Received: 17 April 2020 | Accepted: 12 November 2020

DOI: 10.1111/jvim.15977

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Serum interleukin 17 concentrations in dogs with immune-mediated hemolytic anemia

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Funding information
OVC Pet Trust Fund, Grant/Award Number: 052574

Abstract

Background: Increased serum interleukin 17 (IL-17) concentration has been associated with the immunopathogenesis of autoimmune hemolytic anemia in humans. No data are available about IL-17 in immune-mediated hemolytic anemia (IMHA) of dogs.

Objectives: Monitor changes in serum IL-17 concentration during the acute stages of IMHA in dogs, compared with results in healthy dogs, and its relationship with outcome.

Animals: Thirty-one client-owned dogs with primary IMHA and 27 healthy dogs.

Methods: Quantification of serum IL-17 concentration using a commercially available ELISA kit at the time of admission (D0), after 48 hours (D2) and after 96 hours (D4) as compared to concentration in healthy dogs. The IMHA dogs were classified as survivors if discharged from hospital, or nonsurvivors for any cause of in-hospital mortality.

Results: Mean serum IL-17 concentration was higher in dogs with IMHA on admission compared with healthy dogs (D0), but this difference was not significant (mean, 19.52 pg/mL vs 10.52 pg/mL, respectively, $P = .17$). Throughout hospitalization, serum IL-17 concentration significantly decreased in survivors. Serum IL-17 concentration at D0 was not different between survivors and nonsurvivors, but surviving dogs had significantly lower serum IL-17 concentration at D2 and D4 ($P = .04$ and $P = .004$, respectively) compared with nonsurviving dogs. No correlation was found between serum IL-17 concentration and serum total bilirubin or lactate concentrations or CBC parameters.

Abbreviations: AIHA, autoimmune hemolytic anemia; D0, day 0; D2, day 2; D4, day 4; DAT, direct antiglobulin test; IFN-γ, interferon gamma; IL, interleukin; IMHA, immune-mediated hemolytic anemia; Th1, type 1 T helper cell; Th17, T helper 17 cells; Th2, type 2 T helper cell; $\Delta_{IL\text{-}17}$, IL-17 variation between D0 and D2.

Conclusion and Clinical Importance: Serum IL-17 concentration remained significantly higher in nonsurviving IMHA dogs whereas it significantly decreased during hospitalization in survivors, making serum IL-17 concentration a potential biomarker for severity and response to treatment in IMHA.

KEYWORDS

autoimmune disease, canine, cytokines, inflammation

1 | INTRODUCTION

Primary immune-mediated hemolytic anemia (IMHA) is a disorder that causes acute and marked destruction of red blood cells leading to life-threatening anemia in dogs.[1-4] Although IMHA is considered the most common hematological immune-mediated disorder in dogs, its pathogenesis and the factors that contribute to its severity remain largely unknown.[1-4] Immune mediated hemolytic anemia occurs when anti-erythrocyte antibodies bind to the surface of erythrocytes, leading to hemolysis. However, the exact mechanism leading to the formation of these autoantibodies is not known. Autoimmune hemolytic anemia (AIHA) is a disease of humans that shares some similarities with IMHA in dogs.[5] In both species, life-threatening anemia occurs secondary to immune-mediated erythrocyte hemolysis,[5-7] often necessitating blood transfusion as supportive treatment.[4,8] Similar to veterinary medicine, thromboembolic events are reported as complications in up to 11% of cases in affected humans, including deep vein thrombosis, pulmonary embolism, and a patient with disseminated intravascular coagulation,[1,8,9] but the rate of thromboembolic complication seems much higher in veterinary medicine, affecting 80% of the dogs with IMHA that underwent necropsy in 1 study (27% of the entire study population).[1] The mortality rate associated with AIHA overall is lower in humans: 3.6%-30%[6-8] vs 25.7%-58% reported in canine patients[1-4,10] and disease typically evolves over months in humans as compared with the very acute life threatening presentation in dogs.[3,4,8] An increase in serum interleukin (IL)-17 concentration recently was found to be correlated with disease severity in humans with AIHA.[11,12]

Interleukin (IL)-17 is a pro-inflammatory cytokine involved in recruiting and activating neutrophils, monocytes, and endothelial cells.[13] The T helper (T_h) type 17 lymphocytes, that secrete IL-17, and IL-17 itself also have been associated in vitro and in vivo with B cell activation and antibody production, having direct B-cell helper properties.[12,14-16] Several studies have confirmed the importance of IL-17 in the pathogenesis of autoimmune diseases in humans, and specifically AIHA.[11,12,17] In a murine model of AIHA, IL-17-secreting cells were significantly increased in the diseased mice, and targeted treatment that decreased IL-17 concentration decreased the incidence of AIHA.[11] Data in humans supported an IL-17-mediated production of anti-RBC antibodies, instead of interferon γ (INF-γ), after B-cell activation by T_h17 cells.[12] Anti-IL-17 treatment also decreased the severity of clinical signs, slowed disease progression and decreased the incidence of disease in murine models of other autoimmune diseases, specifically encephalomyelitis, and arthritis.[17] Currently available immunosuppressive treatment in veterinary medicine broadly inhibits the immune system,[3,18-21] but more targeted treatment options might improve outcomes in dogs with IMHA. However, further understanding of the immunopathogenesis of primary IMHA in dogs is needed before such targeted treatment options can be developed.

We hypothesized that serum IL-17 concentration would be increased in dogs with IMHA and that its concentration would decrease in response to treatment. Our objectives were: (a) to determine serum IL-17 concentrations in dogs with IMHA and to compare them with concentrations in healthy dogs; (b) to monitor changes in serum IL-17 concentrations during the acute stages of disease; and (c) to examine relationships between serum IL-17 concentrations and clinicopathological data as well as disease severity scores.[4,22]

2 | MATERIALS AND METHODS

2.1 | Case selection

Dogs were considered to have IMHA if they were anemic, hematocrit <35% on CBC (Advia 2120i; Siemens Canada, Oakville, Ontario, Canada), and fulfilled at least 2 of the following criteria:

1. Signs of immune destruction of red blood cells by observation of ghost cells or spherocytes on a fresh blood smear stained with modified Wright's stain, as determined by a board-certified clinical pathologist.
2. Positive saline agglutination test: Persistent macroscopic or microscopic agglutination after mixing 1 drop of blood with 4 drops of saline.
3. Direct antiglobulin test (DAT) ≥1:16. The DAT was performed at 37°C with polyvalent antisera (Canine Coombs Reagent, Veterinary Medical Research and Development, Pullman, Washington).

Because primary IMHA is a diagnosis of exclusion, all dogs included in the study had no historical evidence or clinical signs of hemorrhage or exposure to oxidative toxins and no other disease detected on serum biochemical profile, infectious disease screening (SNAP 4Dx PLUS, IDEXX Laboratories Canada Corp., Markham, Ontario, Canada), thoracic radiographs, and abdominal ultrasound examination. Dogs were excluded from the study if they had received any blood product or received >48 hours of immunosuppressive

treatment before their presentation to the Ontario Veterinary College - Health Sciences Center (OVC-HSC).

Blood was collected upon admission to OVC-HSE (D0), and again after day 2 (D2) and day 4 (D4) of hospitalization, if the patient remained hospitalized. After a 30-minute clotting period at room temperature, serum was obtained by centrifugation (Sorvall ST 16R, Thermo Fisher Scientific, Waltham, Massachusetts) at 2200*g* for 15 minutes and stored at −80°C until further analysis. Serum total bilirubin and lactate concentrations at the time of diagnosis were recorded. Patients canine hemolytic anemia objective score (CHAOS) and acute patient physiologic and laboratory evaluation (APPLE) scores were calculated at presentation using previously published models.[4,22] Treatments with immunosuppressant drugs, anti-platelet or anti-coagulant drugs, and supportive care with administration of fluids, blood products and gastro-intestinal protectant and anti-nausea medications were recorded.

Twenty-seven clinically healthy, staff- and student-owned dogs were prospectively enrolled as the control population. The control dogs were not receiving any medications, excluding parasite prophylaxis, and had not been vaccinated within the 6 weeks before enrollment in the study. Inclusion criteria were age >1 year, weight >5 kg, unremarkable physical examination, no known chronic conditions including allergies, and data from CBC, urine specific gravity (by refractometer), urine dipstick chemistry (Chemistrip 9, Roche, Basel, Switzerland), serum biochemistry profile (Cobas 6000 c501; Roche) and coagulation profile: pro-thrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration (STA Compact, Diagnostica Stago, Asniere sur Seine, France) within reference intervals. Aliquots of serum were obtained concurrently and prepared as described above until IL-17 analysis. Serum was stored for batched analysis, at −80°C, up to 20 months for both dogs with IMHA and healthy dogs.

Owner consent was obtained before enrollment of any dog in the study. Standards established by the National Council on Animal Care and the Ontario Animals for Research Act (1980) were followed and the University of Guelph Animal Care Committee approved the research protocol.

2.2 | IL-17 measurement

Serum IL-17 concentration was measured for each sample in duplicate, using a commercially available IL-17 ELISA kit for dogs (Canine IL-17/IL-17A Quantikine ELISA Kit, R&D Systems Inc, Minneapolis, Minnesota) and following the manufacturer's recommendations. The manufacturer previously had validated the kit using bilirubin-spiking experiments, up to a serum bilirubin concentration of 1710 mmol/L without interference on the ELISA results (personal communication). No information was available with regard to potential interactions with hemoglobin in serum.

Samples were allotted randomly to the plates and the operator was blinded to the dog's group. Results were obtained using a commercially available spectrophotometer (Epoch Microplate Spectrophotometer, BioTek, Winooski, Vermont) and software interface (Gen5 Software, BioTek).

2.3 | Statistical analysis

Commercial software systems were used for statistical analyses (SAS 9.4, SAS Institute Inc, Cary, North Carolina) and graph generation (GraphPad Prism, GraphPad Software Inc, La Jolla, California). Data below the limit of detection were handled as left-censored data and were analyzed using the multiple imputation method as recently described.[23] Data were assessed for normality using a Shapiro-Wilk test. No outliers were removed from the data sets. An analysis of variance using a type III model of fixed effect was performed to assess the combined effect of breed, age, and sex. A logistic regression was performed including serum IL-17 concentrations, CBC parameters (hematocrit, white blood cell count [WBC], neutrophil count, lymphocyte count, reticulocyte count, and platelet count), as well as serum total bilirubin concentration, serum lactate concentration, CHAOS and APPLE scores at D0, and outcome in the model. A mixed linear model using a type III model of fixed effect analysis followed by a Tukey test were used to assess the variation of serum IL-17 concentration over time in both survivors and nonsurvivors. Mann-Whitney *U* tests were used to compare the IMHA groups (survivors vs nonsurvivors) to each other and to the healthy dogs at each time point.

The difference ($\Delta_{IL\text{-}17}$) between serum IL-17 concentrations at D0 ($[IL\text{-}17]_{D0}$) and D2 ($[IL\text{-}17]_{D2}$) was calculated for both survivors and nonsurvivors: $\Delta_{IL\text{-}17} = [IL\text{-}17]_{D2}\text{-}[IL\text{-}17]_{D0}$. Mann-Whitney *U* tests were used to compare $\Delta_{IL\text{-}17}$ between survivors and nonsurvivors. The level of statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | Demographic data

Thirty-one dogs with primary IMHA were consecutively enrolled in the study between February 2015 and September 2016. All dogs meeting inclusion criteria during this period were enrolled. Median age was 7 years and ranged from 1.5 to 11.9. Seventeen (2 intact and 15 neutered) dogs were female, and 14 (1 intact and 13 neutered) were male. Breed distribution included American Cocker Spaniel (4), Shih Tzu (4), Dachshund (3), Airedale Terrier (2), Maltese Terrier (2), mixed-breed (5, including 3 Cocker Spaniel - Poodle mixed-breeds), and 1 of each of the following breeds: Australian Shepherd, Golden Retriever, Greyhound, Siberian Husky, Jack Russell Terrier, Kerry Blue Terrier, Pomeranian, Standard Poodle, Rottweiler, Scottish Terrier, and Yorkshire Terrier.

Dogs with IMHA were separated into 2 groups: 19 dogs were survivors, defined as discharged to the care of their owners. Twelve dogs were nonsurvivors (Table 1); these dogs were euthanized within 0.5 to 6 days of their presentation (median, 2.5 days). The duration of clinical signs before referral did not differ between survivors (median,

TABLE 1 Demographic data from 31 dogs with immune-mediated hemolytic anemia at the time of admission. Data is separated between survivors and nonsurvivors. Data are presented as medians (range)

	n	Age	Weight (kg)	TBILI (0-4 mmol/L)	Lactate (mmol/L)	Urea (3.5-9 mmol/L)	Creat (20-150 µmol/L)	HCT (39-56%)	#Transfusions	#Hospitaldays (days)	APPLE	CHAOS
Survivors	19	7 (1.5-12)	11.4 (4-42.5)	19.4 (6-494)	3 (1.3-7.9)	7.65 (4.6-20)	62 (21-84)	14 (5-22)	1 (0-10)	4 (1-26)	0.28 (0.03-0.67)	5 (2-6)
Nonsurvivors	12	7.3 (5-9)	14.7 (4.8-27)	46 (4-532)	3.55 (1.9-6.2)	11.1 (4.9-38.3)	57.5 (26-108)	15 (9-24)	1 (0-3)	2.5 (1-6)	0.41 (0.02-0.71)	5 (1-6)

Abbreviations: #Hospitaldays, length of hospitalization; #Transfusions, number of transfusions; APPLE, Acute Patient Physiologic and Laboratory Evaluation Score; CHAOS, Canine Hemolytic Anemia Objective Score; Creat, serum creatinine concentration; HCT, hematocrit; Lactate., plasma lactate concentration; n, total number of individuals; TBILI, total serum bilirubin; Urea, serum urea concentration.

3 days; range, 1-7) and nonsurvivors (median, 2 days; range, 1-5). Twelve dogs had received corticosteroids before enrollment in the study: 5 of these were in the nonsurvivor group. Subjectively, attending clinicians reported that each nonsurvivor in the study was euthanized because of worsening condition, including: severe, progressive, life-threatening dyspnea (8/12); marked deterioration in neurological status compatible with central neurological signs (7/12); continued severe hemolysis (6/12); sustained hypertension (2/12); abdominal effusion (1/12), and retinal hemorrhage (1/12). No instance of euthanasia could be directly attributed to adverse effects of immunosuppressant medications. Necropsy was performed in 5 of the 12 nonsurvivors. Evidence of thrombosis was reported in the spleen (3/5), lungs (2/5), liver (2/5), kidney (1/5), small intestine (1/5), myocardium (1/5), and adrenal glands (1/5). Hemorrhage also was reported in some dogs in the adrenal glands (2/5), pleura (1/5), liver (1/5), kidney (1/5), and brain (1/5). One dog had necrotizing pancreatitis suspected to be secondary to multifocal infarction of the pancreas. No underlying causes were identified, confirming the diagnosis of primary IMHA. There was no effect of breed, sex, or age on outcome. The APPLE and CHAOS scores did not differ between survivors (median scores, 0.28 and 5, respectively) and nonsurvivors (median scores, 0.41 and 5.5, respectively) and there was no association between the scores and outcome (Table 1).

All dogs with IMHA were treated using an immunosuppressive dose of corticosteroids (dexamethasone; median dose, 0.25 mg/kg IV q24h; range, 0.23-0.29 mg/kg) in combination with cyclosporine (median PO dose, 5.0 mg/kg q12h; range, 4.31-10.4 mg/kg and median IV dose, 6.05 mg/kg q24h; range, 5-10 mg/kg) as an adjunctive immunosuppressant treatment in 9 dogs, mycophenolate (median PO dose, 10.28 mg/kg q12h; range, 8.93-13.89 mg/kg and an IV dose of 10 mg/kg q24h in 1 dog) in 6 dogs, and azathioprine (median PO dose, 2.09 mg/kg q24h; range, 1.75-2.3 mg/kg) in 2 dogs. A combination of 2 adjunctive immunosuppressants was used in 10 dogs (cyclosporine and mycophenolate in 6 dogs, and cyclosporine and azathioprine in 4 dogs). All dogs received thromboprophylaxis using clopidogrel (median dose, 2.33 mg/kg; range, 1.1-4.1 mg/kg) in 25 dogs, aspirin (median dose, 0.93 mg/kg; range, 0.53-1.8 mg/kg) in 2 dogs, or a combination of both (4 dogs).

Twenty-seven dogs were enrolled in the control group between February and April 2015, including 16 neutered males and 11 spayed females. The median (range) age was 6 (3-10.5) years and the median (range) weight was 25.7 (6-42) kg. Mixed-breed dogs (n = 11) were overrepresented, followed by Golden Retriever (4), Border Terrier (2), Jack Russell Terrier (2), Labradors Retriever (2), and 1 of each: Border Collie, Siberian Husky, Dachshund, Vizsla, Greyhound, and Shetland Sheepdog.

3.2 | Serum IL-17 concentrations in dogs with IMHA and healthy dogs

In dogs with IMHA, serum IL-17 concentrations were measured in 31 dogs at D0, 29 dogs at D2, and 19 dogs at D4 (Table 2). No effects

of breed, age or sex on serum IL-17 concentration were identified. Logistic regression at D0 failed to identify any correlation between serum IL-17 concentration and any of the CBC parameters, serum total bilirubin or lactate concentrations, number of transfusions, duration of hospitalization, APPLE or CHAOS scores, and survival.

At the time of presentation (Table 2), serum IL-17 concentration was increased in dogs with IMHA compared with that of healthy dogs, but this difference did not reach statistical significance (P = .17; Figure 1). Furthermore, no significant differences in serum IL-17 concentrations were found between the healthy dogs and survivors at any time point (P = .24, P = .46 and P = .53, respectively; Figure 2A). Similarly, nonsurviving dogs with IMHA had significantly higher serum IL-17 concentrations only at D2 (P = .01) but not at D0 (P = .29) or D4 (P = .14) when compared to concentrations measured in healthy dogs (Figure 2B).

TABLE 2 Serum IL-17 concentration in healthy dogs and dogs with IMHA

	Healthy dogs	IMHA D0	IMHA D2	IMHA D4
n	27	31	29	19
Median (pg/L)	7.89	13.32	11.65	7.88
25% IQ	0.02	3.41	4.14	0.02
75% IQ	16.93	26.42	26.36	18.73

Abbreviations: 25% IQ, first interquartile; 75% IQ, third interquartile; D, day; IMHA, immune-mediated hemolytic anemia; n, total number of individuals.

Significant variation in serum IL-17 concentration was observed over the course of hospitalization (Table 2): serum IL-17 concentration was significantly lower at D4 in all dogs with IMHA when compared to D2 (P = .05), but no significant difference in serum IL-17 concentration was found between D0 and D2 or between D0 and D4. In survivors, a significant decrease in serum IL-17 concentration was found from D2 to D4 (P = .02; Figure 2A). However, no significant difference in serum IL-17 concentration was found over the 4-day course of hospitalization in nonsurvivors (P = .08; Figure 2B).

No statistical differences in the serum IL-17 concentration were found at D0 (P = .77) between survivors and nonsurvivors (Table 3), but serum IL-17 concentration remained significantly higher in the nonsurvivors at D2 (P = .04) and D4 (P = .004) when compared to serum IL-17 concentrations in survivors on the same day (Figure 3).

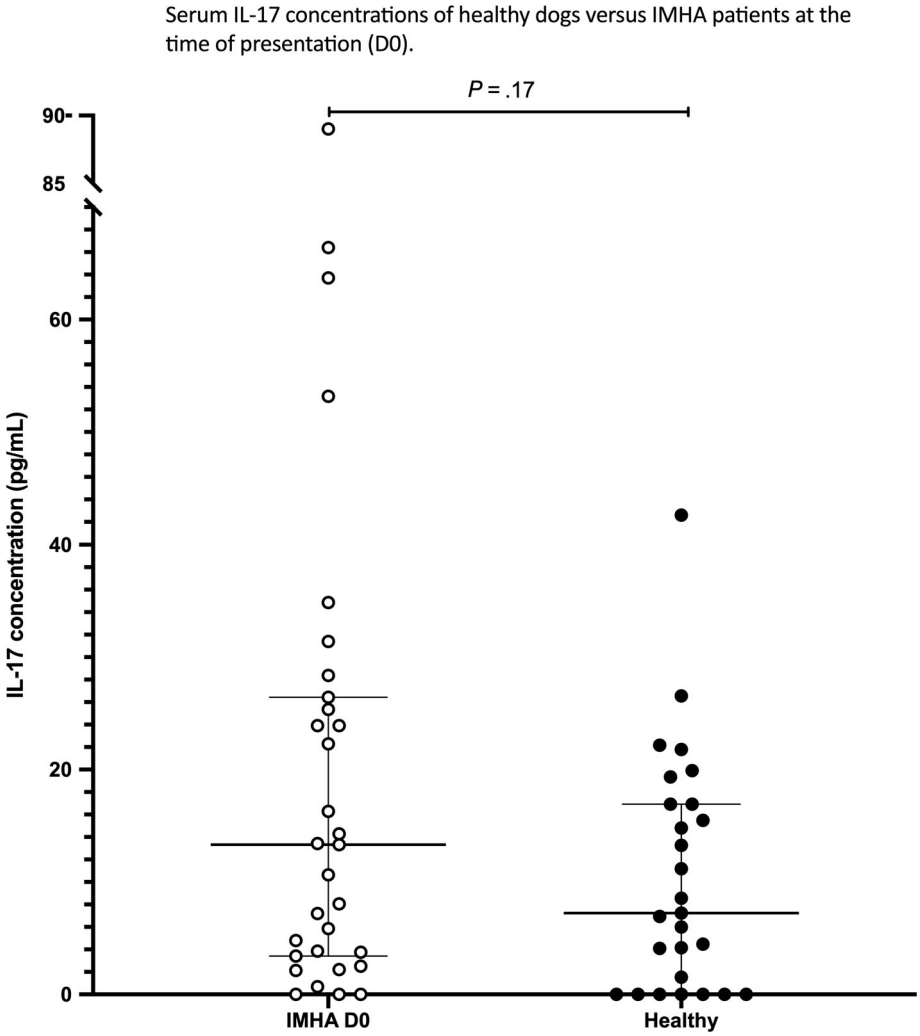

FIGURE 1 Serum IL-17 concentrations of healthy dogs vs dogs with IMHA at the time of presentation (D0). Dots represent individual serum IL-17 concentrations associated with median with 25% and 75% interquartiles

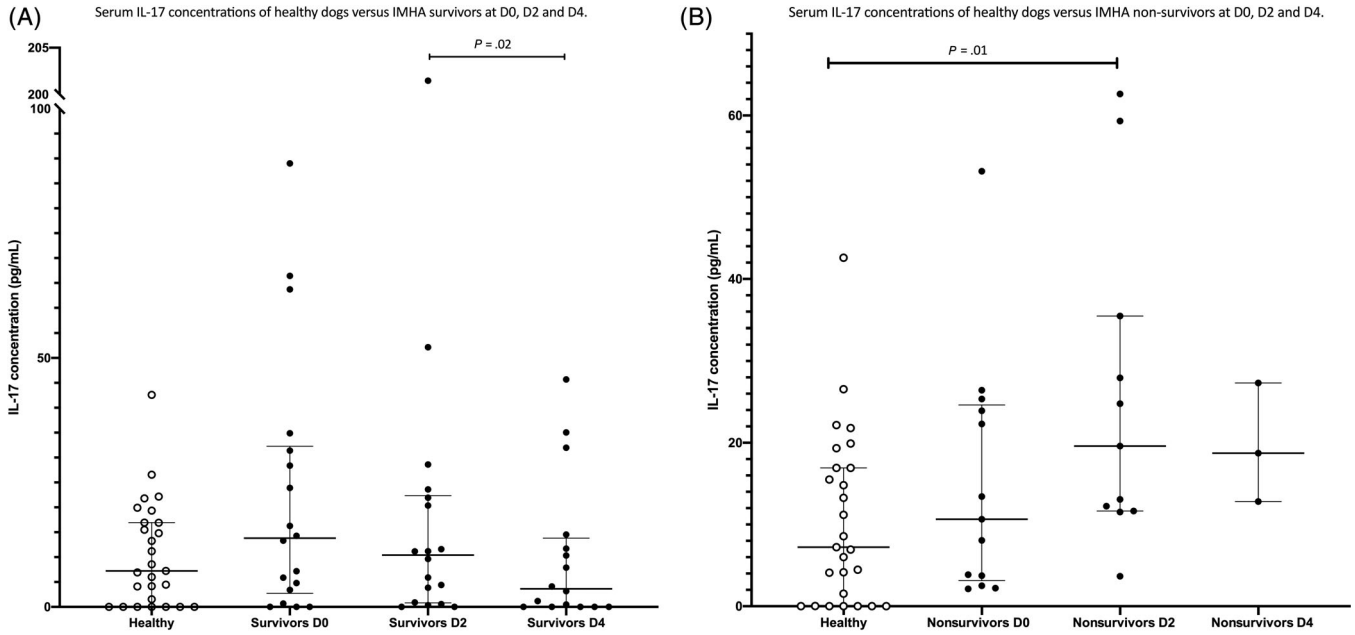

FIGURE 2 Serum IL-17 concentrations of healthy dogs vs survivors (A) and healthy dog vs nonsurvivors (B). Dots represent individual serum IL-17 concentrations associated with median with 25% and 75% interquartiles

TABLE 3 Serum IL-17 concentration in dogs with IMHA based on their discharge status

	Nonsurvivors D0	Nonsurvivors D2	Nonsurvivors D4	Survivors D0	Survivors D2	Survivors D4
n	13	11	3	18	18	16
Median (pg/L)	10.65	19.60	18.73	13.8	10.40	3.63
25% IQ	3.13	11.65	12.82	2.73	0.81	0.01
75% IQ	24.63	35.46	27.32	32.27	22.35	13.81

Abbreviations: 25% IQ, first interquartile; 75% IQ, third interquartile; D, day; IMHA, immune-mediated hemolytic anemia; n, total number of individuals.

The $\Delta_{IL\text{-}17}$ did not differ (P = .07) between survivors and non-survivors (Figure 4), which was attributed to more variability of $\Delta_{IL\text{-}17}$ in survivors (median, −0.06; range, −56.77 to 137.8; Table 4) when compared to nonsurvivors (median, 5.20; range, −2.7 to 55.58).

4 | DISCUSSION

We determined serum IL-17 concentrations in 31 dogs with IMHA from the time of presentation through day 4 of hospitalization. Serum IL-17 concentration was increased in dogs with IMHA compared with healthy dogs at the time of presentation, but not significantly different when compared to healthy dogs. Dogs that survived the acute phase of IMHA experienced a significant decrease in serum IL-17 concentrations over time, whereas nonsurvivors had persistently increased IL-17 concentrations during hospitalization.

Investigations of T helper (T_h) cell subsets in humans led to discovery of a role for T_h17 cells and IL-17 in autoimmune diseases of humans including AIHA.[11,12,17] Two studies found significant increases in serum IL-17 concentrations in patients with AIHA compared to healthy volunteers.[11,12] In people, serum interferon γ (IFN-γ) concentration, the signature cytokine of the T_h1-type response, was not different between people with AIHA and healthy volunteers but serum IL-17 concentration, the signature cytokine of the T_h17-type response, was significantly higher in the AIHA group.[12] This finding suggests that the T_h17 response is more involved in the pathogenesis of AIHA than are T_h1-type cytokines. Previous limited veterinary studies found contradictory results regarding the involvement of pro- and anti-inflammatory cytokines in dogs with IMHA. Variable increases in serum IL-2, IL-6, IL-8, IL-10, tumor necrosis factor α, keratinocyte chemoattractant, and monocyte chemoattractant protein-1 concentrations were found in dogs with IMHA compared with controls.[24,25] The latter study also found no difference in RNA expression for the genes coding for IFN-γ or IL-10 when comparing dogs with IMHA to dogs with other inflammatory diseases or to healthy dogs.[25] These results suggested that, as in people with AIHA,[12] T_h1-type cytokines are not upregulated in dogs with IMHA and therefore other pathways might be involved.[25] Our study suggests that the pro-inflammatory cytokine IL-17 has a role in the pathophysiology of IMHA in dogs.

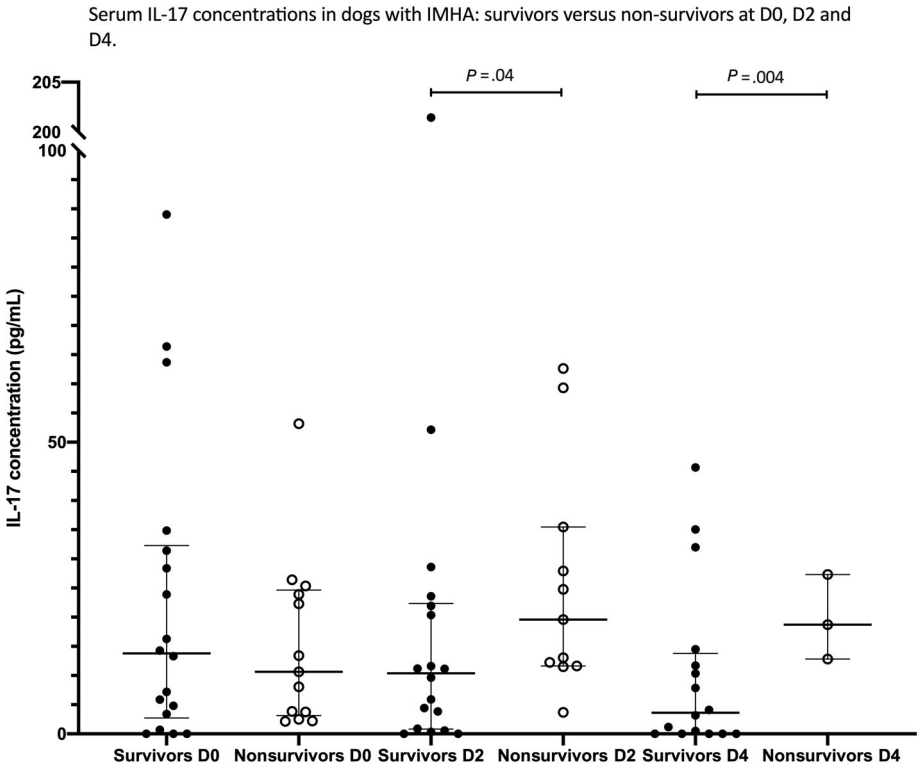

FIGURE 3 Serum IL-17 concentrations of survivors vs nonsurvivors at the time of presentation (D0) and over the initial course of hospitalization (48 hours post admission: D2 and 96 hours post admission D4). Dots represent individual serum IL-17 concentrations associated with median with 25% and 75% interquartiles

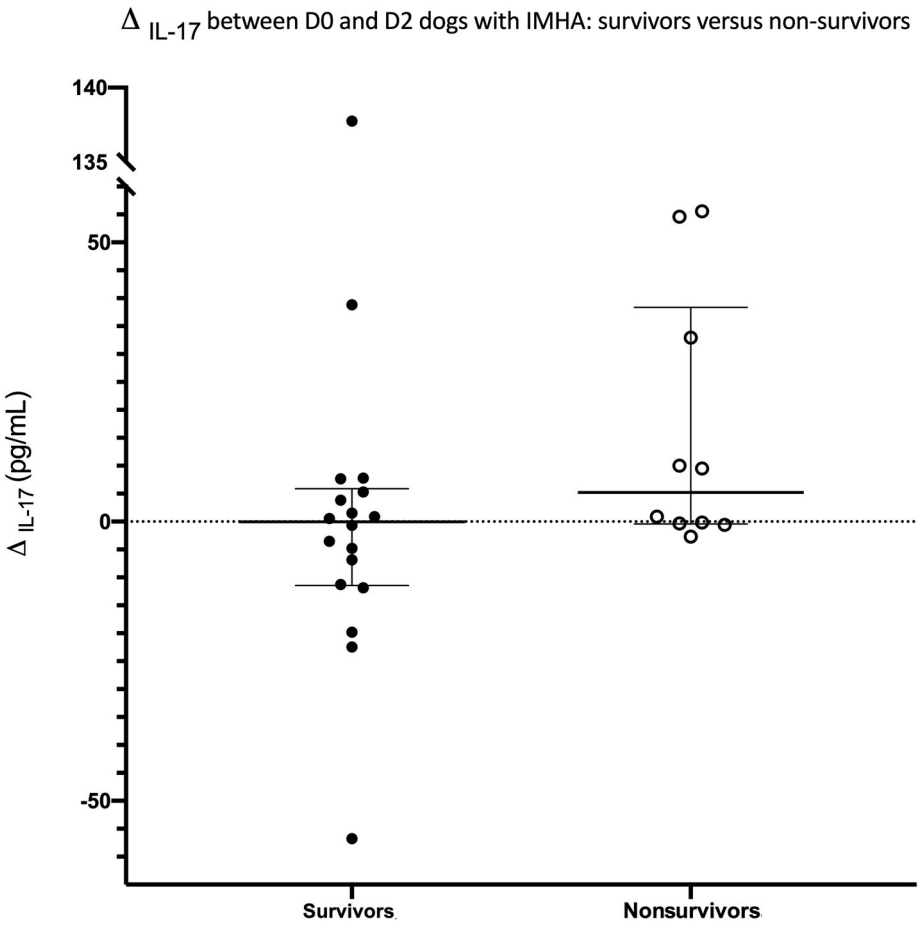

FIGURE 4 Serum IL-17 concentration variation between D0 and D2 ($\Delta_{IL\text{-}17}$) in dogs with IMHA based on their discharge status. Dots represent individual serum IL-17 concentrations associated with median with 25% and 75% interquartiles

TABLE 4 Serum IL-17 concentration variation between D0 and D2 ($\Delta_{IL\text{-}17}$) in dogs with IMHA based on their discharge status

	Survivors	Nonsurvivors
n	18	10
Median (pg/L)	−0.06	5.2
25% IQ	−11.42	−0.41
75% IQ	5.88	38.36
Min	−56.77	−2.7
Max	137.8	55.58

Abbreviations: 25% IQ, first interquartile; 75% IQ, third interquartile; D, day; IMHA, immune-mediated hemolytic anemia; Max, maximum; Min, minimum; n, total number of individuals.

Previous studies in humans suggest an association between serum IL-17 concentration and the severity of AIHA.[11,12] Serum IL-17 concentration was significantly higher in human patients with severe anemia compared to those with low-grade anemia and healthy controls in 1 study.[12] Additionally, a positive correlation between serum IL-17 concentration and anti-red blood cell IgG autoantibodies has been reported.[11] Although in our study IL-17 concentration did not correlate with any CBC parameters, serum IL-17 concentration remained significantly higher in nonsurvivors over time, suggesting sustained higher serum IL-17 concentrations are present with more severe disease. Furthermore, our study suggests that a decrease in serum IL-17 concentration occurs with successful immunosuppressive and supportive treatment, approaching concentrations measured in healthy dogs. This finding indicates that IL-17 potentially could be used as a disease severity marker in dogs with IMHA as well as a possible mean to assess response to treatment. This possibility is of particular interest in a disease with high mortality such as IMHA, because such a marker could provide information about treatment and prognosis for individual patients.[1-4] Previously reported negative prognostic factors such as azotemia and hyperbilirubinemia[4,26] were not associated with outcome in our study, nor were illness severity scores. However, because of the limited number of cases and number of variables included in the model, the power of the regression analysis was limited. Furthermore, we found no differences between APPLE or CHAOS scores between survivors and nonsurvivors, nor were correlations between APPLE or CHAOS scores and serum IL-17 concentrations identified. Moreover, individual variations of serum IL-17 concentration between D0 and D2 in our study were not different between survivors and nonsurvivors, although $\Delta_{IL\text{-}17}$ in the survivors was highly variable, and group sizes were likely underpowered. However, median $\Delta_{IL\text{-}17}$ was positive in nonsurvivors, confirming an increase in serum IL-17 concentration in that group. Because of the limited size of the different groups, multiplicity corrections were not performed, and a larger cohort would be necessary to confirm the magnitude of serum IL-17 concentration variations over time and between groups. Moreover, no power calculation was performed before enrollment to determine sample size.[27]

Because our institution is a tertiary referral center, affected dogs were presented with variable durations of clinical signs and treatments before enrollment. Some dogs were enrolled after receiving 1 or 2 doses of corticosteroids before referral. Previous corticosteroid administration may have impaired our ability to show larger differences in serum IL-17 concentrations between the healthy dogs and dogs with IMHA, or between survivors and nonsurvivors at D0 in dogs with IMHA. Our results are similar to those of another study investigating serum IL-17 concentration in dogs with steroid-responsive meningitis-arteritis (SRMA).[28] Dogs with relapsing SRMA (before initiating treatment) had significantly higher serum IL-17 concentrations than did dogs with SRMA receiving corticosteroids. Studies measuring serum IL-17 concentration in larger cohorts therefore are warranted.

Another limitation was a possible hemoglobin interaction with the ELISA, which was not investigated in our study despite IMHA being a hemolytic disease. Such an interaction has been described for TNF-α ELISA assays in humans,[27] but not for serum IL-17,[11] and this possibility was not investigated in our study and no data were available from the manufacturer. Because of the individual variations in hemolysis among patients, any direct interaction could have influenced the results. A previous study found that the frequency of T_h17 cells in the peripheral blood of people with AIHA correlated with the severity of the anemia and serum hemoglobin concentration as well as with anti-RBC antibodies.[11] Therefore, similar studies evaluating IL-17-producing cells, namely T_h17 cells, in dogs would be necessary to understand better the possible contribution of a T_h17 response in the pathophysiology of IMHA in dogs.

In summary, serum IL-17 concentration remained significantly increased at the time of presentation in the subset of dogs with IMHA that did not survive initial hospitalization, whereas a significant decrease in serum IL-17 concentration was noted in dogs with IMHA that survived acute hospitalization. These results suggest that serum IL-17 concentration could be a marker for disease severity and response to treatment in dogs with IMHA. A larger prospective pre-treatment study would be necessary to investigate the role of IL-17 in the pathogenesis of IMHA and validate serum IL-17 concentration as a biomarker of disease severity and response to treatment.

ACKNOWLEDGMENTS

Funding provided by OVC Pet Trust Fund, 052574. The results were partially presented as an oral abstract at the 2017 ACVIM Forum in National Harbor, Maryland. The authors thank Michelle Beaudoin-Kimble (Dept of Clinical Studies, University of Guelph) for her technical assistance with the IL-17 ELISA assays.

CONFLICT OF INTEREST DECLARATION

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

Animal Utilization Protocol #3236, University of Guelph, Guelph, Ontario, Canada.

HUMAN ETHICS APPROVAL DECLARATION

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

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How to cite this article: Cuq B, Blois SL, Bédard C, et al. Serum interleukin 17 concentrations in dogs with immune-mediated hemolytic anemia. *J Vet Intern Med*. 2021;35:217–225. https://doi.org/10.1111/jvim.15977