs, but no significant differences in CD3+CD4+ lymphocyte concentrations were found between the patient and healthy dog groups. The percentage of CD3+CD8+ lymphocytes was significantly higher in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$) compared with healthy controls, and the CD3+CD8+ lymphocyte concentration was significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .05$). The CD3+CD4+/CD3+CD8+ ratio was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$) compared with healthy dogs. The CD3+CD4+CD8+ and CD3+CD4−CD8− lymphocyte percentages and concentrations were not significantly different between cardiac patient and healthy dog groups. The percentages and concentrations

TABLE 1 Baseline characteristics of 65 dogs with MMVD and 13 healthy dogs

Group	ACVIM B2	ACVIM C stable	ACVIM C and D unstable	Healthy dogs
Number	20	24	21	13
Sex (f/m)	6/14	7/17	11/10	8/5
Spayed/neutered	6/6	2/7	9/2	4/3
Age (years)				
Mean ± SD	10.0 ± 2.1[a]	11.2 ± 1.8[a]	10.7 ± 1.8[a]	7.9 ± 2.2
Range (min-max)	6.0-14.2	7.6-14.3	7.4-13.5	5.0-12.4
Weight (kg)				
Median (IQR)	7.9 (6.1-12.3)	8.3 (5.5-12.5)	7.6 (5.0-10.9)	7.0 (5.1-13.3)
Range (min-max)	3.0-16.8	4.4-33.0	2.4-44.7	2.8-13.5
HR (bpm)				
Mean ± SD	116.0 ± 17.6	128.3 ± 21.2	140.2 ± 20.2	111.5 ± 32.6
Range (min-max)	80.0-150.0	90.0-180.0	100.0-180.0	70.0-200.0
Breeds	4 CKCS, 2 MB, 2 CHI, 2 SHI, 2 TT, 2 WH, 1 PEK, 1 YT, 1 MLT, 1 ECS, 1 STF, 1 CC	8 MB, 4 CKCS, 3 MP, 2 PEK, 1 CHI, 1 YT, 1 TT, 1 MLT, 1 ECS, 1 POM, 1 APBT	7 CKCS, 3 MB, 2 CHI, 1 PEK, 1 YT, 1 MLT, 1 AT, 1 JCH, 1 COTON, 1 IG, 1 DH, 1 GSD	6 MB, 4 SHI, 1 YT, 1 TS, 1 MSCH
Treatment				
Pimobendan	9	24	21	—
ACE inhibitor	6	24	21	—
Furosemide/ torasemide	—	24	21	—
Spironolactone	3	11	9	—
Theophylline	—	2	1	—
Sildenafil	—	1	1	—
Amlodipine	—	1	1	—
Digoxin	—	1	1	—
Potassium chloride	—	1	—	-

Abbreviations: ACE inhibitor, angiotensin-converting enzyme inhibitor; ACVIM, American College of Veterinary Internal Medicine; APBT, American Pit Bull Terrier; AT, Airedale Terrier; bpm, beats per minute; CC, Chinese Crested Dog; CHI, Chihuahua; CKCS, Cavalier King Charles Spaniel; COTON, Coton de Tulear; DH, Dachshund; ECS, English Cocker Spaniel; f, female; GSD, German Shepherd; HR, heart rate; IG, Italian Greyhound; IQR, interquartile range; JCH, Japanese Chin; m, male; MB, mixed breed dog; MLT, Maltese; MMVD, myxomatous mitral valve disease; MP, Miniature Poodle; MSCH, Miniature Schnauzer; PEK, Pekingese; POM, Pomeranian; SHI, Shi Tzu; STF, Staffordshire Terrier; TS, Tibetan Spaniel; TT, Tibetan Terrier; WH, Whippet; YT, Yorkshire Terrier.

[a]Significant difference in comparison to healthy dogs.

TABLE 2 Lymphocytes and their subtype's percentages and concentrations in dogs with MMVD and healthy dogs

Group	ACVIM B2	ACVIM C stable	ACVIM C and D unstable	Healthy dogs
Lymphocytes, total (%)				
Mean ± SD	21.84 ± 6.62	20.19 ± 6.38	21.19 ± 7.09	22.60 ± 4.87
Range (min-max)	11.10-31.70	7.30-32.70	6.80-32.90	15.30-32.40
Lymphocytes, total ($\times 10^9$/L)				
Mean ± SD	1.74 ± 0.64	2.01 ± 0.70	2.11 ± 0.61	1.80 ± 0.60
Range (min-max)	0.80-3.00	0.86-3.87	1.25-3.26	0.98-2.97
CD3 (%)				
Median (IQR)	69.05 (60.38-75.00)	67.25 (51.95-74.00)	68.00 (52.65-74.30)	60.10 (53.50-69.50)
Range (min-max)	38.70-82.70	25.10-83.50	12.60-82.70	41.80-76.00
CD3+ ($\times 10^9$/L)				
Mean ± SD	1.16 ± 0.47	1.24 ± 0.46	1.27 ± 0.53	1.10 ± 0.40
Range (min-max)	0.39-2.23	0.36-2.07	0.35-2.43	0.61-1.81
CD3+CD4+ (%)				
Mean ± SD	47.91 ± 11.66	43.55 ± 8.48[a]	41.78 ± 12.49[a]	55.40 ± 8.91
Range (min-max)	17.00-70.70	31.00-58.50	17.10-65.80	42.80-71.80
CD3+CD4+ ($\times 10^9$/L)				
Mean ± SD	0.56 ± 0.28	0.54 ± 0.22	0.50 ± 0.21	0.60 ± 0.20
Range (min-max)	0.18-1.28	0.14-0.99	0.17-0.93	0.37-1.00
CD3+CD8+ (%)				
Mean ± SD	35.62 ± 12.67	39.66 ± 11.47[a]	40.67 ± 15.70[a]	26.05 ± 8.48
Range (min-max)	16.50-73.20	20.90-61.60	17.70-76.70	13.70-41.50
CD3+CD8+ ($\times 10^9$/L)				
Mean ± SD	0.40 ± 0.19	0.50 ± 0.24	0.55 ± 0.41[a]	0.29 ± 0.15
Range (min-max)	0.12-0.78	0.17-0.93	0.10-1.86	0.08-0.58
CD3+CD4+/CD3+CD8+				
Median (IQR)	1.36 (1.04-2.08)	1.00[a] (0.75-1.71)	1.14[a] (0.60-1.84)	2.28 (1.45-3.20)
CD3+CD4+CD8+ (%)				
Median (IQR)	1.65 (1.10-2.55)	1.35 (0.83-3.15)	1.10 (0.90-1.80)	1.20 (0.90-1.55)
CD3+CD4+CD8+ ($\times 10^9$/L)				
Median (IQR)	0.02 (0.01-0.04)	0.02 (0.01-0.03)	0.01 (0.01-0.02)	0.01 (0.01-0.03)
CD3+CD4−CD8− (%)				
Mean ± SD	14.64 ± 5.52	14.20 ± 5.37	16.20 ± 7.77	17.15 ± 4.66
Range (min-max)	6.20-26.30	4.30-22.50	5.30-36.10	9.20-24.80
CD3+CD4−CD8− ($\times 10^9$/L)				
Median (IQR)	0.14 (0.09-0.26)	0.17 (0.11-0.24)	0.14 (0.11-0.30)	0.15 (0.10-0.32)
CD45+CD21+ (%)				
Mean ± SD	16.70 ± 6.68	18.63 ± 9.08	16.74 ± 7.08	15.08 ± 6.39
Range (min-max)	8.40-36.60	4.80-41.20	3.30-27.90	7.00-28.50
CD45+CD21+ ($\times 10^9$/L)				
Mean ± SD	0.30 ± 0.17	0.39 ± 0.27	0.35 ± 0.19	0.28 ± 0.17
Range (min-max)	0.11-0.67	0.06-1.19	0.09-0.91	0.09-0.68

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; IQR, interquartile range; MMVD, myxomatous mitral valve disease.
[a]Significant difference ($P < .05$) in comparison to healthy dogs.

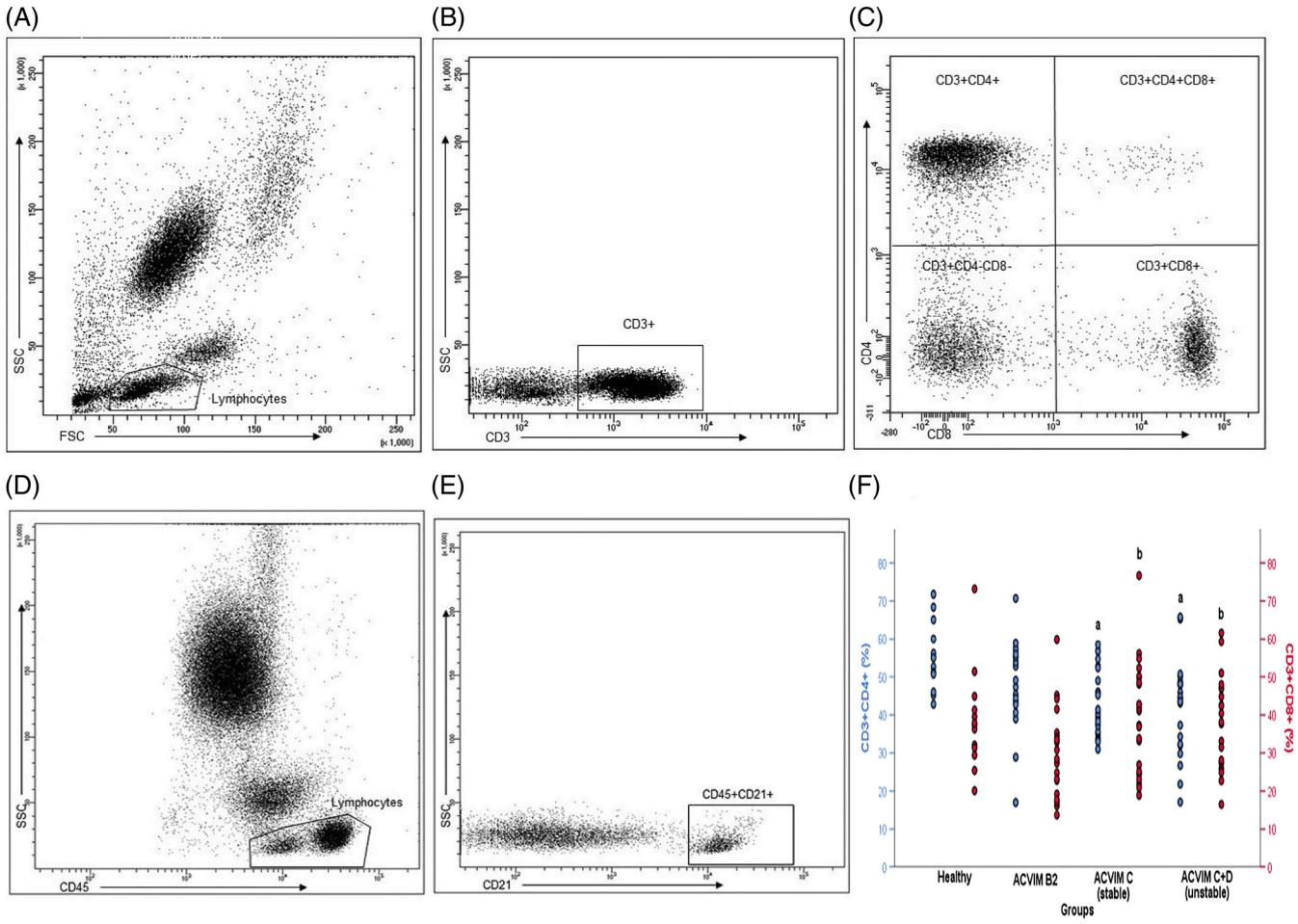

FIGURE 1 Immunophenotyping of canine PBL subtypes (representative sample) with gating strategies for total lymphocytes (SSC/FSC) (A), CD3+ T lymphocytes (B), CD3+CD4+ T helper lymphocytes, CD3+CD8+ cytotoxic T lymphocytes, CD3+CD4+CD8+ DP T lymphocytes, CD3+CD4−CD8− DN T lymphocytes (C), CD45+ lymphocytes (D), CD45+CD21+ B lymphocytes (E) are presented. Scatter plots showing CD3+CD4+ T helper lymphocyte and CD3+CD8+ cytotoxic T lymphocyte percentages in healthy controls and dogs in different stages of MMVD (F). [a]The median values of the stable and unstable CHF patients were significantly lower ($P < .05$) than that of the healthy dogs. [b]The median values of the stable and unstable CHF patients were significantly higher ($P < .05$) than that of the healthy dogs. CD, cluster of differentiation; CHF, congestive heart failure; DN T, double negative T; DP T, double positive T; FSC, forward scatter; MMVD, myxomatous mitral valve disease; PBL, peripheral blood lymphocyte; SSC, side scatter

of CD3+ and CD45+CD21+ lymphocytes and the percentages and concentrations of total lymphocytes did not differ significantly between the patient and healthy dog groups.

The percentages of neutrophils and monocytes (Table 3) did not differ significantly between the patient and healthy dog groups. Neutrophil concentration (Table 3) was significantly higher in unstable ACVIM C and D patients compared with ACVIM B2 patients ($P = .03$), and monocyte concentration (Table 3) was significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .03$) and ACVIM B2 patients ($P = .04$). Total WBC concentration (Table 3) was significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .04$) and ACVIM B2 stage dogs ($P = .02$).

Plasma NT-proBNP concentrations (Table 3) differed significantly between all groups of patients and healthy dogs with higher concentrations in advanced disease, except for ACVIM B2 compared to healthy dogs. Dogs in stable ACVIM C had significantly higher NT-proBNP concentrations than did healthy dogs ($P = .003$) and dogs in ACVIM B2 ($P = .002$). Dogs in the unstable ACVIM C and D groups had higher NT-proBNP concentrations than did healthy dogs ($P < .001$), ACVIM B2 patients ($P < .001$), and stable ACVIM C patients ($P = .04$). Serum cTnI concentrations (Table 3) were significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .01$) and ACVIM B2 patients ($P = .001$). Serum CRP concentrations (Table 3) did not differ significantly between groups of MMVD patients and healthy dogs.

The concentration of CD3+CD4+ lymphocytes correlated significantly and negatively with the percentage of neutrophils in CHF patients (combined stages stable ACVIM C and unstable ACVIM C and D; $r = -0.36$, $P = .01$) and in ACVIM B2 patients ($r = -0.533$, $P = .02$), but not in healthy dogs, and positively with monocyte concentration ($r = 0.318$, $P = .03$) in CHF dogs, but not in ACVIM B2 and

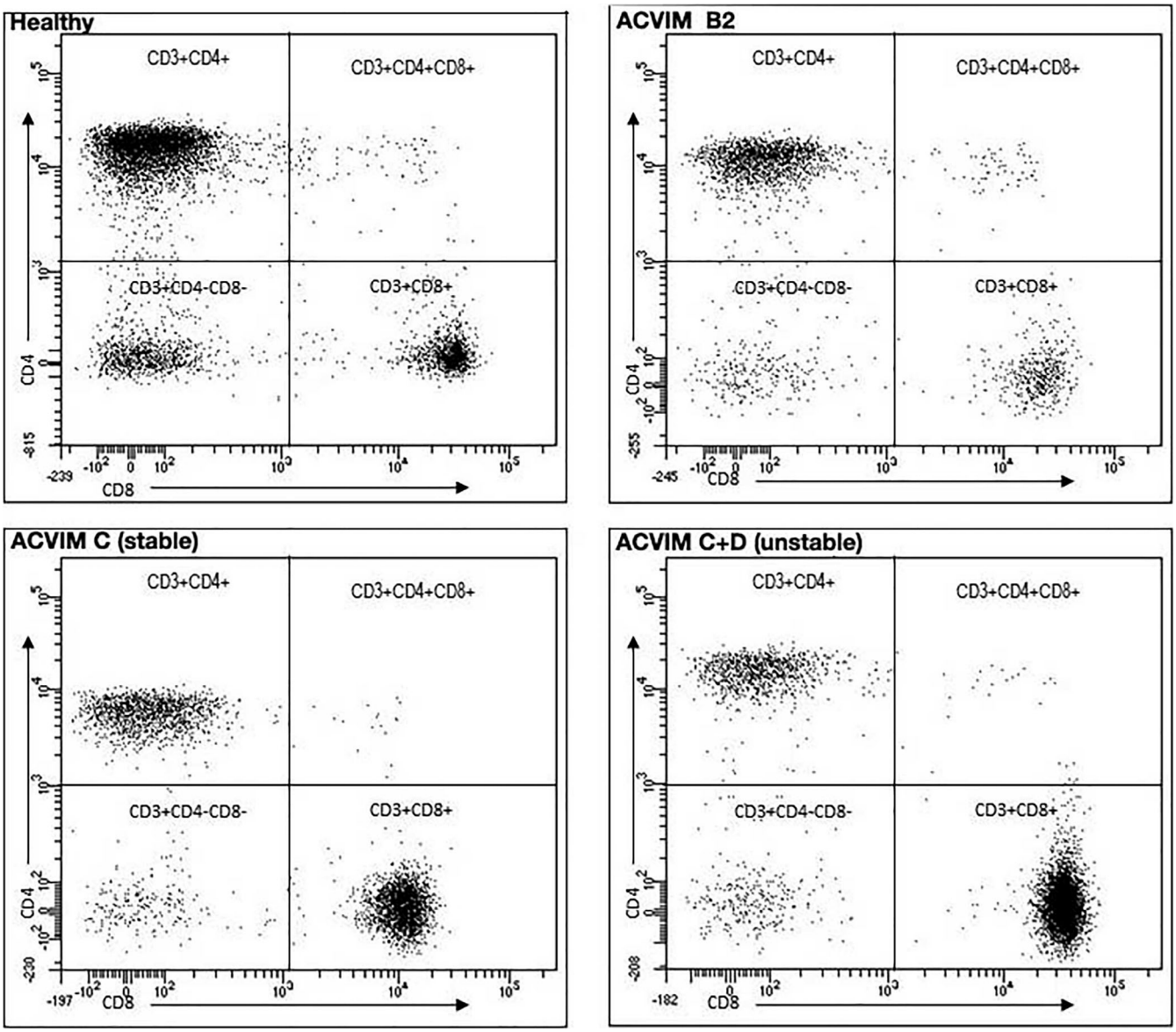

FIGURE 2 Representative flow cytometry plots showing CD3+CD4+ T helper lymphocytes, CD3+CD8+ cytotoxic T lymphocytes, CD3+CD4+CD8+ DP T lymphocytes, and CD3+CD4−CD8− DN T lymphocytes in groups of dogs included in our study—healthy control dogs and dogs with different stages of myxomatous mitral valve disease. DN T, double negative T (lymphocytes); DP T, double positive T (lymphocytes)

healthy controls. The CD3+CD4+CD8+ lymphocyte concentration correlated positively with serum CRP concentration ($r = 0.343$, $P = .02$) in CHF patients, whereas no such correlation was found in ACVIM B2 and healthy controls. The CD3+ lymphocyte concentration correlated negatively with neutrophil percentage in CHF dogs ($r = -0.397$, $P = .01$) and in ACVIM B2 stage ($r = -0.672$, $P = .001$), but not in healthy controls, and positively with monocyte concentration in CHF ($r = 0.328$, $P = .03$), but not in ACVIM B2 or healthy subjects. A significant negative correlation was found between CD45+CD21+ lymphocyte concentration and neutrophil percentage in CHF dogs ($r = -0.406$, $P = .01$) and ACVIM B2 dogs ($r = -0.564$, $P = .01$), but not in healthy dogs. Total lymphocyte percentage correlated negatively with serum CRP concentration in CHF dogs ($r = -0.370$, $P = .01$) and ACVIM B2 dogs ($r = -0.555$, $P = .01$), but not in healthy dogs, and with WBC concentration ($r = -0.563$, $P = .00$) and heart rate ($r = -0.352$, $P = .02$) only in CHF dogs, but not in ACVIM B2 or healthy dogs. Total lymphocyte concentration correlated positively with E wave velocity ($r = 0.316$, $P = .04$) and A wave velocity ($r = 0.330$, $P = .04$) in dogs with CHF, but not in ACVIM B2 or healthy dogs.

In our study, correlations between PBL subtypes and NT-proBNP were not found in any of the MMVD groups or in healthy control dogs. Cardiac troponin I correlated significantly and negatively with the percentage of CD3+CD4−CD8− lymphocytes ($r = -0.451$, $P = .05$) and the concentration of CD45+CD21+ lymphocytes ($r = -0.464$, $P = .04$) in the ACVIM B2 group, but no correlations were found between cTnI and PBL subtypes in dogs with heart failure (combined groups of stable and unstable CHF patients) or in healthy subjects.

TABLE 3 Markers of MMVD progression and inflammation in dogs with MMVD and healthy dogs

Group	ACVIM B2	ACVIM C stable	ACVIM C and D unstable	Healthy dogs
NT-proBNP (pmol/L)				
Median	1324	3122[a,b]	10112[a,b,c]	1428
IQR	880-2107	2284-5599	4888-16586	1031-1742
Cardiac TnI (μg/L)				
Median	0.044	0.082	0.132[a,b]	0.025
IQR	0.032-0.082	0.043-0.130	0.067-0.259	0.012-0.212
CRP (mg/L)				
Median	1.653	2.704	3.559	1.681
IQR	1.022-2.750	1.403-5.485	1.656-6.555	1.164-3.122
WBC ($\times 10^9$/L)				
Mean ± SD	8.11 ± 2.10	10.23 ± 2.56	11.10 ± 4.83[a,b]	8.05 ± 2.26
Range (min-max)	4.01-11.89	5.77-16.20	5.55-23.65	5.14-12.09
Neutrophils (%)				
Mean ± SD	68.09 ± 8.13	70.18 ± 7.63	69.13 ± 7.74	68.55 ± 5.56
Range (min-max)	47.50-79.70	57.50-84.00	57.80-83.90	59.60-77.80
Neutrophils ($\times 10^9$/L)				
Mean ± SD	5.51 ± 1.64	7.27 ± 2.27	7.93 ± 4.30[b]	5.51 ± 1.52
Range (min-max)	2.96-9.32	3.53-11.36	3.31-19.80	3.21-7.86
Monocytes (%)				
Mean ± SD	4.83 ± 1.66	5.16 ± 1.09	5.69 ± 1.36	4.92 ± 2.67
Range (min-max)	2.10-8.80	2.50-6.90	3.10-8.00	2.50-10.60
Monocytes ($\times 10^9$/L)				
Median	0.36	0.48	0.49[a,b]	0.33
IQR	0.23-0.51	0.38-0.69	0.39-0.83	0.25-0.48

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; cardiac TnI, cardiac troponin I; CRP, C-reactive protein; IQR, interquartile range; MMVD, myxomatous mitral valve disease; NT-proBNP, N-terminal pro B-type natriuretic peptide; WBCs, white blood cells.
[a]Significant difference ($P < .05$) in comparison to healthy dogs.
[b]Significant difference in comparison to dogs in ACVIM B2 stage.
[c]Significant difference in comparison to dogs in stable ACVIM C stage.

In addition, serum cTnI concentration correlated positively with plasma NT-proBNP concentration ($r = 0.474$, $P = .001$), serum CRP concentration ($r = 0.641$, $P = .00$), monocyte concentration ($r = 0.358$, $P = .02$), and left atrium to aorta ratio ($r = 0.329$, $P = .03$) in dogs with CHF, but not in ACVIM B2 stage and healthy dogs. Serum CRP concentration correlated positively with WBC concentration ($r = 0.456$, $P = .002$), neutrophil percentage ($r = 0.369$, $P = .01$), neutrophil concentration ($r = 0.477$, $P = .001$), and monocyte concentration ($r = 0.430$, $P = .003$) in CHF patients whereas no such correlations were found in the other groups of dogs in our study.

4 | DISCUSSION

Our study showed that MMVD dogs in CHF (in stable ACVIM C stage and in unstable ACVIM stages C and D) had a significantly lower percentage of CD3+CD4+ (T helper) lymphocytes, a significantly higher percentage of CD3+CD8+ (cytotoxic T) lymphocytes, and consequently a significantly lower CD4/CD8 ratio compared with healthy dogs, while CD3+CD4+CD8+ (DP T), CD3+CD4−CD8− (DN T), and CD21+ (B) lymphocyte percentages or counts were not altered. Besides, dogs in unstable CHF had a significantly higher concentration of CD3+CD8+ (cytotoxic T) lymphocytes, monocytes, and total WBC compared with healthy dogs. These changes were not present in dogs with MMVD without CHF (ACVIM B2 stage). Therefore, we can consider these results indicative of a systemic immune and inflammatory response specifically associated with CHF in dogs with MMVD. In addition, we found several correlations suggesting that systemic inflammation is present in dogs with MMVD when they are in CHF. Our findings facilitate understanding the nature of MMVD and CHF in dogs.

Previous studies have shown that T lymphocytes recruited to the heart can support chronic inflammation and promote cardiomyocyte death, cardiac fibrosis, and adverse cardiac remodeling with subsequent deterioration of cardiac function.[13,14,23-27] The distinct roles of helper (CD4+) and cytotoxic (CD8+) T lymphocytes, which are the 2

major T lymphocyte subsets, have been investigated previously in experimental research, and it has been shown in a mouse model that under pathological conditions both CD4+ and CD8+ lymphocytes infiltrate the heart[14,23] and spread in the circulation.[14] The CD4+ lymphocytes have been shown to promote fibrosis and adverse cardiac remodeling,[12] in both ischemic[14] and nonischemic[13,23] heart failure. The B lymphocytes also play a role in the deterioration of cardiac function because large amounts of autoantibodies associated with increased apoptosis rates and increased circulating components of the complement system have been found in both ischemic and nonischemic heart failure.[15,24,28,29]

Previous studies in human cardiovascular patients showed alterations in CD4+ and CD8+ PBL subtypes. One study of end-stage CHF caused by coronary artery disease and DCM found an increased percentage of CD4+ lymphocytes and CD4+/CD8+ ratio in both groups of patients.[17] Another study found a lower percentage of CD4+ lymphocytes in CHF and an increased percentage of CD8+ lymphocytes in elderly CHF patients, compared to healthy controls.[19] As evidenced by research in humans, the changes in lymphocyte subpopulations are not uniform, which may reflect disease-specific changes, but some investigators suggest these changes may be the result of CHF rather than a specific disease.[17]

Alterations of these lymphocyte subtypes in peripheral blood also have been found in cardiac canine patients. In the first study, performed on 13 German Shepherd dogs with mitral regurgitation (most dogs in this study did not have mitral valve lesions typical of MMVD, as stated by the authors), similar findings to those of our study were seen: a lower percentage of CD4+ lymphocytes, a higher percentage of CD8+ lymphocytes, and a significantly lower CD4+/CD8+ ratio compared to healthy controls.[21] However, it is not clear from the data how many dogs were asymptomatic or in CHF. In contrast to our results, another study found that the percentages of both CD4+ and CD8+ cells were decreased and the CD4+/CB8+ ratio was not changed in dogs in heart failure compared to healthy controls.[20] The difference between these results and our results could be a consequence of the different populations studied, because they included dogs with MMVD and DCM, the latter typically being younger and of different breeds than dogs with MMVD, and the fact that the investigators stated as a limitation that the gated CD4+ cells could not be lymphocytes only, because they did not use antibody against CD3. However, the authors of that study found no differences between DCM and MMVD dogs in any variables and suggested that changes in CD4+ and CD8+ lymphocytes may be a consequence of heart failure and not a specific disease.[20] The mechanisms driving these changes in PBL subtypes have not been studied in canine CHF patients and have been studied in human medicine mainly in coronary artery disease, in which T-cell immunosenescence is a risk factor. The changes in CD4+ and CD8+ lymphocyte subtypes found in our study may be explained by immunosenescence characterized by decreases in some subtypes of T lymphocytes (such as naïve T-cells) and expansion of other T lymphocyte subtypes (such as CD8+ effector memory subsets).[30]

The CD3+ (T) and CD21+ (B) lymphocytes did not differ between the patient and healthy dog groups in our study. A decreased percentage of B lymphocytes was shown in human CHF patients.[19] In dogs with cardiac disease, the percentages of T and B lymphocytes have not been studied extensively, and in the only study[21] in which antibodies against T lymphocytes (CD3) and B lymphocytes (CD21) were used, no changes in these subpopulations were reported. It can therefore be assumed that the disproportions of lymphocyte populations are mainly seen in the T lymphocyte subtypes, whereas the total concentrations and percentages of T and B lymphocytes are not affected.

In contrast to studies in humans that showed either decreased total lymphocyte concentration or decreased lymphocyte percentage and found that lymphopenia correlated with disease severity and mortality in cardiovascular patients,[16-18,31,32] we found no decreased total lymphocyte concentration compared to healthy controls. Previous studies in dogs with CHF did not find lower total blood lymphocytes when comparing CHF patients (all classes combined) and control dogs.[11,20] However, when individual CHF classes were compared with healthy dogs, dogs with severe CHF were found to have significantly lower total lymphocyte counts than healthy dogs and dogs with mild and moderate CHF.[20] The difference between the results of that study and our results could be a result of different populations studied and different methods used.

In our study, we measured concentrations and percentages of CD3+CD4+CD8+ (DP T) and CD3+CD4−CD8− (DN T) lymphocytes in dogs with MMVD. These data obtained in canine cardiac patients have not been reported previously in the veterinary literature. And although these PBL subtypes did not differ between patient groups and control dogs, a significant positive correlation was found between DP T lymphocytes and serum CRP concentration in dogs with heart failure. The DP T lymphocytes have not been studied in cardiovascular disease in humans or dogs and are a cell subset whose role generally is poorly studied.[33] The DP T-cells in dogs have a phenotype, typical of activated T lymphocytes,[34,35] and they have characteristics of both CD4+ (either suppressor or regulatory phenotype) and CD8+ cells (cytotoxic phenotype).[36] They may play a role in autoimmune diseases, viral diseases and cancer,[35] and the correlation found in our study may indicate their role in inflammation in MMVD patients with CHF. Furthermore, it may be of interest that the percentage of DN T lymphocytes found both in cardiac patients and healthy dogs in our study was much higher than in healthy mice and humans, which typically have <5% of DN T-cells.[37,38] Two studies in healthy dogs[39,40] also found a high percentage of DN T lymphocytes in peripheral blood, approximately 10% in 1 study[39] and 15% in another study.[40] The reason for this species-specific difference remains to be investigated. It is known that DN T lymphocytes play a role in host defense, inflammation,[38] autoimmunity,[37] and immune tolerance by suppressing other T lymphocytes and possibly regulating B lymphocytes,[38] although to our knowledge, their role has not been studied in human or canine cardiovascular patients.

We found numerous correlations between various blood variables, including immune cells, and those associated with inflammation and the disease progression. Some of the correlations were only found in dogs with CHF and not in non-CHF (ACVIM B2) or healthy

dogs. Namely, in CHF group, serum CRP concentration correlated positively with DP T lymphocyte concentration and serum cTnI concentration, and total lymphocyte concentration correlated positively with mitral inflow E wave and A wave velocity. These associations may further support the hypothesis of the inflammatory nature and concurrent inflammatory and immunomodulatory changes in CHF.

One of the limitations of our study was the significantly younger age of healthy control dogs compared to the MMVD dogs, but all included healthy dogs were >5 years of age. Because of possible effects of age on the relative and absolute numbers of lymphocytes and their subtypes, with increasing age resulting in lower absolute numbers of all lymphocyte subtypes, lower percentages of CD3+CD4+ and CD21+ lymphocytes, and higher percentages of CD3+CD8+ lymphocytes,[41-44] we aimed to include age-matched control dogs. Initially, 28 age-matched, clinically healthy dogs were recruited for the study, but 15 were excluded after screening because of mitral or tricuspid regurgitation or both (n = 13), arrhythmias (n = 1), or eosinophilia (n = 1). It proved very difficult to find healthy dogs that matched MMVD dogs by age, breed, and weight. It is difficult to determine to what extent the age difference between control and MMVD dogs may have influenced the results of our study. The results of previous studies of PBL changes with age in dogs have been inconsistent regarding which PBL subtypes change with age and which do not. For example, some studies found that the percentage of CD4+ lymphocytes in healthy dogs decreased significantly with age,[41,42] whereas another study found no significant difference.[43] Similarly, the percentage of CD8+ lymphocytes was significantly higher in old dogs compared with young dogs in some studies,[41,44] whereas in other studies this difference was not reported[42] or was reported only for females.[43] Similar discrepancies exist for the absolute concentrations of these PBL subtypes and for the percentages and absolute concentrations of CD3+ (T), CD21+ (B) lymphocytes, and total lymphocytes. Moreover, in some of the studies reporting differences in CD4+ and CD8+ T lymphocytes,[41,42] these differences were significant only when young dogs were compared with old dogs, whereas our control group did not consist of young dogs but of middle-aged and old dogs (all dogs were >5 years of age, and the mean age of the group was 7.9 years).

In addition, unlike dogs with MMVD, our control group consisted of more females than males, although the difference was not significant. Previously, it was found that females of the Labrador Retriever breed had a significantly higher percentage of CD8+ cells than did males of the same breed.[41] However, our healthy dogs had a significantly lower percentage of CD8+ compared to dogs with MMVD, arguing against a possible influence of the sex difference between the dog groups on this variable.

In summary, our study showed that CD4+ (helper) and CD8+ (cytotoxic) T lymphocyte subtypes and CD4+/CD8+ ratio were altered in MMVD dogs with CHF, in contrast to total CD3+ (T), double positive CD3+CD4+CD8+ (T), double negative CD3+CD4−CD8− (T) and CD21+ (B) lymphocyte counts and percentages, and total lymphocyte counts that were not altered. Dogs with CHF had a lower CD4+ percentage, higher CD8+ percentage and concentration (the latter only in unstable CHF), and lower CD4+/CD8+ ratio, supporting the hypothesis that these cell subtypes are involved in the disease processes in dogs with MMVD and CHF. Future studies to elucidate the mechanisms behind the changes found in our study, to explore the prognostic value of these changes and possible therapeutic corrections, and to clarify the role of T helper lymphocytes and cytotoxic T lymphocytes in the pathogenesis and progression of MMVD and CHF in dogs are warranted.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All procedures complied with the applicable Slovenian governmental regulations (Animal Protection Act, The Official Gazette of the Republic of Slovenia, 43/2007).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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