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Dogs with osteosarcoma have altered pro- and anti-inflammatory cytokine profiles

Sandra M. Axiak-Bechtel[1] | **Leanne M. Mathew**[1,2] | **Juliana R. Amorim**[2] | **Amy E. DeClue**[2]

[1]Comparative Oncology Laboratory, Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA

[2]Comparative Internal Medicine Laboratory, Department of Veterinary Medicine and Surgery, College of Veterinary Medicine, University of Missouri, Columbia, MO, USA

Correspondence
Sandra M. Axiak-Bechtel, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, 2015 SW 16th Ave, PO Box 10016, Gainesville, FL 32610.
Email: sbechtel@ufl.edu

Present address
Sandra M. Axiak-Bechtel, Department of Small Animal Clinical Sciences, College of Veterinary Medicine, University of Florida, Gainesville, FL

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Abstract

Background: Current advances in immunotherapy are an exciting area of study in canine osteosarcoma (OSA). The objective of this study was to determine the immune response in dogs with osteosarcoma by measuring stimulated leukocyte production of tumor necrosis factor (TNF), interleukin (IL)-6, IL-10 and TNF and IL-6 to IL-10 ratios.

Methods: Whole blood was collected from dogs with osteosarcoma receiving non-steroidal anti-inflammatory drugs (NSAIDs, n = 11), dogs with osteosarcoma not receiving NSAIDs (n = 14) and healthy dogs (n = 5).

Results: No difference in TNF production was found among healthy and OSA dogs regardless of NSAID administration following stimulation with lipopolysaccharide (LPS) (p = .410), lipoteichoic acid (LTA) (p = .693) or PBS (p = .120). Leukocyte IL-6 production was greater in all dogs with OSA after stimulation with LPS (p = .015), LTA (p = .014) and PBS (p = .034) with no difference between OSA dogs receiving NSAIDs and those not. No differences in IL-10 were found among healthy controls and dogs with OSA regardless of NSAID use. There was no difference among groups for LPS-stimulated TNF to IL-10 ratios (p = .407). For LTA-stimulated leukocytes, the TNF to IL-10 ratio was lower in dogs with OSA than in healthy dogs (p = .031) with no difference between OSA NSAID dogs compared to OSA non-NSAID dogs (p = .059). No differences were found in LPS (p = .310)- or LTA (p = .265)-stimulated leukocyte IL-6 to IL-10 production ratios among groups.

Conclusions: Dogs with osteosarcoma have an altered pro- and anti-inflammatory immunologic profile compared to healthy dogs regardless of NSAID use. Further study is indicated to determine the potential prognostic and therapeutic implications of these findings.

KEYWORDS
cytokine production, dog, immunology, osteosarcoma

1 | INTRODUCTION

Dysregulation of the immune response is implicated in the progression of a number of cancers, including osteosarcoma, and factors such as immune elements in circulation—cytokines—ultimately determine prognosis and response to therapy (Finn, 2012; Lippitz & Harris, 2016; Rutkowski, Kamińska, Kowalska, Ruka, & Steffen, 2003). Subsequently, assessment of the activity of the immune system is helpful in determining the status of immune system responses, defining prognostic biomarkers, identifying predictors of response to therapy and selecting therapeutic targets (Finn, 2012). Osteosarcoma (OSA) is a common and often fatal cancer in dogs due to progressive local disease or metastatic disease. The median survival times reported with amputation of the affected limb are 4–5 months; carboplatin chemotherapy following amputation of the affected limb increases median survival times to 10 months (Saam, Liptak, Stalker, & Chun, 2011; Selmic, Burton, Thamm, Withrow, & Lana, 2014; Skorupski et al., 2016). Interestingly, dogs with osteosarcoma suffering from post-operative wound infection following limb-sparing surgery have improved survival times compared to dogs treated similarly but not experiencing the complication of osteomyelitis (Sottnik, U'Ren, Thamm, Withrow, & Dow, 2010). Further study using a mouse model revealed that stimulation of innate immunity, specifically natural killer cells and monocytes, inhibited the growth of OSA (Sottnik et al., 2010). Additional studies in dogs with OSA revealed that immunotherapy, in combination with surgery and chemotherapy, provided improved survival times compared to surgery and chemotherapy alone (Mason et al., 2016; Wycislo & Fan, 2015). These data suggest that the pathogenesis of OSA in dogs could be related to immune system dysregulation and immunomodulation could result in improved prognosis. Therefore, further delineation of immune function in dogs with OSA is necessary to assist in the understanding of the immunopathogenesis of OSA and provide a better understanding of prognosis, immunotherapy failures and how to target manipulation of the immune system.

Cytokines are chemical messengers responsible for communication within the innate and between the innate and adaptive immune systems. Cytokines bind cell receptors and induce changes in cell growth, development, and activity (Finn, 2012; Lippitz & Harris, 2016; Müller, Herner, Stagg, Bendtzen, & Woo, 1998; Rutkowski et al., 2003). Tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 are important pro-inflammatory cytokines that activate neutrophils, monocytes and macrophages, which then initiate bacterial and tumour cell killing (Calder, 2007; Müller et al., 1998). They also increase adhesion molecule expression on the surface of neutrophils and endothelial cells, stimulate proliferation of T- and B-lymphocytes and initiate the production of other proinflammatory cytokines (Calder, 2007). In cancer, the acute production of these pro-inflammatory cytokines is thought to be destructive to tumours by assisting with immune stimulation and recognition of tumour-associated antigens leading to tumour destruction. However, when inflammation is chronic and uncontrolled, tumour promotion can be stimulated by cellular DNA damage from free radicals and stromal remodelling required for invasion and metastasis (Rutkowski et al., 2003).

While inflammation and cytokine production in dogs with cancer is not well studied, particularly in dogs with OSA, one study found that dogs with OSA had increased plasma TNF concentrations compared to healthy dogs (Tuohy, Lascelles, Griffith, & Fogle, 2016). Other studies investigated leukocyte cytokine secretion in dogs with lymphoma and soft tissue sarcoma (Axiak-Bechtel et al., 2014; Fowler, Axiak, & DeClue, 2011; Zhang et al., 2017), but these immunologic parameters have not been investigated in dogs with OSA. The objective of this study was to determine the leukocyte production of TNF, IL-6 and IL-10 in addition to the TNF and IL-6 to IL-10 ratios at the time of OSA diagnosis following stimulation with pathogen-associated molecular pathogens. The hypothesis was that dogs with OSA would have decreased stimulated TNF and IL-6 production and simultaneous increased stimulated IL-10 production compared to healthy dogs.

2 | MATERIALS AND METHODS

Client-owned dogs presenting to the University of Missouri with a diagnosis of OSA prior to definitive therapy were eligible for enrolment following informed client consent (University of Missouri Animal Care and Use Protocol #7334). Diagnosis was made prior to presentation or at the referral institution. Inclusion criteria included a diagnosis of OSA using cytology reviewed by a board-certified clinical pathologist or histopathology reviewed by a board-certified anatomic pathologist. A history, physical examination and complete blood count (CBC) were also required of all dogs as part of routine diagnostics and staging. Dogs with a history of glucocorticoid therapy or immunosuppressive/immunomodulatory therapy within 3 months of enrolment, vaccination in the month prior to presentation or prior therapy for their OSA (including chemotherapy, radiation therapy, surgery or immunotherapy) were excluded from the study. Other staging procedures recommended, but not mandatory for study enrolment, included a biochemical panel, urinalysis and three-view thoracic radiographs.

Non-steroidal anti-inflammatory drugs (NSAIDs) have the potential to effect leukocyte cytokine production (Page et al., 2010; Tsuboi, Tanaka, Nakao, Shichijo, & Itoh, 1995), consequently medication history was documented and dogs were separated into two groups, those not receiving NSAIDs and those receiving NSAIDs. In addition, a control population of healthy dogs was recruited for enrolment following informed written client consent for dogs to participate (University of Missouri Animal Care and Use Protocol #7334). Control dogs were deemed healthy based on a health history, normal physical examination and CBC with values in the reference range. Control dogs were required to have a history free of illness within the past 3 months, vaccination or medication administration (with the exception of routine parasitic preventative) within the month prior to sample collection.

Ten mL of whole blood was collected into potassium ethylenediaminetetraacetic acid (EDTA) and sodium heparin tubes for a CBC and immunologic evaluation, respectively, via jugular venipuncture. Blood was processed within 2 hr of collection. Whole blood culture and leukocyte cytokine analysis were performed as previously described and validated in dogs (DeClue, Cohn, Lechner, Bryan, & Dodam, 2008; Fowler et al., 2011). Five millilitres of whole blood were diluted 1:2 with complete RPMI 1640 medium containing 200 U/ml of penicillin and 200 mg/ml of streptomycin (Gibco®, Invitrogen), placed in 12-well plates and stimulated with one pathogen-associated molecular pattern motif: lipopolysaccharide (LPS) from *Escherichia coli* O127:B8 at a final well concentration of 100 ng/ml (Sigma-Aldrich) or lipoteichoic acid (LTA) from *Streptococcus faecalis* at a final well concentration of 1,000 ng/ml (Sigma-Aldrich); PBS was used as a control. The 12-well plates were then gently mixed on a plate rocker for 5 min and incubated for 24 hr at 37°C in 5% CO_2. Following the 24-hr incubation time, supernatant was collected and frozen at −80°C for batch analysis. A canine-specific multiplex bead based assay (Millipore) (Karlsson et al., 2012) was used to measure TNF, IL-6 and IL-10 in supernatant. All samples were analysed in duplicate with controls and associated data analysis software to determine the median fluorescence intensity and cytokine concentration in pg/ml. The lower limit of detection of this assay was 48.8 ng/ml, the intra-assay coefficient of variation was <5%, and the inter-assay coefficient of variation was <15% under our laboratory conditions.

Statistical analysis was performed using commercially available software (SigmaStat, Systat Software Inc.). A Shapiro–Wilk was used to test normality assumptions and the Brown–Forsythe test was used to test for equal variance. One-way repeated measures analysis of variance (ANOVA) and post hoc Fisher least significant difference method or a Kruskal–Wallis ANOVA and post hoc Dunn's multiple comparison procedure were then used to evaluate for differences among groups. Extreme outliers were defined as any datum that fell outside a range defined as 3 times the interquartile range either greater than or less than the median. Extreme outliers were removed from graphical representations of data, but were not removed from statistical analysis. For cytokine concentrations below the limit of clinical detection, the lower limit of detection (48.8 ng/ml) was used for statistical analysis. A $p < .05$ was considered statistically significant.

3 | RESULTS

Fourteen dogs with OSA not receiving NSAIDs were enrolled. The median age was 7 years (range 5–11) and the median weight was 38.5 kg (range 13.1–47.8). Six dogs were neutered females and eight were neutered males; breeds were Labrador retriever ($n = 6$) and one each of the following: Great Pyrenees, Doberman, Saint Bernard, Catahoula leopard dog, Welsh Corgi, Great Dane, Rottweiler and mixed breed. The OSA locations were as follows: right distal radius ($n = 4$), left distal radius ($n = 4$) and one each in the following locations: left distal tibia, left ilial wing, left distal femur, left proximal humerus, right proximal humerus and left proximal tibia. Eight dogs had OSA diagnosed utilizing cytology and six were diagnosed using histopathology. Thirteen of the 14 dogs had three-view thoracic radiographs reviewed by a board certified radiologist and none had evidence of metastasis at the time of diagnosis. Complete blood counts are presented in Table 1. Medications dogs were receiving at the time of diagnosis and sample collection other than routine parasitic prevention were gabapentin ($n = 2$) and tramadol ($n = 4$). Two dogs had concurrent disease: one dog had heartworm disease and one dog had cutaneous mast cell tumour. Treatment pursued following sample collection was variable and included clinical trial enrolment ($n = 7$), no further treatment ($n = 5$), carboplatin chemotherapy ($n = 1$) or amputation followed by carboplatin chemotherapy ($n = 1$). Demographics are presented for study dogs in Table 1.

Eleven dogs with OSA that were receiving NSAIDs were enrolled. The median age was 8.5 years (range 2–11) and the median weight was 47.1 kg (range 28.3–60.6). Six dogs were neutered females, four were neutered males and one was an intact male; breeds were Great Pyrenees ($n = 2$), Great Dane ($n = 2$), Rottweiler, mixed breed, Border Collie, Greyhound, Saint Bernard, Australian shepherd and German shepherd dog. The OSA locations were the left distal radius ($n = 2$), right distal radius ($n = 2$), right proximal humerus ($n = 2$), left proximal humerus ($n = 2$), and one each of the following: left distal tibia, right distal tibia and right distal femur. Nine dogs had the diagnosis confirmed with histopathology and two dogs were diagnosed with cytology. All 11 dogs had three-view thoracic radiographs reviewed by a board-certified radiologist with no evidence of metastasis. Dogs were receiving the following NSAIDs at the time of diagnosis and sample collection: carprofen ($n = 4$), deracoxib ($n = 4$), meloxicam ($n = 2$) or firocoxib ($n = 1$). Additional medications included cefpodoxime ($n = 1$), tramadol ($n = 4$), gabapentin ($n = 4$) and amantadine ($n = 1$). One dog had a concurrent urinary tract infection and no other dog had concurrent disease. Treatment pursued following sample collection was variable and included clinical trial enrolment ($n = 5$), amputation alone ($n = 2$), radiation therapy followed by carboplatin chemotherapy ($n = 2$) or amputation followed by carboplatin chemotherapy ($n = 2$).

Five healthy dogs were enrolled. Health status was determined based on history, physical examination and normal CBC. All dogs were greater than 10 kg and were neutered adults greater than 2 years of age (specific ages were not available in some dogs due to adoption history).

Complete blood count results are presented in Table 2. There was no difference between groups between neutrophils and monocytes. Healthy dogs had an increased number of lymphocytes compared to dogs with osteosarcoma ($p = .010$) and osteosarcoma with NSAIDs ($p = .013$).

There was no difference in TNF production between the leukocytes of healthy controls and dogs with OSA regardless of NSAID administration when stimulated with LPS ($p = .410$), LTA ($p = .693$) or PBS ($p = .120$) (Figure 1). Leukocyte IL-6 production was greater in dogs with OSA after LPS ($p = .015$), LTA ($p = .014$) or PBS ($p = .034$)

TABLE 1 Demographic data of study dogs

Parameter	No NSAID	NSAID
Number of dogs	14	11
Median age (years)	7 (range 5–11)	8.5 (range 2–11)
Median weight (kg)	38.5 (13.1–47.8)	47.1 (range 28.3–60.6)
Sex		
Male	0	1
Male neutered	8	4
Female	0	0
Female neutered	6	6
Breed		
Labrador retriever	6	0
Great Pyrenees	1	2
Doberman	1	0
St. Bernard	1	1
Catahoula leopard dog	1	0
Welsh Corgi	1	0
Great Dane	1	2
Rottweiler	1	1
Mixed Breed	1	1
Border Collie	0	1
Greyhound	0	1
Australian Shepherd	0	1
German shepherd	0	0
Tumour location		
Right distal radius	4	2
Left distal radius	4	2
Left distal tibia	1	1
Right distal tibia	0	1
Left ilial wing	1	0
Left distal femur	1	0
Right distal femur	0	1
Left proximal humerus	1	2
Right proximal humerus	1	2
Left proximal tibia	1	0
Method of diagnosis		
Cytology	8	2
Histopathology	6	9
Thoracic radiographs (note that all thoracic radiographs showed no evidence of metastasis)	13	11
Therapy		
Clinical trial	7	5
Amputation + chemotherapy	1	2
Amputation alone	0	2
Chemotherapy alone	1	0
Radiation + chemotherapy	0	2
No treatment	5	0
Concomitant medications		
Gabapentin	2	4
Tramadol	3	4
Carprofen	0	4
Deracoxib	0	4
Meloxicam	0	2
Firocoxib	0	1
Amantadine	0	1
Cefpodoxime	0	1

stimulation (Figure 2). Dogs with OSA receiving NSAIDs and not receiving NSAIDs had no difference in LPS (p = .971)- or LTA (p = 0.314)-stimulated IL-6 production. There were no differences between IL-10 production among healthy controls, dogs with OSA receiving NSAIDs and dogs with OSA not receiving NSAIDs, when stimulated with LPS (p = .282), LTA (p = .357) or PBS control (p = .408) (Figure 3).

There were no differences among healthy dogs, OSA dogs receiving NSAIDs and OSA dogs not receiving NSAIDs for LPS-stimulated TNF to IL-10 (p = .407) ratios (Figure 4). The LTA-stimulated TNF to IL-10 ratio was lower in dogs with OSA receiving NSAIDs and not receiving NSAIDs than in healthy dogs (p = .031); there was no difference between OSA NSAID dogs compared to OSA non-NSAID dogs (p = .059) (Figure 4). There were no differences between LPS (p = .310)- or LTA (p = .265)-stimulated leukocyte IL-6 to IL-10 production ratios among groups (Figure 5). The PBS-stimulated TNF to IL-10 ratios were not significantly different among groups (median, Q1, Q3; Healthy, 1.0, 1.0, 1.7; OSA, 3.3, 1.0, 5.8; OSANSAID, 5.1, 1.1, 9.0; p = .078). Similarly, PBS-stimulated IL-6 to IL-10 ratios were not significantly different among groups (median, Q1, Q3; Healthy, 1.0, 1.0, 1.0; OSA, 1.6, 1.0, 2.1; OSANSAID, 2.1, 1.0, 2.6; p = .109).

4 | DISCUSSION

Dogs with OSA have an altered cytokine production profile compared to healthy dogs that is not affected by the use of NSAIDs. The whole blood cytokine pattern of dogs with OSA revealed increased IL-6 production compared to healthy dogs following stimulation, with no differences in TNF or IL-10 production. In addition to cytokines, the balance of cytokines was investigated using the pro-to-anti- inflammatory cytokine ratios. These ratios can detect an imbalance between pro- and

TABLE 2 White blood cell data in dogs at the time of sample collection and processing. There was no difference between groups between neutrophils and monocytes. Healthy dogs had an increased number of lymphocytes compared to dogs with osteosarcoma (p = .010) and osteosarcoma with NSAIDs (p = .013)

Dog group	Median neutrophils per μl (range)	Median lymphocytes per μl (range)	Median monocytes per μl (range)
Healthy	6,400 (2,870–9,000)	2,280 (1,180–3,780)	250 (240–410)
Osteosarcoma	5,859 (3,800–11,380)	1,027 (480–2,860)	480 (220–930)
Osteosarcoma with NSAIDs	5,020 (3,622–15,480)	1,178 (280–2,642)	482.5 (133–1,390)

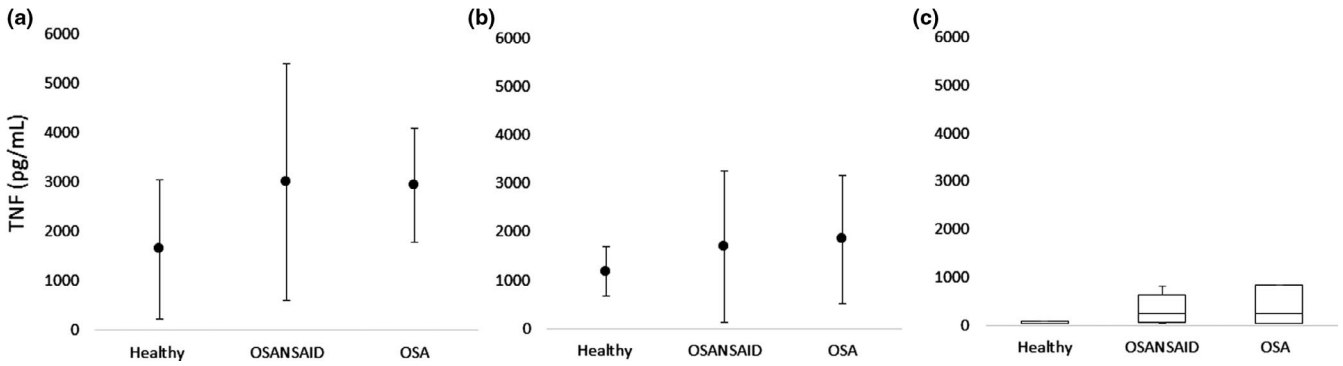

FIGURE 1 (a) Lipopolysaccharide (LPS)-, (b) lipoteichoic acid (LTA)- and (c) PBS (control)-stimulated production of tumor necrosis factor (TNF) in healthy dogs, dogs with osteosarcoma not on non-steroidal anti-inflammatory drugs (OSA) and on non-steroidal anti-inflammatory drugs (OSANSAID). Data for (a) LPS and (b) LTA stimulation is parametric and therefore represented as mean ± *SD*. Data for (c) PBS (control) is non-parametric data and therefore represented as a box and whisker plot with the median (line in box), first and third quartiles (top and bottom of box) and range (whiskers). Extreme outliers are not represented graphically but were included in statistical analysis

anti-inflammatory cytokines that may be missed if investigating cytokine patterns alone. Dogs with OSA had a decreased TNF to IL-10 production ratio compared to healthy dogs following LTA-stimulation suggesting alterations in the pro- and anti-inflammatory cytokine profile. By measuring stimulated leukocyte cytokine production, this study provides insight into the innate immune system in dogs with OSA.

In this study, whole blood was stimulated with LPS, LTA or PBS (control). Lipopolysaccharide is an endotoxin and component of the outer cell membrane of Gram-negative bacteria and LTA is a component of Gram-positive bacterial cell walls. When LPS or LTA is released by bacteria (due to bacterial cell division or death), these components, called pathogen-associated molecular patterns, are recognized by pattern recognition receptors such as the toll like receptor (TLR). Pattern recognition receptors are expressed on cells of the innate immune system and upon activation a cascade of signalling begins, resulting in pro-inflammatory transcription factors and upregulation of genes controlling the host immune response (Chandler & Ernst, 2017). The outcome is increased phagocytosis and the release of pro-inflammatory cytokines, namely TNF and IL-6 (Rosenfeld & Shai, 2006). The objective of this study was to determine how well the immune system was capable of responding to stimulation in dogs with OSA by measuring stimulated leukocyte production of TNF, IL-6 and IL-10 and the TNF or IL-6 to IL-10 ratios at the time of OSA diagnosis. Stimulating the leukocytes of dogs diagnosed with OSA and comparing their cytokine production to that of healthy dogs provides an assessment of the innate immune system's ability to respond to pathogen-associated molecular pattern motifs.

Although cytokine patterns are not well studied in dogs with cancer, some studies do provide background for the current investigation. For example, dogs with lymphoma have decreased TNF, IL-6 and IL-10 production following stimulation with pathogen-associated molecular pattern motifs (Fowler et al., 2011). Additional study showed that induction of remission did not alter the blunted innate immune response in dogs with lymphoma (Axiak-Bechtel et al., 2014). These data suggested that dogs with lymphoma have a decreased ability to respond appropriately to infectious stimuli that is independent of remission status, and dogs with OSA were hypothesized to have a similar cytokine pattern. However, in contrast to dogs with lymphoma, leukocyte production of TNF and IL-10 following stimulation was not different between healthy dogs and dogs with OSA. Furthermore, no difference was detected between dogs with OSA that were receiving NSAIDs and those that were not. Dogs with OSA did have a decreased LTA-stimulated TNF to IL-10 ratio compared to healthy dogs, again with no difference between dogs with OSA receiving NSAIDs and those with OSA not receiving NSAIDs. This indicates a potential imbalance in pro- and anti-inflammatory cytokines favouring an anti-inflammatory response to pathogen-associated molecular pattern motif stimulation. This difference was seen following stimulation with LTA, but not following stimulation with LPS. Studies in people have shown differences in cytokine/chemokine release both in vitro and in vivo with LPS and

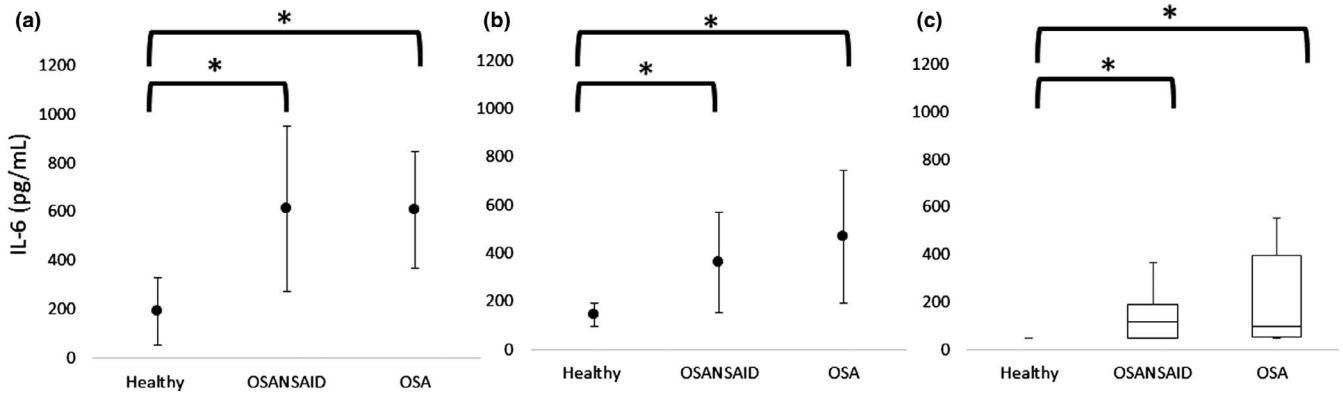

FIGURE 2 (a) Lipopolysaccharide (LPS)-, (b) lipoteichoic acid (LTA)- and (c) PBS (control)-stimulated production of interleukin (IL)-6 in healthy dogs and dogs with osteosarcoma not on non-steroidal anti-inflammatory drugs (OSA) and on non-steroidal anti-inflammatory drugs (OSANSAID). Data for (a) LPS and (b) LTA stimulation is parametric and therefore represented as mean ± *SD*. Data for (c) PBS (control) is non-parametric data and therefore represented as a box and whisker plot with the median (line in box), first and third quartiles (top and bottom of box) and range (whiskers). Extreme outliers are not represented graphically but were included in statistical analysis. Samples from healthy dogs for IL-6 detection following PBS (control) were below the limit of detection for the assay, thus no box plot is presented. *Indicates significant difference between groups

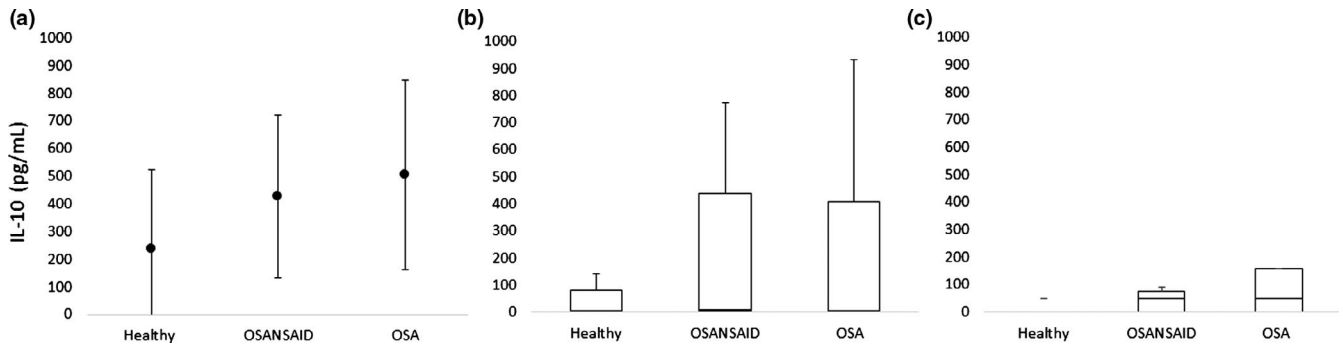

FIGURE 3 (a) Lipopolysaccharide (LPS)-, (b) lipoteichoic acid (LTA)- and (c) PBS (control)-stimulated production of interleukin (IL)-10 in healthy dogs and dogs with osteosarcoma not on non-steroidal anti-inflammatory drugs (OSA) and on non-steroidal anti-inflammatory drugs (OSANSAID). Data for (a) LPS-stimulation is parametric and therefore represented as mean ± *SD*. Data for (b) LTA stimulation and (c) PBS (control) is non-parametric data and therefore represented as a box and whisker plot with the median (line in box), first and third quartiles (top and bottom of box) and range (whiskers). Extreme outliers are not represented graphically but were included in statistical analysis. Samples from healthy dogs for IL-10 detection following PBS (control) incubation were below the limit of detection for the assay, thus no box plot is presented. The lower limit of the SD is 0 in all graphs

LTA, in particular decreased IL-10 and increased IL-6 with Gram-positive bacterial infections or stimulation with LTA. These differences were noted to be distal to NF-κB/AP-1 activation and may include chromatin remodelling and mRNA stability (Finney, Leaver, Evans, & Burke-Gaffney, 2012). Thus, the differential results in stimulation between LPS and LTA could be due to differences in response to Gram-negative and Gram-positive bacteria and should be further studied and clarified in dogs with OSA. A blunted immune response to Gram-positive bacterial insult in dogs with OSA may result in a decreased ability to respond to pathogens compared to normal dogs and thus have implications in chemotherapy dose intensification. This anti-inflammatory profile may also support the findings of previous studies, in which dogs with OSA suffering from osteomyelitis had longer overall survival times compared to dogs without osteomyelitis (Lascelles et al., 2005; Sottnik et al., 2010). In the mouse model of canine osteosarcoma, osteomyelitis resulted in the stimulation of monocytes and natural killer cells which suppressed tumour angiogenesis (Sottnik et al., 2010). Perhaps monocyte inactivity is, in part, reflected in dogs with naturally occurring osteosarcoma by the altered cytokine profiles found in this study. The alteration in pro-inflammatory cytokine to anti-inflammatory cytokine production following LTA stimulation could also contribute to the complex and poorly understood immunodysfunction in dogs with OSA, and offer insight into prognostic and predictive markers, in addition to representing potential additional therapeutic interventions in immunotherapeutic studies.

Interleukin-6 is an inflammatory cytokine that functions in both the innate and adaptive arms of the immune system. It stimulates acute-phase protein synthesis and stimulates production of neutrophils. The whole blood leukocyte cytokine production of IL-6 was greater in dogs with OSA compared to healthy dogs. This is in contrast

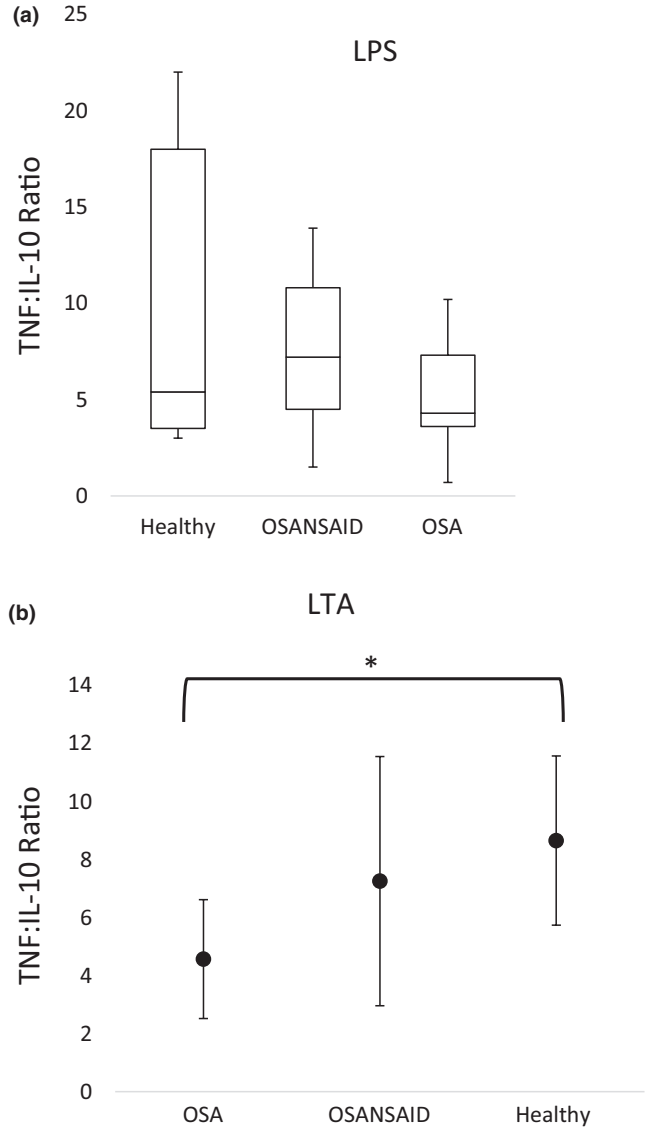

FIGURE 4 (a) Lipopolysaccharide (LPS)- and (b) lipoteichoic acid (LTA)-stimulated tumor necrosis factor to interleukin-10 ratio (TNF:IL-10) in healthy dogs and dogs with osteosarcoma not on non-steroidal anti-inflammatory drugs (OSA) and on non-steroidal anti-inflammatory drugs (OSANSAID). Non-parametric data (a) is represented as a box and whisker plot with the median, first and third quartiles and range; parametric data (b) is represented as mean ± *SD*. *Indicates a significant difference between groups. Following LTA stimulation, the TNF: IL-10 was lower in dogs with OSA than in healthy dogs (p = .031)

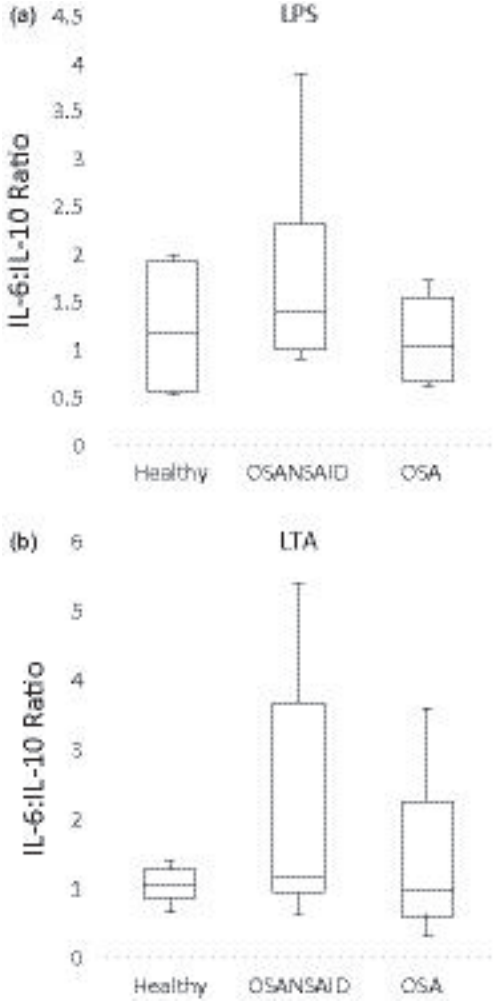

FIGURE 5 (a) Lipopolysaccharide (LPS) and (b) lipoteichoic acid (LTA) interleukin-6 to interleukin-10 ratio (IL-6:IL-10) in healthy dogs and dogs with osteosarcoma not on non-steroidal anti-inflammatory drugs (OSA) and on non-steroidal anti-inflammatory drugs (OSANSAID). Data are represented as a box and whisker plot with the median (line in box), first and third quartiles (top and bottom of box) and range (whiskers). The OSA group for LPS-stimulated IL-6:IL-10 ratio had an extreme outlier not graphically represented; however, this outlier was included in the statistical evaluation. No differences were found in LPS (p = .310)- or LTA (p = .265)-stimulated leukocyte IL-6:IL-10 production ratios among groups

to the anti-inflammatory cytokine production shift noted in TNF to IL-10 ratios. Increased serum IL-6 concentrations are associated with a negative prognosis in multiple human cancer types, including bone sarcoma (Rutkowski et al., 2003). Interestingly, the source of increased IL-6 in some studies of human cancer types was not the tumour cells but instead circulating monocytes. It would be interesting to evaluate if monocytes predominantly produce IL-6 in dogs with OSA. Increased production of IL-6 could have many consequences, including recruitment of monocytes to the tumour microenvironment and differentiation to tumour-associated macrophages. Not only is increased IL-6 production a negative prognostic factor in human cancer patients, a decrease in IL-6 production correlates positively with survival (Lippitz & Harris, 2016). Although this correlation has not yet been studied in dogs with cancer, evidence of increased IL-6 production from whole blood leukocytes from dogs with osteosarcoma warrants further study as a prognostic and predictive biomarker.

Many dogs in this study were on concurrent medications including gabapentin and tramadol; healthy dogs were on no medications other than routine parasitic prevention. This accurately reflects the clinical scenario in which multiple modalities of pain control are

utilized to provide quality of life pending definitive localized therapy. Gabapentin was the most commonly prescribed analgesic in addition to NSAIDs. The effects of gabapentin on cytokine production have not been studied in the dog and are minimally studied in other species. In rats, intrathecal administration of gabapentin may increase IL-10 and inhibit pro-inflammatory cytokine production including IL-6 and TNF (Lee, Jun, Kim, & Park, 2013). Thus, it is possible that gabapentin may have affected cytokine production following oral administration in these dogs. If an effect occurred, based on previous research it would likely have resulted in decreased concentrations of IL-6 and TNF with increased concentrations of IL-10 (Lee et al., 2013). Further investigation into the immunologic effects of gabapentin in dogs is warranted. The other common analgesic administered to dogs with OSA in this study was tramadol. The immunologic effects of tramadol have been investigated in dogs, and tramadol did not alter cytokine production; therefore, it is hypothesized that tramadol was unlikely to affect the results of this study (Axiak-Bechtel et al., 2015).

Three dogs in this study had concurrent disease—two dogs in the non-NSAID group (heartworm disease and mast cell tumour) and one dog in the NSAID group (urinary tract infection). The mast cell tumour and urinary tract infection were localized to the skin and urinary bladder, respectively, and likely had minimal, if any, impact on systemic leukocyte cytokine production. This expectation is based on research in human medicine showing compartmentalization of infection and inflammation, in particular when comparing local leukocyte cytokine production to systemic using whole blood culture (Abraham & Miao, 2015). One dog had asymptomatic heartworm disease, considered Stage I, and not treated at the time of sample collection. An investigation of inflammatory markers in dogs with heartworm disease that IL-6 was not increased compared to normal dogs at the time of diagnosis, and increased following therapy (Yoon et al., 2017). Based on this data, the impact of untreated Stage I heartworm disease in one dog in this study is expected to be minimal.

Of note is that no metastasis was found in any of the 24 dogs that had thoracic radiographs. This is consistent with published literature, which reports <10% visible metastatic disease at the time of diagnosis (Finn, 2012; Lippitz & Harris, 2016; Rutkowski et al., 2003; Skorupski et al., 2016). The association between cytokine production and prognosis was not evaluated since this was not a goal of the study. Furthermore, as the treatments pursued by dog owners were variable and a number of dogs were enrolled in various clinical trials following sample collection, progression free intervals and survival times were not included. Given the results of this study and the potential for IL-6 to be involved in cancer progression, further evaluation of IL-6 as a biomarker and its association with prognosis is warranted in dogs with OSA.

Whole blood was used in this study rather than isolating individual immune cell populations or cytokine production on a per cell basis. This method was chosen because it is most relevant for clinical assessment of functional immunity in dogs with OSA. Whole blood contains all the components of blood including a complete cell population, proteins, hormones and other compounds that better represents the complex in vivo immune response in blood compared to isolated cells. This technique also minimizes handling compared to cell isolation, reducing the risk of cellular activation or contamination and maintaining normal cell to cell interactions (Calder, 2007; Müller et al., 1998).

In conclusion, the leukocytes of dogs with osteosarcoma had altered cytokine production in response to pathogen-associated molecular pattern motifs compared to healthy dogs with increased IL-6 production and decreased LTA-stimulated TNF to IL-10 ratios. This pattern of cytokine production found in OSA dogs was different than that reported in dogs with lymphoma (Fowler et al., 2011). Future investigations should monitor leukocyte function through a defined treatment protocol and determine the prognostic significance and potential therapeutic targeting of these changes. These factors, with other immunologic parameters, should also be considered when investigating immunotherapy as potential predictive and prognostic markers.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

ETHICAL 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. The US National Research Council's guidelines for the Care and Use of Laboratory Animals were followed.

ORCID

Sandra M. Axiak-Bechtel https://orcid.org/0000-0002-7773-5959

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