ORIGINAL ARTICLE

Serum IL-6 and MCP-1 concentrations in dogs with lymphoma before and after doxorubicin treatment as a potential marker of cellular senescence

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Abstract

Background: Chemotherapy can induce cellular senescence and a secretory phenotype characterized by an increased expression of inflammatory cytokines, such as IL-6 and MCP-1. Increased IL-6 and MCP-1 serum concentrations have been documented in dogs with lymphoma, but no studies have evaluated the effects of chemotherapy on cytokine concentrations.

Objectives: To measure IL-6 and MCP-1 in 16 client-owned dogs with lymphoma, at baseline and before and after doxorubicin, as a potential marker for senescence and correlate cytokine concentrations with treatment response and toxicities.

Methods: Serum IL-6 and MCP-1 concentrations at baseline, 0-h, 3-h, 6-h, 24-h and 1 week post doxorubicin were measured using a canine ELISA. We hypothesized that IL-6 and MCP-1 concentrations would increase following doxorubicin as a result of induction of cellular senescence.

Results: IL-6 concentrations were unchanged from baseline to 0-h but significantly decreased 1 week post doxorubicin ($p = 0.001$) compared to 0–6 h ($p = 0.045$) and 24-h ($p = 0.001$) time points. MCP-1 concentrations significantly decreased from baseline to 0-h ($p = 0.003$). Compared to 0–6 h, MCP-1 concentrations transiently increased at 24-h ($p = 0.001$) and decreased at 1 week ($p = 0.014$) post doxorubicin. Changes in IL-6 and MCP-1 concentrations did not correlate with leukocyte count, response to treatment or chemotherapy toxicities.

Conclusions: Changes in IL-6 and MCP-1 concentrations did not support doxorubicin-induced cellular senescence or correlate with leukocyte count, response to treatment or chemotherapy toxicity. However, our results suggest that remission status and doxorubicin treatment may influence cytokine concentrations and future studies are warranted to investigate the role of these cytokines as biomarkers.

KEYWORDS
canine, chemotherapy, cytokine, toxicity

1 | INTRODUCTION

Cellular senescence is a phenomenon whereby cells undergo terminal growth arrest and occurs in response to oncogenic stressors, including endogenous cellular processes (e.g. repeated cell division, telomere shortening, DNA damage and mutations) and exogenous stimuli (e.g. genotoxic drug exposure, radiation and injury) (Campisi, 2013; Jeyapalan & Sedivy, 2008). Senescence has been historically thought to be protective due to loss of replicative capacity. However, senescent cells remain metabolically active and can acquire a senescence-associated secretory phenotype (SASP), a pro-inflammatory state characterized by increased secretion of cytokines, chemokines, growth factors and proteases (Campisi, 2013; Freund et al., 2010; Jeyapalan & Sedivy, 2008). These factors act locally and systemically to promote inflammation, angiogenesis and cellular proliferation and can lead to progression of age-related diseases and tumourigenesis (Bitto et al., 2010; Campisi, 2013, 2001; Freund et al., 2010; Jeyapalan & Sedivy, 2008; Liu & Hornsby, 2007; Shane Anderson & Loeser, 2010).

Cellular senescence and the SASP have far reaching consequences in cancer treatment. A variety of drugs, including the chemotherapy drug doxorubicin, have been shown to induce cellular senescence, termed therapy-induced senescence (TIS), in primary cell culture and in vivo (Demaria et al., 2017; J. Ewald et al., 2008; J. A. Ewald et al., 2010; Fallah et al., 2019; Gonzalez et al., 2016; Mikuła-Pietrasik et al., 2020; Silwinska et al., 2009;). Although senescent cells undergo terminal arrest following chemotherapy, they may still contribute to tumour progression via secretion of tumour promoting growth factors induced by the SASP. Such factors can act in a paracrine fashion to promote survival of neighbouring cells that have endured chemotherapy (Faget et al., 2019; Guillon et al., 2019). In addition to chemotherapy resistance, human and murine studies also have shown that TIS may contribute to toxicity and influence outcome (Campisi, 2013; Demaria et al., 2017; J. A. Ewald et al., 2010; Gonzalez et al., 2016; Mikuła-Pietrasik et al., 2020). Thus, a better understanding of cellular senescence may help improve quality of life and outcomes in cancer patients.

Lymphoma represents 7%–24% of canine neoplasias. Although multi-agent chemotherapy induces clinical remission in greater than 80% of patients, more than half of dogs succumb to disease within a year due to chemotherapy resistance (Vail et al., 2020). Additionally, dogs experience chemotherapy toxicities similar to humans, with approximately 50% developing gastrointestinal or haematologic adverse events during multi-agent CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy. (Tomiyasu et al., 2010). While chemotherapy toxicities are often low grade and self-limiting, it can negatively impact patient's quality of life, outcome and owner compliance.

While senescence has been documented in mouse models and humans with cancer, little is known about senescence in tumour-bearing dogs. Several studies have measured serum cytokine concentrations at diagnosis in dogs with lymphoma and found that interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) are elevated compared to healthy control dogs (Calvalido et al.,

2016). Additionally, increased MCP-1 concentrations were associated with a shortened disease-free interval in dogs treated with CHOP chemotherapy (Perry et al., 2011). However, the influence of chemotherapy on cytokine concentrations has not been evaluated.

The aim of this study was to measure serum IL-6 and MCP-1 concentrations in dogs with lymphoma at baseline and before and after doxorubicin, as surrogate markers for induction of cellular senescence. We hypothesized that doxorubicin would induce senescence in dogs, resulting in increased serum cytokine concentrations. The candidate cytokines, IL-6 and MCP-1, were chosen based on in vitro and in vivo evidence suggesting that they are important signalling molecules in the SASP as well as the aforementioned studies demonstrating altered cytokine expression in dogs with lymphoma (Calvalido et al., 2016; Demaria et al., 2017; Jin et al., 2016; Kojima et al., 2013; Ortiz-Montero et al., 2017; Perry et al., 2011; Rolt et al., 2019). Additional objectives of this study included comparing IL-6 and MCP-1 in naïve and treated dogs and determining if changes in cytokines correlated with leukocyte count, chemotherapy response or toxicity.

2 | MATERIALS AND METHODS

2.1 | Study design

For this prospective study, client-owned dogs were enrolled from March 2018 through September 2019 at The Ohio State University Veterinary Medical Center. For eligible enrollment, dogs must have had a cytologic or histopathologic diagnosis of multicentric, high grade lymphoma and owner intent to treat with chemotherapy. Additional criteria included weight (>8 kg), adequate organ function as indicated by standard laboratory tests and performance status of 0 or 1 (according to the modified Eastern Cooperative Oncology Group performance score) (Oken et al., 1982). Dogs were not eligible for enrollment if the patient had non-multicentric lymphoma (e.g. cutaneous, gastrointestinal), previous lymphoma treatment, evidence of inflammatory or infectious disease on exam, bloodwork or imaging, or concurrent use of medications that could affect cytokine concentrations (e.g. oclacitinib; Apoquel, Zoetis Inc., Kalamazoo, Michigan), herbal supplements and antioxidants (Gonzales et al., 2014; Härtel et al., 2004). A 24-h washout was required for dogs receiving exogenous corticosteroids prior to enrollment. All dogs were required to have a physical examination and recent (<14 days) complete blood count (CBC) and biochemistry profile at screening. Additional staging tests were at clinician discretion but not required for enrollment. Stage and substage determination were based on the World Health Organization (WHO) clinical staging system (Carbone et al., 1971).

2.2 | Study schedule

Blood was collected for serum cytokine concentrations; lymph node measurements and quality of life surveys were obtained at baseline and before and after doxorubicin as shown in Tables 1–3. The

TABLE 1 Single agent doxorubicin chemotherapy protocol

Treatment	Week					
	1[*]	4	7[*]	8[*]	10	13
Prednisone PO	X	X				
Doxorubicin IV	X	X	X		X	X
Complete blood count	X	X	X	X	X	X
Serum and plasma collection	X		X[+]	X		
Lymph node measurements	X	X	X	X	X	X
QOL survey	X		X	X		

Abbreviation: QOL, quality of life.
*Denotes study visit.
[+]Serum and plasma collection occurred immediately before (0 h) and 3, 6 and 24 h after doxorubicin treatment.

TABLE 2 Single agent doxorubicin chemotherapy protocol with vincristine induction

Treatment	Week						
	0[*]	1	4	7[*]	8[*]	10	13
Prednisone PO	X	X	X				
Vincristine IV	X						
Doxorubicin IV		X	X	X		X	X
Complete blood count	X	X	X	X	X	X	X
Serum and plasma collection	X			X[+]	X		
Lymph node measurements	X	X	X	X	X	X	X
QOL survey	X			X	X		

Abbreviation: QOL, quality of life.
*Denotes study visit.
[+]Serum and plasma collection occurred immediately before (0 h) and 3, 6 and 24 h after doxorubicin treatment.

doxorubicin study visit occurred at week 7 or 9, depending on the patient's chemotherapy protocol. At this visit, blood collection was performed prior to (0-h) and 3-h, 6-h and 24-h after completion of doxorubicin. An owner diary was provided to document concurrent medications and adverse events, including anorexia, diarrhoea and/or nausea or vomiting, that occurred in the subsequent week. Following the doxorubicin study visit, patients returned one week later for a CBC. Following that visit, patients were deemed off study and continued their respective chemotherapy protocols.

2.3 | Chemotherapy

Chemotherapy schedules are outlined in Tables 1–3. All patients were treated with single agent doxorubicin $\pm$ vincristine induction, or a 25-week CHOP protocol. The protocol was chosen by the owner. Doxorubicin was administered via intravenous catheter in a peripheral vein (25-30 mg/m^2 or 1 mg/kg for dogs <15 kg; over 30 min). Start and stop time and location of the infusion were recorded.

2.4 | Corticosteroid and concomitant medications

Patients were eligible to start or restart prednisone after baseline blood collection but were required to discontinue at least 7 days prior to the doxorubicin study visit. Concomitant medications to prevent or treat toxicities were used via clinical discretion and included anti-emetics (maropitant (Cerenia, Zoetis Inc., Kalamazoo, Michigan, USA), ondansetron), anti-diarrheals (metronidazole), probiotics (Fortiflora, Nestle Purina, St. Louis, Missouri, USA) and fibre supplementation. Behavioural modification drugs including gabapentin and trazodone were also permitted.

2.5 | Tumour response

Lymph node measurements and response assessments were performed at time points shown in Tables 1–3. Responses were characterized according to the Veterinary Cooperative Oncology Group (VCOG) Response Evaluation Criteria for Peripheral Nodal Lymphoma (Nguyen et al., 2015). For purposes of statistical analysis, tumour response was defined as patient's response to therapy at the doxorubicin study visit.

2.6 | Adverse events

Haematologic adverse events were assessed via CBC performed one week after doxorubicin administration. For study purposes, haematological toxicity was defined as the development of or increase in grade of a pre-existing anaemia, neutropenia and/or thrombocytopenia following doxorubicin. Gastrointestinal adverse events were assessed via owner diary and physical exam and were defined as the development of anorexia, diarrhoea and/or nausea and vomiting in the week following doxorubicin. Adverse events were characterized according to the VCOG Common Terminology Criteria for Adverse Events (VCOG, 2016).

2.7 | Sample collection and processing

Six millilitres of blood was sampled from the jugular vein or intravenous catheter and placed in an EDTA and red top tube. The EDTA tube was stored at 4°C and the red top tube at room temperature until processing 60 min after collection. Samples were centrifuged for 20 min at 1000 g (or 3000 rpm) at 4°C for EDTA tubes and room temperature for red top tubes. Serum and plasma were aliquoted into cryovials ($\geq$500 μl per vial) and stored at −80°C. The plasma samples were banked.

2.8 | Serum IL-6 and MCP-1 ELISA

Serum IL-6 and MCP-1 were measured in triplicate with a commercially available quantitative ELISA kit (R&D Systems, Minneapolis,

TABLE 3 CHOP 25-week chemotherapy protocol

Treatment	Week									
	1[*]	2	3	4	5	6	7	8	9[*]	10[*]
Prednisone PO	X	X	X	X						
Doxorubicin IV				X					X	
Vincristine IV	X		X			X		X		
Cyclophosphamide		X					X			
Complete blood count	X	X	X	X	X	X	X	X	X	X
Serum and plasma collection	X								X[+]	X
Lymph node measurements	X	X	X	X	X	X	X	X	X	X
QOL survey	X								X	X

Abbreviation: QOL, quality of life.

[*]Denotes study visit.

[+]Serum and plasma collection occurred immediately before (0h) and 3, 6 and 24 h after doxorubicin treatment.

Minnesota, USA) according to the manufacturer's instructions. The absorbance of each well was measured at 450 nM and was subtracted from the absorbance reading at 570 nM with a microplate reader (Spectra Max; Molecular Devices, Sunnyvale, California, USA). A standard curve was generated for each plate using the provided standard solutions and a control sample was performed in duplicate for each plate.

2.9 | Statistical analysis

Multiple marginal linear mixed regression models with generalized estimating equations were utilized to investigate statistical associations between cytokine concentrations, categorical independent risk factors (time, treatment, response, toxicity and body weight) and clinically relevant interactions among these risk factors. The individual patient was included in the model as a cluster variable to account for correlation between measurements within each patient. Linear regression models were used to assess correlations between cytokine concentrations and white blood cell counts (neutrophils, lymphocytes and monocytes) at baseline and 1 week post treatment. Cytokine concentrations were log transformed on the natural logarithmic scale to approximate a normal distribution. Zero values were replaced with the lowest mean measured concentration detected in this study to minimize lost observations due to missing values. The normality of the data and residuals were visualized using common graphical measures (Dohoo, 2009). Models were constructed using a Gaussian distribution with an identity link. The covariance matrix for all constructed models was analyzed using residual correlation plots, and exchangeable and unstructured covariance matrices were determined most appropriate for models investigating dichotomous risk factors and categorical risk factors with three or more levels. Models investigating difference between time points and time point interactions with three or more categorical levels utilized an initial post-therapy time point where time points '0-h', '3-h' and '6-h' were collapsed and averaged because of minimal quantitative differences between cytokine values. Coeffi-

cients and their 95% confidence intervals were presented and all significant associations were characterized by 95% confidence intervals that did not include the value of 0 ($p < 0.05$). All statistical analyses were conducted using STATA version 15.1 software (StataCorp LLC, College Station, TX, USA).

3 | RESULTS

3.1 | Study population

Twenty-one dogs with multicentric lymphoma were enrolled and 16 dogs completed the study. Five dogs de-enrolled and withdrew prior to the doxorubicin study visit. Two dogs withdrew due to progressive disease, resulting in a change to the prescribed chemotherapy protocol. The third dog withdrew due to receiving concurrent oclacitinib. The fourth dog withdrew to chemotherapy discontinuation by the owner and the fifth dog withdrew due to patient temperament. Thirteen dogs were naïve to steroids at enrollment and the remaining three required a 24-h washout.

Patient characteristics are summarized in Table 4 and Table S1. Nine breeds were represented, with mixed breed ($n = 8$) being the most common. Twelve of 16 study dogs were considered at least stage 3 based on the WHO clinical staging system (Carbone et al., 1971). The remaining four dogs were classified as stage 4 or 5, with two dogs in each stage. The stage 4 dogs were classified based on the presence of organomegaly on abdominal palpation. For the stage 5 dogs, one was due to the presence of neoplastic pleural effusion documented prior to screening and the other was due to circulating neoplastic cells on CBC.

3.2 | Adverse events

Adverse event reporting was available for 15 dogs. In the week following doxorubicin administration, two of 15 dogs (13.3%) developed nausea and/or vomiting, seven of 15 dogs (46.6%) developed diarrhoea and

TABLE 4 Patient characteristics

Variable	Study dogs (n = 16)
Age (years)	Median: 10.3 Range: 5.0–13.4
Sex	Female spayed (8)
	Male castrated (7)
	Male intact (1)
Body weight (kg)	Average: 27.79
Breed	Mixed breed (8)
	Beagle (1)
	Boucheron (1)
	Cocker Spaniel (1)
	Doberman Pinscher (1)
	German Shepherd dog (1)
	Golden Retriever (1)
	Labrador Retriever (1)
	Petit Bassett Griffon Vendeen (1)
Immunophenotyping	B-cell (5)
	T-cell (0)
	Not tested (11)
Stage	At least stage 3 (12)
	Stage 4 (2)
	Stage 5 (2)
Substage	a (12)
	b (4)
Chemotherapy protocol	CHOP (8) SA Dox w Vinc induction (3)
	SA Dox (5)
Dox dose	1 mg/kg (3)
	25 mg/m^2 (4)
	27.5 mg/m^2 (2)
	30 mg/m^2 (7)
Response to therapy	CR = 10
	PR = 4
	SD = 2
	PD = 0

Abbreviations: CR, complete response; DOX, doxorubicin; kg, kilogram; m^2, metered squared; mg, milligram; PD, progressive disease; PR, partial response; SA, single agent; SD, stable disease.

FIGURE 1 Mean log transformed cytokine concentrations at baseline and prior (0-h) to doxorubicin. IL-6 (a) and MCP-1 (b). The central line represents the mean log transformed cytokine concentrations and the whiskers represent 95% confidence intervals. Symbols represent individual patient concentrations

eight of 15 dogs (53.3%) developed anorexia. All gastrointestinal toxicities were grades 1 or 2.

All dogs had a CBC performed 1 week after doxorubicin. Nine of 16 dogs (56.2%) developed at least one haematological adverse event (anaemia, neutropenia and/or thrombocytopenia). All haematologic toxicities were grade 1 or 2, with the exception of one grade 4 neutropenia. Five dogs developed neutropenia, with four dogs having a grade 1 and one dog having a grade 4. The dog with grade 4 neutropenia was afebrile and recovered without hospitalization. Three dogs had a grade 1 thrombocytopenia. Five of the 16 dogs developed a grade 2
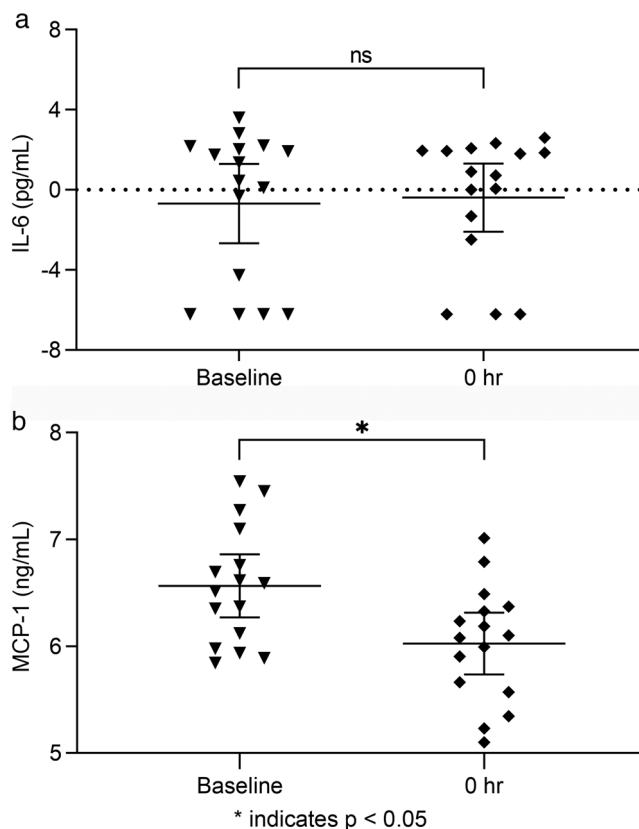
anaemia. In four of the five dogs, a grade 1 anaemia was present at the time of doxorubicin administration.

3.3 | Tumour response

At the time of the doxorubicin study visit, 10 dogs had a complete response (CR), five dogs had a partial response (PR) and one dog had stable disease (SD). No dogs that completed the study had progressive disease (PD).

3.4 | Interleukin-6

IL-6 concentrations are summarized in Table 5 and Figures 1a and 2a. There was no difference in mean log IL-6 from baseline to 0-h, which represents cytokine concentrations in naïve and treated patients ($p = 0.526$). For comparison of time points before and after doxorubicin administration, there were no significant differences in mean log IL-6 concentrations between the 0-h, 3-h and 6-h time points; therefore, these values were averaged for statistical analysis and represented by the 0-6-h time point. There was a nearing significance decrease in mean

TABLE 5 Mean IL-6 (pg/ml) for each time point with standard deviations, minimums and maximums

Time point	Mean	Standard deviation	Minimum	Maximum
Baseline	6.199	9.660	0.002	36.880
0 h	4.085	4.275	0.002	13.395
3 h	5.656	5.742	0.002	19.210
6 h	8.345	10.195	0.002	39.653
24 h	5.163	6.281	0.002	22.714
1 week	2.693	3.962	0.002	11.473
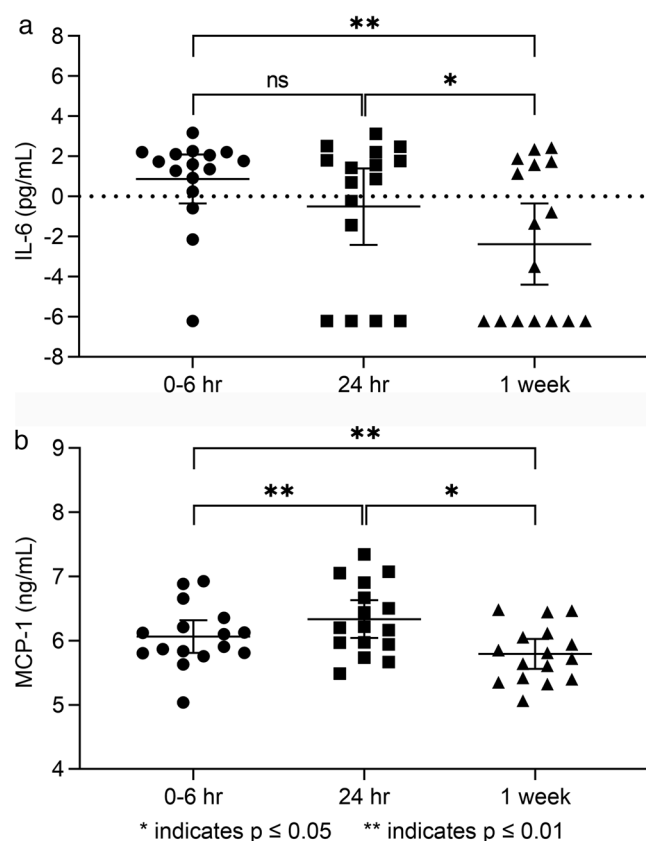
FIGURE 2 Mean log transformed cytokine concentrations at various time points before and after doxorubicin. IL-6 (A) and MCP-1 (B). The central line represents the mean log transformed concentrations and the whiskers represent 95% confidence intervals. Symbols represent individual patient concentrations. There were no significant differences between cytokine concentrations collected at 0-h, 3-h and 6-h post doxorubicin; these values were averaged for analysis and are represented by the 0–6h data points

log IL-6 concentrations at 24-h ($p = 0.055$) and a significant decrease at 1 week ($p = 0.001$) post doxorubicin, when compared to the 0-6-h time point. Comparing 24-h and 1 week post doxorubicin, there was a significant decrease in mean log IL-6 concentrations ($p = 0.045$). Mean log IL-6 concentrations at 1 week post doxorubicin treatment were not statistically associated with leukocyte counts (neutrophils: β: 0.45; 95% CI: −0.39, 1.3; $p = 0.272$; monocytes: β: 0.37; 95% CI: −5.67, 6.41;

P$p = 0.898$; lymphocytes: β: −2.55; 95% CI: −7.77, 2.67; $p = 0.313$) (Table S2).

3.5 | Monocyte chemoattractant protein-1

MCP-1 concentrations are summarized in Table 6 and Figures 1b and 2b. There was a significant decrease in mean log MCP-1 from baseline to 0-h ($p = 0.003$). As with IL-6 there were no significant differences in mean log MCP-1 concentrations between 0-, 3- and 6-h time points; therefore, these values were averaged for statistical analysis and represented by the 0-6-h time point. There was a significant increase in mean log MCP-1 cytokine concentrations at 24-h ($p = 0.001$) and a significant decrease at 1 week ($p = 0.014$) post doxorubicin, when compared to the 0- to 6-h time point. Comparing 24-h and 1 week post doxorubicin, there was a significant decrease in mean log MCP-1 cytokine concentrations ($p < 0.001$). Mean log MCP-1 concentrations at 1 week post doxorubicin treatment were not statistically associated with leukocyte counts (neutrophils: β: −0.03; 95% CI: −0.13, 0.07; $p = 0.573$; monocytes: β: −0.54; 95% CI: −1.16, 0.08; $p = 0.084$; lymphocytes: β: −0.39; 95% CI: −0.97, 0.19; $p = 0.174$) (Table S2).

3.6 | Cytokine concentrations, tumour response and adverse events

Changes in mean log IL-6 and MCP-1 were not significantly different between patients that obtained a CR and those that did not (PR and SD). Changes in cytokine concentrations were not significantly different between patients that developed chemotherapy toxicities (any grade haematological and/or gastrointestinal) and those that did not.

4 | DISCUSSION

Canine lymphoma is a multicentric disease treated with systemic chemotherapy and is a useful model to study the impact of chemotherapy on cellular senescence. Doxorubicin, an anthracycline chemotherapeutic, was chosen as it is commonly used in the treatment of canine lymphoma (Al-Nadaf et al., 2018; Mutsaers et al., 2002; Vail et al., 2020). Doxorubicin causes cytotoxicity via multiple mechanisms including DNA intercalation, topoisomerase I and II inhibition,

TABLE 6 Mean MCP-1 (ng/ml) for each time point with standard deviations, minimums and maximums

Time point	Mean	Standard deviation	Minimum	Maximum
Baseline	823.675	489.785	345.338	1888.870
0 h	486.620	255.944	164.091	1112.160
3 h	479.346	253.640	154.399	1131.420
6 h	487.106	250.046	143.340	1027.300
24 h	652.399	381.884	241.015	1548.760
1 week	358.820	161.936	157.875	652.321

generation of reactive oxygen species and is a known inducer of cellular senescence (Demaria et al., 2017; J. Ewald et al., 2008; J. A. Ewald et al., 2010; Fallah et al., 2019; Gonzalez et al., 2016; Mikuła-Pietrasik et al., 2020; Silwinska et al., 2009; Tacar et al., 2013). Furthermore, doxorubicin induces the expression of pro-inflammatory cytokines, which may contribute to development and propagation of the SASP (Demaria et al., 2017; Fallah et al., 2019; Sauter et al., 2011; Tacar et al., 2013). Additionally, doxorubicin can cause a systemic inflammatory response in mice and human cancer patients, and contributes to adverse effects such as mucositis, cardiomyopathy and phlebitis (Mauldin et al., 1992; Ogilvie et al., 1989; Sauter et al., 2011; Tacar et al., 2013). IL-6 and MCP-1 were selected based on their role in the SASP and previous studies demonstrating altered expression in dogs with lymphoma, including elevated concentrations at the time of diagnosis (Campisi, 2013; Calvalido et al., 2016; Dias et al., 2019; Jin et al., 2016; Perry et al., 2011; Rolt et al., 2019). Potential explanations for elevated cytokines in dogs with lymphoma, includes primary production by neoplastic cells, and lymphoma-induced changes to host immune and cytokine environment (Calvalido et al., 2016; Dias et al., 2019).

In the current study, we found that serum IL-6 levels did not significantly change from baseline to the doxorubicin study visit; however, IL-6 levels significantly decreased 1 week post doxorubicin administration compared to 0–6 and 24-h time points. MCP-1 levels decreased from baseline to the doxorubicin study visit. Interestingly, when compared to the 0-6-h time point, MCP-1 concentrations were significantly increased at 24-h and significantly decreased at 1 week post doxorubicin. Finally, changes in systemic cytokine levels did not correlate with leukocyte counts (neutrophils, lymphocytes and monocytes), tumour response or the development of chemotherapy-induced toxicity (gastrointestinal and haematologic). While our results are not supportive of doxorubicin-induced senescence, they suggest that both remission status and doxorubicin treatment may influence systemic cytokine concentrations in dogs with lymphoma.

IL-6 is a cytokine produced by a variety of cells, including lymphocytes, monocytes, neutrophils and senescent cells, and is critical for production of acute phase proteins, antibodies and effector T cells (Rolt et al., 2019; Tanaka et al., 2014). IL-6 signalling occurs through the Janus Kinase-Signal Transducer and Activator of Transcription Proteins pathway (JAK-STAT) and leads to transcription of genes involved in inflammation, cellular proliferation and survival (Kojima et al., 2013; Lankskron et al., 2014; Rolt et al., 2019; Tanaka et al., 2014). IL-6 is upregulated in the SASP and may reinforce senescence via autocrine

feedback loops (Ortiz-Montero et al., 2017). Increased IL-6 concentrations are associated with the presence of constitutional signs and a poorer prognosis in human non-Hodgkin's lymphoma (NHL) (Kurzrock et al., 2013; Niitsu et al., 2002). IL-6 is produced in part by lymphoma cells, suggesting that IL-6 could serve as a biomarker to assess treatment efficacy and may represent a target for novel therapeutics (Kato et al., 1996; Kurzrock et al., 2013; Niitsu et al., 2002).

In our study, IL-6 did not significantly change from baseline to the 0-h time point, suggesting there was not a significant difference in IL-6 between naïve and treated dogs with large cell or lymphoblastic lymphoma. The lack of observed decrease in IL-6 from baseline suggests that IL-6 concentrations do not directly correlate with remission status and that cytokine dysregulation persists throughout chemotherapy. This finding is consistent with a recent study evaluating the prognostic value of pro-inflammatory cytokines in humans with lymphoma. In this study, no significant difference in IL-6 was observed following multi-agent chemotherapy in patients with NHL; in contrast, a decrease in IL-6 was present in patients with Hodgkin's lymphoma (HL), which has been demonstrated in other studies (Gaiolla et al., 2011; Hamed Anber et al., 2019). While most cases of high-grade canine lymphoma are analogous to human NHL, it is important to note that lymph node biopsy and histopathologic assessment of nodal architecture were not performed in our study (Ito et al., 2014). In addition, immunophenotyping was available for 5/16 dogs evaluated in the current study; therefore, potential influence of B- versus T-cell lymphoma classification on IL-6 concentrations is unclear. Prior studies have demonstrated higher serum IL-6 levels in dogs with T-cell lymphoma compared to B-cell lymphoma (Calvalido et al., 2016). As such, future studies in a more uniform population of canine lymphoma patients with standardized immunophenotypic and histologic and/or flow cytometry characteristics is necessary to better understanding the impact of chemotherapeutics on circulating IL-6 levels.

We found that IL-6 significantly decreased following doxorubicin, with concentrations at one week being significantly lower than the 0–6 and 24-h time points. This unanticipated finding is of interest, as it does not support our initial hypothesis that doxorubicin would cause an increase in IL-6. This finding may be due, in part, to the influence of chemotherapy induced myelosuppression on circulating IL-6 concentrations. Hematopoietic cells, including lymphocytes, neutrophils and monocytes are sources of IL-6, and doxorubicin is known to cause myelosuppression, particularly impacting the white blood cell lineage (Tanaka et al., 2014; Wittenburg et al., 2019). In our study, 12 (75%),

14 (88%) and 9 (56%) of dogs experienced a decrease in their neutrophil, lymphocyte and monocyte counts, respectively, 1 week post doxorubicin. While this decrease in leukocyte counts is consistent with the myelosuppressive properties of doxorubicin, we failed to detect a statistically significant correlation between individual leukocyte cell counts (neutrophils, monocytes and lymphocytes) and serum IL-6 levels in this patient population. These data suggest that alterations in absolute leukocyte counts are not responsible for the observed decrease in IL-6 levels following doxorubicin administration.

MCP-1, also known as CCL2, is a chemokine responsible for monocyte recruitment. MCP-1 is produced mainly by macrophages/monocytes as well as tumour cells, either constitutively or in response to stress. MCP-1 functions in immune surveillance and is a documented component of the SASP (Deshmane et al., 2009; Jin et al., 2016). The role of MCP-1 in cancer is complex as MCP-1 can promote tumourigenesis by recruiting immunosuppressive tumour-associated macrophages, and myeloid-derived suppressor cells; conversely, MCP-1 also functions to suppress tumourigenesis via the recruitment of neutrophils and non-tumour associated monocytes/macrophages to tumour sites (Conti & Rollins, 2004; Niiya et al., 2003; Nokihara et al., 1999; Perry et al., 2011). MCP-1 increases with senescence induction and similar to IL-6, MCP-1 may function to maintain senescence through autocrine/paracrine feedback loops between MCP-1 and its receptor, CCR2 (Jin et al., 2016).

Increased MCP-1 levels have been shown to negatively influence prognosis in a variety of human and canine cancers, including lymphoma (Li et al., 2019; Perry et al., 2011). In our study, MCP-1 significantly decreased from baseline to the 0-h time point, which may reflect decreased tumoural production of MCP-1 in treated patients with a reduced disease burden. Production of MCP-1 by lymphoma cells is supported by a study examining MCP-1 and CCR2 expression in lymph node biopsies from humans with diffuse large B cell lymphoma (DLBCL). This study found that the majority of DLBCL biopsies showed high MCP-1 and CCR2 expression and that high expression correlated with decreased overall survival and progression free interval (Li et al., 2019). Moreover, the decrease in MCP-1 in our study is consistent with the aforementioned study, which showed that MCP-1 significantly decreased post chemotherapy in both NHL and HL patients (Hamed Anber et al., 2019).

Interestingly, we observed a significant increase in MCP-1 concentrations at 24-h and a significant decrease at 1 week post doxorubicin, when compared to the 0-6-h time point. This acute increase of MCP-1 at 24 h may reflect cell damage or death caused by doxorubicin. Doxorubicin has been shown to induce MCP-1 expression in human lung carcinoma cell lines, and MCP-1 induction has also been reported following treatment with other chemotherapeutics, such as platinum compounds and taxanes (Geller et al., 2010, 2004; Niiya et al., 2003; Shibakura et al., 2003). The transient increase in MCP-1 observed in our study may be multifactorial but likely reflects doxorubicin cytotoxicity. Despite an increase in MCP-1 at 24-h, a significant decrease was seen at 1 week post doxorubicin when compared to both the 0–6 h and the 24-h time points. In murine models of senescence, increased expression of SASP cytokines and chemokines are detectable 7 or more

days following doxorubicin administration. Furthermore, other markers of senescence, such as bioluminescent p16INK4-positive cells, persist for weeks following treatment (Demaria et al., 2017). Other studies evaluating the impact of doxorubicin in xenograft murine models of prostatic cancer demonstrate that senescent cells persist as long as 5 weeks post doxorubicin (J. Ewald et al., 2008). Based on this data, we would expect senescence-associated MCP-1 to remain elevated 7 days post-doxorubicin treatment; however, the transient increase observed in our study may be due to an acute inflammatory response or cell death, rather than the induction of senescence. Furthermore, absolute leukocyte counts (neutrophils, monocytes and lymphocytes) were not found to be significantly associated with systemic MCP-1 levels suggesting that the observed decrease in MCP-1 following doxorubicin treatment is not directly linked to reduced leukocyte counts.

A secondary aim of this study was to determine if changes in cytokine concentrations correlated with development of chemotherapy toxicity. While little is known about the role of senescence in mediating chemotherapy toxicity, studies conducted in women with breast cancer undergoing chemotherapy found that severe fatigue was correlated with increased expression of the senescence marker *p16*INK4a in peripheral blood T-cell lymphocytes (Demaria et al., 2017). Similar to humans, doxorubicin has been shown to cause significant gastrointestinal toxicity in dogs, that can result in treatment delays or discontinuation (Mutsaers et al., 2002; Ogilvie et al., 1989). Our study found that changes in IL-6 and MCP-1 did not differ between dogs with and without chemotherapy toxicities. It is important to note that not all dogs experienced chemotherapy toxicity, and toxicities in our population were mild (grades 1 and 2), with no dogs becoming febrile or requiring hospitalization. This lack of high-grade chemotherapy toxicities may have impacted changes in cytokines concentrations and thereby, statistical power. The lack of high grade toxicities is further explained by the fact that several patients did not receive a maximally tolerated dose of 30 mg/m2 of doxorubicin chemotherapy due to low patient body weight, owner's risk adversity to chemotherapy side effects or dose reductions from previous treatments. While there was no difference in cytokine concentrations between dogs that received 30 mg/m^2 and those that did not, this negative finding may be due to small sample sizes. Additionally, the use of concomitant medications at the time of chemotherapy administration may have reduced adverse events and contributed to the low incidence of chemotherapy toxicity in this patient cohort.

An additional aim of our study was to determine if treatment response had any correlation with cytokine concentrations. We found that changes in IL-6 and MCP-1 did not correlate with clinical response to treatment. This is limited by the fact that the vast majority of study patients (15/16) were already classified as having a CR or PR at the doxorubicin study visit. To increase statistical power, we chose to compare patients with a CR to those that did not attain a CR (PR, SD), although it may be more biologically relevant to compare responders to non-responders. However, non-responders (*n* = 2) were removed from the study as they required a change in their chemotherapy protocol prior to the doxorubicin study visit. To more accurately determine whether systemic cytokine levels correlate with response

to treatment and remission status, future longitudinal studies are necessary to investigate serum IL-6 and MCP-1 concentrations at the time of diagnosis, throughout therapy, and 2- to 4-weeks following completion of chemotherapy until relapse in a larger population of dogs that are both responsive and non-responsive to chemotherapy.

Finally, it is possible that in patients undergoing chemotherapy that have achieved a clinical remission, senescent cells represent a small fraction of the cell population. Therefore, senescent-dependent secretion may have a very minor influence on systemic cytokine levels. We chose to investigate senescence in patients that were in remission, as changes in cytokine concentrations following doxorubicin in naïve dogs may reflect doxorubicin-induced tumour cell death, rather than senescence induction. There are, however, other drugs besides doxorubicin that can affect IL-6 and MCP-1 including glucocorticoids, antioxidants, anti-atopic drugs and maropitant (Brattsand & Linden, 1996; Gonzales et al., 2014; Härtel et al., 2004; Tasukamoto et al., 2018). The study prohibited the administration of several drugs known to influence cytokine production including oclacitinib, anti-oxidants and other supplements (Gonzalez et al., 2014; Härtel et al., 2004); however, a number of concomitant medications were used to manage chemotherapy toxicities or treat comorbidities and thus it is difficult to determine what influence, if any, these drugs had on cytokine concentrations. We also required patients to have a 24-h and 7-day washout prior to enrollment and their doxorubicin study visit, thus minimizing any potential confounding influence of exogenous corticosteroids on systemic cytokine levels (Brattsand & Linden, 1996). While it is unlikely that a 24-h period prior to enrollment was sufficient to mitigate the effects of corticosteroids on cytokine concentrations, a longer washout period was not required due to concern for a decline in patient's condition that could potentially prohibit enrollment. Importantly, there was no significant difference in IL-6 and MCP-1 between dogs that were steroid-naïve and those requiring a 24-h washout, although there were only three dogs in the latter group. Future studies should only include glucocorticoid naïve dogs to further elucidate the influence of exogenous steroid administration on serum cytokine levels.

It is also important to note that sources of IL-6 and MCP-1 may include a variety of normal and neoplastic cells within and outside the tumour micro-environment. We attempted to limit potential non-neoplastic cytokine sources by excluding sick patients or patients with evidence of inflammatory disease; however, one patient had acral lick granulomas and another developed hypertension and protein losing nephropathy. It is unknown how these comorbidities may influence systemic cytokine concentrations. Additionally, given the complexity of cytokine production in health and disease, it is impossible to determine if cytokine changes reflect induction of senescence or other biological processes. While outside of the scope of the current study, a better understanding of the process of cellular senescence induced by doxorubicin in canine lymphoma cells would require characterizing the expression of other markers of senescence such as p16NK4a and p21Cip1 and/or assessing the activity of senescence-associated β-galactosidase (SA-β-gal) in lymphoma cells obtained from serial lymph node biopsies or cytologic samples from canine lymphoma patients.

In addition to the aforementioned study limitations, the study population of dogs with lymphoma evaluated represents a relatively small sample size ($n = 16$) and patients did not receive standardized chemotherapy protocols at consistent doxorubicin dosing schemes. At the authors' institution, it is common practice to begin doxorubicin treatment at 25–27.5 mg/m^2 and dose escalate subsequent doses to 30 mg/m^2 depending on patient tolerability. Additionally, dogs receiving CHOP chemotherapy had seven doses of chemotherapy prior to the doxorubicin study visit, while dogs receiving single agent doxorubicin received on average two to three chemotherapy doses. The extra chemotherapy doses given in the CHOP treatment group could have influenced tumour burden, haematopoietic cells and possibly cytokine concentrations. While cytokine concentrations were not different among the various chemotherapy protocols and doxorubicin dosing groups, this may be due to the relatively low number of cases within each group, and it is possible that these factors do influence cytokine concentrations. Future studies should include a larger sample size with consistent chemotherapy dosing and protocols to limit possible variables influencing cytokine production.

In conclusion, to the author's knowledge, this is the first study to examine the effects of chemotherapy on cytokine concentrations in dogs with lymphoma. We hypothesized that serum IL-6 and MCP-1 would increase following doxorubicin as a result of induction of cellular senescence. While our study failed to support this hypothesis, the changes in IL-6 and MCP-1 concentrations suggest that serum cytokines are influenced by disease status and treatment, among other factors. Additionally, changes in IL-6 and MCP-1 did not correlate with chemotherapy toxicities and response to therapy although few toxicities were documented and non-responders were removed from the study. Further investigation of these and other cytokines to serve as biomarkers for induction of senescence, toxicity or response to treatment is recommended.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. This study was approved by the OSU Institutional Animal Care and Use Committee (IACUC) and all patients enrolled had signed owner consent.

AUTHOR CONTRIBUTIONS

Data curation (lead), investigation (lead), visualization, project administration, writing original draft (lead) & writing review & editing (lead): Brittany L. Evans. Methodology, resources, visualization, writing original draft & writing review & editing, project administration, supervision: Joelle M. Fenger.

Formal analysis, methodology, resources, software, validation, visualization, writing-original draft and writing-review & editing: Greg Ballash. Conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, supervision, writing-original draft and writing-review & editing: Megan Brown.

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