Clinical Use of Lymphocyte Subset Analysis: As a Prognostic Marker for Dogs with Pyometra

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Research

Keywords: dogs, pyometra, lymphocyte subset analysis

Posted Date: October 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-948363/v1>

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Abstract

Background

Lymphocyte subset analysis is clinically applied in human medicine. However, lymphocyte subset analysis is rarely used in small animal practice. We hypothesized that lymphocyte subsets analysis was useful in small animal practice as a biomarker for evaluating immune competence and predicting the disease prognosis. Lymphocyte subset analysis was performed prospectively for pyometra, a common disease in dogs, to assess its clinical usefulness in small animal practice.

Results

This study included 29 dogs diagnosed with pyometra. They were classified into group 1 and group 2 on the basis of clinical course postoperatively. Sixteen dogs were classified in group 1 with no adverse events postoperatively. Thirteen dogs experienced adverse events such as increase in C-reactive protein concentration and white blood cell count, discharge from operation site, and hypoglycemia. These dogs were classified as group 2. Nine dogs were below the reference interval for the lymphocyte subset, eight of which were in group 2. Group 2 included significantly more dogs with lymphocyte subset abnormalities ($p = 0.005$). In the multivariable logistic regression analysis, only the result of lymphocyte subset analysis was significantly associated with adverse events ($p = 0.02$, 95% confidence interval = 1.68–192). Most dogs in group 2 were successfully treated.

Conclusions

These results indicate that lymphocyte subset analysis is useful as a prognostic tool for pyometra. Further studies are necessary for evaluating the clinical usefulness of lymphocyte subset analysis in pyometra and other diseases.

Plain English Summary

Although lymphocyte subset analysis is commonly applied in human clinical medicine, it is rarely used in small animal practice. We aimed to demonstrate that lymphocyte subset analysis is also useful for small animals. We performed lymphocyte subset analysis for 29 dogs diagnosed with pyometra, a common canine disease. Of these 29 dogs, 13 experienced adverse events postoperatively. We then compared dogs with or without adverse events to understand the factors that may affect the occurrence of adverse events. The results revealed that dogs with any lymphocyte subset depletion were significantly more prone to adverse events. Eight out of nine dogs with a decreased lymphocyte subset experienced adverse event. This finding supports that lymphocyte subset analysis is useful as a prognostic tool for pyometra. Further studies are necessary to evaluate the usefulness of lymphocyte subset analysis in small animal practice.

Background

Lymphocyte subset analysis is used to classify lymphocyte components by surface antigen. The clinical usefulness of lymphocyte subset analysis has been reported in human medicine. It is useful in the diagnosis of lymphoid tumors and immunodeficiency. CD4⁺ T-cell count is associated with the mortality of patients with human immunodeficiency virus infection and the infection-related indicators of systemic lupus erythematosus (SLE) (1, 2). In patients with acute myeloid leukemia, lymphocyte subset analysis is useful in the differentiation of specific subtypes and prediction of prognosis (3). In human patients with SLE, peripheral CD4⁺ T-cell count was revealed to be negatively correlated with serum C-reactive protein (CRP) concentration, and patients with CD4⁺ T-cell depletion are more prone to develop infections (1). Lymphocyte subset abnormalities may correlate with decreased immune competence in humans. In recent years, lymphocyte subset analysis for dogs has become possible in a commercial laboratory in Japan. Some studies have also evaluated lymphocyte subsets in dogs. Decreased lymphocyte subsets due to aging or specific diseases (4-7) and immunodeficiency due to congenital B-cell depletion (8, 9) have been reported. Another study proposed that decreased lymphocyte subsets may be useful as a prognostic indicator of mortality (10). However, lymphocyte subset analysis is not often used clinically in small animal practice.

Pyometra is a common disease in intact bitches and affects approximately 19%–25% of all intact bitches before 10 years of age (11, 12). Pyometra has a good prognosis if appropriate treatment is provided. However, some dogs experience a prolonged treatment duration (12). It has been reported that a closed cervix associated with more severe illness and that leukopenia is a risk factor for peritonitis and prolonged hospitalization (12, 13). However, not all dogs with a prolonged treatment duration show a closed cervix or leukopenia. The etiology of pyometra is not fully understood. A previous study suggested that inhibition of mitogen-driven lymphocyte proliferation and suppression of immune system activity may occur in dogs with pyometra (14). Thus, we hypothesized that the prognosis of canine pyometra is affected by the suppression of immune system activity. Lymphocyte subset abnormalities are associated with reductions in immune competence in both veterinary and human medicine. Therefore, lymphocyte subset abnormalities may be associated with adverse secondary complications of pyometra. To evaluate its clinical usefulness, we performed lymphocyte subset analysis in dogs with pyometra and evaluated the association between the results and adverse secondary complications postoperatively for canine pyometra.

Methods

Patient population

Dogs diagnosed with pyometra at Yuki Animal Hospital between July 2018 and July 2021 were prospectively included. All dogs underwent physical examinations as well as CBC, plasma biochemistry profile analysis, thoracic and abdominal radiography, and abdominal ultrasonography. If pyometra was suspected from these analyses, ovariohysterectomy and cytological examination of a fluid sample from the uterus were performed. Clinical diagnosis of pyometra was made based on these clinical findings (22). Bacterial culture of fluid, blood culture, and arthrocentesis was performed when a clinical diagnosis

of pyometra was made. In this study, dogs with underlying diseases were not excluded. This study was designed to evaluate the association between the presence of underlying disease and adverse events. Bacterial culture was outsourced to a commercial laboratory (Animal Medical Technology, Aichi, Japan). Since laboratory results typically take 5–7 days, bacterial culture was also performed using the disk method at our hospital to rapidly obtain sensitivity test results.

Postoperatively, antibacterial treatment was implemented under hospitalization. CBC and CRP concentrations and, in some cases, other plasma biochemistry profiles, were monitored daily. Antibacterial drugs were started during the perioperative period. The initial antibiotic was selected from ampicillin or cephalexin (27). If the clinician deemed it necessary, enrofloxacin was used in combination with these antibiotics. Adequate antibacterial drugs were selected depending on the results of the in-hospital bacterial culture. If the outsourced and in-hospital sensitivity test results differed, the antibacterial drugs were changed in accordance with the outsourced result. Antibacterial drugs were continued until suture removal or the CRP concentration decreased to the reference range (<0.9 mg/dl). The dogs were discharged at the clinician's discretion when continuous decrease of CRP concentration for >2 days postoperatively and improvement of appetite and activity were confirmed. Adverse events were defined as any abnormal clinical presentations such as diarrhea, discharge from operation site, hypoglycemia, and re-increase in CRP concentration or WBC count between surgery and suture removal. If CRP concentration and WBC count were increased from the previous day, they were judged to be re-increased. Monitoring was continued until the CRP concentrations decreased to within the reference interval or the adverse events were cured. Dogs with arthritis were not given immunosuppressive therapy and rechecked for arthrocentesis at the time of suture removal.

Dogs diagnosed with pyometra were classified into group 1 or group 2 depending on the clinical course postoperatively. Dogs that experienced no adverse events until suture removal were classified into group 1, whereas dogs that experienced any adverse event were classified into group 2. The history, signalment, laboratory test results, treatment duration, and lymphocyte subset analysis results were compared between the two groups. Lymphocyte subset analysis was re-performed in dogs that showed a reduction in any lymphocyte subset at the time of re-examination after treatment was completed, as long as the owner's permission was obtained.

Blood collection and quantification

We collected blood samples for CBC, plasma biochemistry profile analysis, lymphocyte subset analysis, and blood bacteriologic culture analysis from all dogs via venipuncture of the cephalic, saphenous, or jugular vein. Samples were placed in tubes with or without an anticoagulant. Plasma was separated from blood within 30 min of collection. These samples were analyzed on the day of collection. For blood bacteriologic culture, 1 mL of whole blood was collected in bottles for aerobic culture (Versa TREK™ REDOX™ 1 EZ Draw™; Thermo Fisher Scientific, Waltham, MA, USA) and anaerobic culture (Versa TREK™ REDOX™ 2 EZ Draw™; Thermo Fisher Scientific, Waltham, MA, USA).

Assays

We measured the CBC using an automated hematology analyzer (IDEXX ProCyt Dx; IDEXX Laboratories, Westbrook, MA, USA). We obtained the plasma biochemical profile by using a dry chemistry analyzer (Fujifilm DRI-CHEM 7000 V; Fujifilm Corporation, Tokyo, Japan). An in-hospital antibacterial susceptibility test was performed by the disk diffusion method. Purulent fluid collected from the uterus was inoculated directly in Mueller Hinton agar plates and antibacterial disks (KB disk, EIKEN CHEMICAL CO., LTD., Tokyo, Japan). After incubation at 37°C for 48 h, the zone diameters were measured. The standard of susceptibility depended on the package insert.

Lymphocyte subset analysis

Lymphocyte subset analysis was outsourced to the Animal Allergy Clinical Laboratories (AACL; Kanagawa, Japan). We collected 1 mL whole blood specimens mixed with EDTA on the same day as ovariohysterectomy. These blood specimens were sent to AACL within 2 days after collection. The percentages of T-cells, B-cells, Th-cells, cytotoxic T-cells (Tc-cells), and natural killer cells (NK-cells) were calculated using flow cytometry. Cells were incubated with each antibody at 4°C for 30 min to identify the surface antigen. The antibodies used to identify T-cells, B-cells, Th-cells, and Tc-cells were fluorescein isothiocyanate (FITC) labeled anti-canine CD3 (CA17.2A12; Bio-Rad Laboratories, inc., Hercules, CA, USA), Phycoerythrin (PE) labeled anti-human CD21 (B-ly4; BD Biosciences, Bedford, MA, USA), allophycocyanin (APC) labeled anti-canine CD4 (YKIX302.9; Thermo Fisher Scientific, Waltham, MA, USA), and FITC labeled anti-canine CD8 (YCATE55.9; Bio-Rad Laboratories, inc., Hercules, CA, USA), respectively. NK-cells were identified as CD3-positive, CD5-slightly positive, or CD8-positive cells (25). The antibodies used to identify NK-cells were FITC labeled anti-canine CD3 (CA17.2A12; Bio-Rad Laboratories, inc., Hercules, CA, USA), APC labeled anti-canine CD8 (YCATE55.9; Thermo Fisher Scientific, Waltham, MA, USA), and PE labeled anti-canine CD5 (YKIX322.3; Thermo Fisher Scientific, Waltham, MA, USA), respectively. FITC labeled anti-mouse IgG1 and PE labeled anti-mouse IgG1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used as isotype controls. Propidium iodide (PI) (BD Biosciences, Bedford, MA, USA) was used to stain DNA of dead cells. Samples were assayed by FACS Canto[®] (BD Biosciences, Bedford, MA, USA) and data were analyzed by FACS Diva (BD Biosciences, Bedford, MA, USA). A total of 1000 PI-negative cells were counted to analyze T-cells, B-cells, Th-cells, and Tc-cells. Similarly, 1000 CD3-positive cells were counted to analyze NK-cells. The ratio of the helper T-cells to the killer T-cells was also calculated. The accuracy of the lymphocyte subset analysis was measured in the AACL itself. The errors during repeated measurements of the same samples by an examiner were within 5% for T-cells and within 3% for the other cells. The error between the examiners ranged from 2% to 3%. Reference intervals were established from the data of 86 healthy dogs. The median age of these healthy dogs was 4 years (range, 6 months to 14 years).

Statistical analyses

The CBC and biochemical analysis results, age, body weight, days from onset to visit in our hospital, and length of hospitalization were compared using the Mann–Whitney *U* test. The presence or absence of anorexia, underlying diseases, lymphocyte subset analysis abnormalities, and arthritis, along with the

results of bacterial culture and blood culture, were compared using Fisher's exact test. Multivariable logistic regression analysis including the results of lymphocyte subset analysis and other variables potentially associated with adverse events was performed. Variables with p -values of <0.05 were considered statistically significant. Statistical analyses were performed using the software Easy R (28).

Results

Patient population

A total of 29 dogs were diagnosed with pyometra during the study period. The median age and body weight of all dogs were 9 years and 4 months (range, 3 years and 11 months to 19 years) and 4.0 kg (range, 1.4–32 kg), respectively. The cohort included Chihuahua ($n = 9$), Pomeranian ($n = 2$), Miniature Dachshund ($n = 2$), Papillon ($n = 2$), Toy Poodle ($n = 2$), Shiba ($n = 2$), Maltese ($n = 1$), Labrador Retriever ($n = 1$), American Cocker Spaniel ($n = 1$), Boston Terrier ($n = 1$), Border Collie ($n = 1$), Miniature Schnauzer ($n = 1$), Norfolk Terrier ($n = 1$), and mix breed dogs ($n = 3$). The common signs of pyometra were anorexia ($n = 21$), lethargy ($n = 17$), vomiting ($n = 4$) and diarrhea ($n = 4$). The median number of days between hospital visit and surgery was 0 days (range, 0–2 days). The median hospitalization duration was 4 days (range, 2–14 days). The median suture removal data was 11 days (range, 10–14 days). The phase of estrous cycle was not heard in most of cases. Sixteen dogs have closed cervix and 13 dogs have open cervix.

Lymphocyte subset analysis

Lymphocyte subset abnormalities were found in 9 dogs. The results of lymphocyte subset analyses are presented in Table 1. Cases 1, 3, and 5 were re-examined with lymphocyte subset analysis after hospital discharge. In case 1, both the B- and helper T-cell (Th-cell) counts remained low on days 23 and 133. In cases 3 and 5, the T-cell count increased to within the reference interval on days 70 and 53, respectively. Eight out of 9 dogs classified as group 2.

Table 1. The results of lymphocyte subset analysis

	Case1		Case2		Case3		Case4		Case5		Case6		Case7		Case8		Case9		Normal	RI
	Day1	Day23	Day133	Day1	Day1	Day73	Day1	Day1	Day1	Day53	Day1	Day1	Day1	Day1	Day1	Day1	Day1	Day1	Median	(range)
T-cell (%)	76.5	82.2	91.9	79.1	54.8	65.4	49.3	53.6	69	52.3	78.1	52.4	53	53	52.4	53	53	70.25	56.2-89.2	
B-cell (%)	1.8	3.3	3.5	1.3	16.9	17	39.9	11	6	13.4	9.8	23.3	30.1	30.1	23.3	30.1	30.1	8.75	4.7-29.6	
Th-cell (%)	11.9	12.9	11	46.6	26.6	28.7	27.5	31.8	38.6	35.5	25.3	25.5	42.3	42.3	25.5	42.3	42.3	42.05	27.1-62.2	
Tc-cell (%)	18.3	30	31.9	23.3	22.2	28.1	10.2	20.1	32.4	9.4	44.9	25.7	11.4	11.4	25.7	11.4	11.4	24.25	10.9-41.4	
Th/Tc	0.65	0.43	0.34	2	1.2	1	2.7	1.6	1.2	3.8	0.6	1	3.2	3.2	1	3.2	3.2	2.0	0.6-3.9	
NK-cell (%)	12.7	22.8	21.9	13.7	10	16.7	5.5	15.7	26.3	1.4	29.5	16.7	6.1	6.1	16.7	6.1	6.1	9.25	1.7-16.2	
																			(4.1-27.3)	

RI, Reference interval; Th-cell, Helper T-cell; Tc-cell, Cytotoxic T-cell; NK-cell, Natural-Killer cell

Normal indicates dogs with no abnormal value of lymphocyte subset analysis.

Bold indicates abnormal value.

Comparison of clinical signs, signalments, and clinical course of 2 groups

Group 1 included 16 dogs and group 2 included 13 dogs. Adverse events in group 2 included increased CRP concentration (n = 8), increased white blood cell (WBC) count (n = 3), hypoglycemia (n = 3), discharge from surgical site (n = 2), diarrhea (n = 2), nonregenerative anemia (n = 1), increased hepatic enzyme activity (n = 1), and delayed improvement of thrombocytopenia (n = 1). Comparisons of the history, signalment, major clinical presentations, and prognosis between group 1 and group 2 are reported

in Table 2. The median treatment duration in group 2 was significantly longer than that in group 1 ($p = 0.003$). Group 2 included significantly more dogs with lymphocyte subset analysis abnormalities ($p = 0.005$). The frequency of underlying disease was higher in group 2 than in group 1, but this was not statistically significant ($p = 0.09$). In a multivariable logistic regression analysis including age, lymphocyte subset analysis abnormalities, and underlying disease, only the presence of lymphocyte subset analysis abnormalities was significantly associated with adverse events ($p = 0.02$, 95% confidence interval = 1.68–192). Underlying diseases included myxomatous mitral valve disease (American College of Veterinary Internal Medicine class B2) ($n = 3$), myxomatous mitral valve disease (American College of Veterinary Internal Medicine class B1) ($n = 1$), mammary gland tumor ($n = 3$), chronic kidney disease (International Renal Interest Society stage 3) ($n = 1$), bladder tumor ($n = 1$), hyperadrenocorticism ($n = 1$), and hypothyroidism ($n = 1$). Most group 2 dogs were successfully treated, and treatment was mainly with antibacterial therapy. Hypoglycemia was treated with glucose infusion. One dog that experienced severe nonregenerative anemia received a blood transfusion.

Table 2. Signalment, history, major clinical presentations, and prognosis of each group

	Group 1 (n = 16) median or number (range or %)	Group 2 (n = 13) median or number (range or %)	<i>p</i> value
Age (years)	7.7 (3.9–14.3)	9.4 (7.1–19)	0.15
Body weight (kg)	4.1 (1.4–32)	3.7 (1.9–9.4)	0.41
Days from onset to visit (days)	3 (1–14)	5.5 (0–30)	1
Anorexia	10 (62.5%)	10 (76.9%)	1
Underlying disease	2 (11.8%)	6 (46.2%)	0.09
Closed cervix	7 (43.8%)	9 (69.2%)	1
Hospitalization (days)	3 (2–8)	5 (3–14)	0.08
Treatment duration (days)	10 (9–14)	18 (12–61)	0.003
Lymphocyte subset abnormalities	1 (6.3%)	8 (61.5%)	0.005

Comparison of clinicopathological results between the normal and abnormal groups

No significant differences were found between group 1 and group 2 in all parameters of the complete blood count (CBC) and plasma biochemical profile. Blood culture was positive in 1 dog in group 1 and 3 dog in group 2 ($p = 0.60$). Arthritis was found in 5 dogs in group 1 and 2 dog in the abnormal group ($p = 0.40$). All cases of arthritis resolved 10 days postoperatively without immunosuppressive therapy. Isolated bacteria did not vary significantly between the two groups ($p = 0.89$) (Table 3).

Table 3. Bacterial species isolated from both groups

	Group 1	Group 2
<i>Escherichia coli</i>	8	5
<i>Enterobacter cloacae</i>	1	1
<i>Enterobacter aerogenes</i>	0	1
Beta-hemolytic streptococcus	2	1
Group G streptococcus	1	1
<i>Pseudomonas aeruginosa</i>	1	1
<i>Proteus mirabilis</i>	1	1
<i>Enterococcus gallinarum</i>	1	0
<i>Enterococcus faecalis</i>	1	0
<i>Staphylococcus pseudintermedius</i>	0	1
<i>Haemophilus spp</i>	1	0
<i>Corynebacterium spp</i>	1	0
<i>Pasteurella spp</i>	1	0
Negative	0	1

Discussion

In the present study, significantly more dogs with decreased B- and/or T-cell at diagnosis of pyometra experienced complicated outcomes. CRP concentration is generally known to rapidly decrease to within the reference interval on the 10th day after surgical treatment for pyometra (15). The treatment duration of group 2 was significantly longer than that of group 1. These results indicate that the decreased B- or/and T-cell counts may be negative prognostic markers of pyometra. As the CRP concentrations in group 2 were decreased with changes in or increased doses of the antibiotic drugs, the re-increase in the CRP concentration was considered to be caused by persistent infection, although no infection site was found in the postoperative examination. In human medicine, increased susceptibility to infection is caused by a decline in lymphocyte counts under the condition of some diseases and aging (2, 16-18). Several studies have been also conducted in dogs about decreases in B- and T-cell counts. The decrease of B- or T-cell counts have been reported in dogs with common variable immunodeficiency, chronic kidney disease, and diabetes mellitus (5, 6, 8, 9). However, these diseases were not found among the cases in the present study. Negative correlations between age and the number of B- and T-cells were reported in

healthy beagles in a previous study (4). In the present study, although data are not shown, the median age of dogs with decreased B- or/and T-cells (12 years and 7 months) was significantly higher than that of the other dogs (8 years and 1 month) ($p = 0.05$). An age-related increase in susceptibility to infections may develop in dogs, as it does in humans. However, age was not associated with the occurrence of adverse events. This result suggests that adverse event cannot be predicted by age alone.

In cases 3 and 5, the T-cell percentage increased after treatment. In human medicine, protein-energy malnutrition (PEM) is a well-known factor of immune-compromised condition by starvation (19). It leads to increased susceptibility to infection. However, only few studies have researched on PEM in dogs despite the fact that nutritional status is also believed to affect the immunity of the animal. In a study with mice, lymphocytes, especially CD4⁺ T-cells, were reduced in the mice fasted for a period as short as 48 h (20). Another study showed that healthy cats fasted for 4 days have reduced CD4⁺ T-cells, which was reversed by refeeding (21). These previous studies suggested that animals as well as humans who go through starvation may develop PEM. Cases 3 and 5 had anorexia for 1 week and 2 weeks, respectively. Although not supported by this study, the temporary decrease in the percentage of T-cells in these two cases could be caused by acute starvation. The decreased immune competence caused by starvation may worsen the prognosis for pyometra.

We evaluated other factors that may be involved in immune response and prognosis. Endotoxemia, sepsis, and reactive polyarthritis are associated with pyometra (22, 23). Although not proven, they are believed to potentially worsen prognosis. However, these factors appear to have a low impact on the lymphocyte subset analysis and prognosis.

Some adverse events in the present study are not considered specific to pyometra. For example, one dog that experienced severe nonregenerative anemia was diagnosed with hyperadrenocorticism, hypothyroidism, and chronic kidney disease as underlying diseases. Nonregenerative anemia may have been caused by these underlying diseases. This dog showed no abnormalities on lymphocyte subset analysis. It should be noted that dogs with underlying diseases were not excluded in the present study. In addition, dogs with pyometra tend to be old. As a result, underlying disease is often diagnosed at the same time as pyometra. Although it was expected that underlying diseases may affect prognosis, no significant association was found. Furthermore, infection of the surgical site and increased WBC count, CRP concentration, and liver enzyme activity may be caused by the surgical procedure or anesthesia itself. If lymphocyte subset analysis can predict these postoperative complications, it may also be useful in predicting prognosis not only for pyometra but also for other diseases that require surgical treatment.

The present study has some limitations. First, the lymphocyte subset analysis is susceptible to errors during flow cytometry analysis. The method used for flow cytometric analysis of peripheral blood lymphocytes has been described previously (24, 25). In the present study, the gating technique and cell surface markers used were similar to these previous studies mentioned. Second, whether the prognosis of pyometra should be evaluated using the numbers or percentages of lymphocytes is arguable. Prognosis was difficult to determine using the number of each lymphocyte component because the number of

lymphocytes varied greatly between the individual cases. We consider that the use of percentages is a useful and simple method. Third, the timing of sampling differed from case to case because of differences in the period from onset and data from follow-ups. Lastly, we did not perform histopathological examination and immunohistochemistry of the uterus. Definitive diagnosis of pyometra is made based on postoperative macroscopic and histopathological examination findings of the uterus as well as microbiological examination of uterine contents (22). However, pyometra can be strongly suspected through the combination of clinicopathological findings and diagnostic imaging without histopathological examination (22, 23). We clinically diagnosed pyometra by comprehensively evaluating the results of all tests we performed. When immunohistochemistry of the uterus is performed, local changes in lymphocyte subsets may be present (26). Since we are a primary care animal hospital, this examination could not be performed due to a lack of equipment.

Conclusion

Lymphocyte subset analysis has been shown to be useful as a tool for predicting the prognosis of canine pyometra. This study demonstrates that lymphocyte subset analysis may be useful for predicting prognosis not only for pyometra but also for other diseases. The usefulness of lymphocyte subset analysis in small animal practice should be further evaluated in cases of other disease and pyometra.

Abbreviations

AACL: Animal Allergy Clinical Laboratories

APC: Allophycocyanin

CBC: Complete blood count

CRP: C-reactive protein

EDTA: Ethylenediaminetetraacetate

FITC: Fluorescein isothiocyanate

NK-cell: Natural killer cell

PE: Phycoerythrin

PEM: Protein-energy malnutrition

PI: Propidium iodide

SLE: Systemic lupus erythematosus

Tc-cell: Cytotoxic T-cell

Th-cell: Helper T-cell

WBC: White blood cell

Declarations

Ethical approval and Consent to participate

In Japan, there is no ethics committee available for private-practice animal hospitals. Nevertheless, this study was conducted according to the ethical codes of the Japan Veterinary Medical Association. The samples in this study were obtained and used after obtaining written consent from each dog owner.

Consent for publication

All authors give their consent for publication.

Availability of supporting data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by AACL. Lymphocyte subset analysis was performed by AACL without charge.

Author contributions

All authors contributed to the study conception and design. SY performed case selection, data analysis, and manuscript preparation under the supervision of MY and KM. SY, TH, EN, DK, HT, and MN performed case collection. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Lu Z, Li J, Ji J, Gu Z, Da Z. Altered peripheral lymphocyte subsets in untreated systemic lupus erythematosus patients with infections. *Braz J Med Biol Res.* 2019;52:e8131.

2. Ren L, Li J, Zhou S, Xia X, Xie Z, Liu P, et al. Prognosis of HIV Patients Receiving Antiretroviral Therapy According to CD4 Counts: A Long-term Follow-up study in Yunnan, China. *Sci Rep*. 2017;7:9595.
3. Park Y, Lim J, Kim S, Song I, Kwon K, Koo S, et al. The prognostic impact of lymphocyte subsets in newly diagnosed acute myeloid leukemia. *Blood Res*. 2018;53:198-204.
4. Fujiwara M, Yonezawa T, Arai T, Yamamoto I, Ohtsuka H. Alterations with age in peripheral blood lymphocyte subpopulations and cytokine synthesis in beagles. *Vet Med (Auckl)*. 2012;3:79-84.
5. Kralova S, Leva L, Toman M. Changes in lymphocyte function and subsets in dogs with naturally occurring chronic renal failure. *Can J Vet Res*. 2010;74:124-9.
6. Mori A, Sagara F, Shimizu S, Mizutani H, Sako T, Hirose H, et al. Changes in peripheral lymphocyte subsets in the type 1 diabetic dogs treated with insulin injections. *J Vet Med Sci*. 2008;70:185-7.
7. Withers SS, Moore PF, Chang H, Choi JW, McSorley SJ, Kent MS, et al. Multi-color flow cytometry for evaluating age-related changes in memory lymphocyte subsets in dogs. *Dev Comp Immunol*. 2018;87:64-74.
8. Kanemoto H, Morikawa R, Chambers JK, Kasahara K, Hanafusa Y, Uchida K, et al. Common variable immune deficiency in a Pomeranian with *Pneumocystis carinii* pneumonia. *J Vet Med Sci*. 2015;77:715-9.
9. Lobetti R. Common variable immunodeficiency in miniature dachshunds affected with *Pneumocystis carinii* pneumonia. *J Vet Diagn Invest*. 2000;12(1):39-45.
10. Watabe A, Hanazono K, Komatsu T, Fu DR, Endo Y, Kadosawa T. Peripheral lymphocyte subsets as a prognostic indicator of mortality and morbidity in healthy dogs. *J Vet Med Sci*. 2012;74:937-43.
11. Egenvall A, Hagman R, Bonnett BN, Hedhammar A, Olson P, Lagerstedt AS. Breed risk of pyometra in insured dogs in Sweden. *J Vet Intern Med*. 2001;15:530-8.
12. Jitpean S, Ström-Holst B, Emanuelson U, Höglund OV, Pettersson A, Alneryd-Bull C, et al. Outcome of pyometra in female dogs and predictors of peritonitis and prolonged postoperative hospitalization in surgically treated cases. *BMC Vet Res*. 2014;10:6.
13. Jitpean S, Ambrosen A, Emanuelson U, Hagman R. Closed cervix is associated with more severe illness in dogs with pyometra. *BMC Vet Res*. 2017;13(1):11.
14. Faldyna M, Laznicka A, Toman M. Immunosuppression in bitches with pyometra. *J Small Anim Pract*. 2001;42:5-10.
15. Yuki M, Itoh H, Takase K. Serum alpha-1-acid glycoprotein concentration in clinically healthy puppies and adult dogs and in dogs with various diseases. *Vet Clin Pathol*. 2010;39:65-71.

16. Caraux A, Klein B, Paiva B, Bret C, Schmitz A, Fuhler GM, et al. Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. *Haematologica*. 2010;95:1016-20.
17. Freitas GRR, da Luz Fernandes M, Agena F, Jaluul O, Silva SC, Lemos FBC, et al. Aging and End Stage Renal Disease Cause A Decrease in Absolute Circulating Lymphocyte Counts with A Shift to A Memory Profile and Diverge in Treg Population. *Aging Dis*. 2019;10:49-61.
18. Yarmohammadi H, Cunningham-Rundles C. Idiopathic CD4 lymphocytopenia: Pathogenesis, etiologies, clinical presentations and treatment strategies. *Ann Allergy Asthma Immunol*. 2017;119:374-8.
19. Savy M, Edmond K, Fine PE, Hall A, Hennig BJ, Moore SE, et al. Landscape analysis of interactions between nutrition and vaccine responses in children. *J Nutr*. 2009;139:2154S-218S.
20. Saucillo DC, Gerriets VA, Sheng J, Rathmell JC, Maciver NJ. Leptin metabolically licenses T cells for activation to link nutrition and immunity. *J Immunol*. 2014;192:136-44.
21. Freitag KA, Saker KE, Thomas E, Kalnitsky J. Acute starvation and subsequent refeeding affect lymphocyte subsets and proliferation in cats. *J Nutr*. 2000;130:2444-9.
22. Hagman R. Pyometra in Small Animals. *Vet Clin North Am Small Anim Pract*. 2018;48:639-61.
23. Stone M. Immune-mediated polyarthritis and other polyarthritides. In: Ettinger SJ, Feldman EC, CÔTÉE, editors. *Textbook of veterinary internal medicine : diseases of the dog and the cat*. Eighth edition. Amsterdam: Elsevier; 2017. pp. 861-866.
24. 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;73:167-82.
25. Huang YC, Hung SW, Jan TR, Liao KW, Cheng CH, Wang YS, et al. CD5-low expression lymphocytes in canine peripheral blood show characteristics of natural killer cells. *J Leukoc Biol*. 2008;84:1501-10.
26. Bartoskova A, Turanek-Knotigova P, Matiasovic J, Oreskovic Z, Vicenova M, Stepanova H, et al. $\gamma\delta$ T lymphocytes are recruited into the inflamed uterus of bitches suffering from pyometra. *Vet J*. 2012;194:303-8.
27. Fransson BA. Ovaries and Uterus. In: Johnston SA, Tobias KM, editors. *Veterinary surgery : small animal*. Second edition. St. Louis, Missouri: Elsevier; 2017. p. 2109-30.
28. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant*. 2013;48:452-8.